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Direct ethanol production from hemicellulosic materials of rice straw by use of an engineered yeast strain codisplaying three types of hemicellulolytic enzymes on the surface of xylose-utilizing *Saccharomyces cerevisiae* cells

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ABSTRACT

The cost of the lignocellulose-hydrolyzing enzymes used in the saccharification process of ethanol production from biomass accounts for a relatively high proportion of total processing costs. Cell surface engineering technology has facilitated a reduction in these costs by integrating saccharification and fermentation processes into a recombinant microbe strain expressing heterologous enzymes on the cell surface. We constructed a recombinant *Saccharomyces cerevisiae* that not only hydrolyzed hemicelluloses by codisplaying endoxylanase from *Trichoderma reesei*, β-xylosidase from *Aspergillus oryzae*, and β-glucosidase from *Aspergillus aculeatus* but that also assimilated xylose through the expression of xylose reductase and xylitol dehydrogenase from *Pichia stipitis* and xylulokinase from *S. cerevisiae*. The recombinant strain successfully produced ethanol from rice straw hydrolysate consisting of hemicellulosic material containing xylan, xylooligosaccharides, and cellooligosaccharides without requiring the addition of sugar-hydrolyzing enzymes or detoxication. The ethanol titer of the strain was 8.2 g/l after 72 h fermentation, which was approximately 2.5-fold higher than that of the control strain. The yield (grams of ethanol per gram of total sugars in rice straw hydrolysate consumed) was 0.41 g/g, which corresponded to 82% of the theoretical yield. The cell surface-engineered strain was thus highly effective for consolidating the process of ethanol production from hemicellulosic materials.

1. Introduction

In response to concerns related to the effects of fossil fuel combustion on climate, interest in the production of biofuels from biomass and their use as a renewable resource has increased considerably in recent years. Of these biofuels, fuel ethanol has received considerable attention, particularly in Brazil and the USA where it is produced from sugarcane and starch-rich grains (Sánchez and Cardona, 2008). However, the use of grains and sugarcane as biomass for fuel production is not considered to be sustainable as it competes with food and animal feed uses. Instead, the use of lignocellulosic biomass, such as agricultural residues (e.g. rice straw, corn stover, and bagasse) and industrial waste, which is cheap, abundant, and does not compete with food production, is considered more desirable.

Lignocellulosic biomass is primarily composed of cellulose, hemicellulose and lignin, and the bioconversion of these raw materials to ethanol consists of three stages: pretreatment, hydrolysis by an enzyme complex, and fermentation (Lynd et al., 2002). Liquid hot water (LHW) treatment is considered to be one of the effective pretreatment processes for solubilizing hemicellulose and for making the cellulose more accessible (Alvira et al., 2010). The slurry generated after pretreatment can be filtered to obtain two fractions: a solid cellulose-enriched fraction and a liquid fraction that is rich in hemicellulose-derived sugars. The economic viability of using lignocellulosic biomass in the ethanol production process is highly dependent upon the bioconversion efficiency of hemicellulose and cellulose.

Hemicellulose is a substituted polysaccharide with a xylan backbone consisting of β-1,4-linked xylose monomers to which substituents and saccharides, such as glucose, galactose and arabinose, are attached (Saha, 2003). In order to directly convert hemicellulosic materials into ethanol, xylanolytic enzymes should be expressed using ethanol-producing fungi and bacteria such as *Saccharomyces cerevisiae*, *Pichia stipitis*, and *Escherichia coli* through genetic engineering (Görgens et al., 2005; Li et al., 2010; Valenzuela et al., 2010). Of the organisms currently used for ethanol production, *S. cerevisiae* is the most widely used for the heterologous production of xylanase and xylosidase (Lee et al., 2009; Yeasmin et al., 2010). However, since a major disadvantage associated

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with using *S. cerevisiae* is its inability to utilize xylose, wild type strains of this yeast have been genetically engineered to assimilate xylose through the introduction of genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) from fungi (Eliasson et al., 2000; Ho et al., 1998; Johansson et al., 2001), or genes encoding xylose isomerase from bacteria and fungi (Karhumaa et al., 2005; Kuyper et al., 2003). While these changes have facilitated the direct fermentation of xylan by yeast (Katahira et al., 2004), the rate of conversion of xylan into xylose is considered to be insufficient and alternative ways to increase hemicellulase activity are required.

In addition, the hemicellulosic material derived from the pretreatment of biomass is not pure, which consists of sugars like glucoxylan, xyloglucan and cellooligosaccharide and xylooligosaccharide. Furthermore, fermentation inhibitors have been reported to accumulate in the hydrolysates due to the over-degradation of lignocelluloses (Almeida et al., 2007). The direct fermentation of the sugar mixture in the presence of inhibitors, such as weak acids, furan compounds, and phenolics is therefore critical to the industrial utilization of hemicellulosic materials.

To examine ethanol production from hemicellulosic materials, we constructed a xylose-assimilating *S. cerevisiae* capable of codisplaying endoxylanase from *Trichoderma reesei*, β-xylosidase from *Aspergillus oryzae*, and β-glucosidase from *A. aculeatus*. Compared to the control strain, ethanol production and sugar consumption were remarkably improved by using the recombinant strain.

2. Materials and methods

2.1. Strains, plasmids and media

Table 1 summarizes the genetic properties of all strains and plasmids used in this study. *E. coli* NovaBlue (Novagen Inc., Madison, WI) was used as the host strain for recombinant DNA manipulation. The yeast strains MT8-1/X and NBRC1440/X were used for cell surface display of the *XYNII* gene from *T. reesei*, *XylA* was from *A. oryzae* and *BGL1* was from *A. aculeatus*.

E. coli transformants were grown in Luria–Bertani medium (10 g/l tryptone, 5 g/l yeast extract and 5 g/l NaCl, pH 7.0) supplemented with 100 μg/ml of ampicillin. Yeast transformants were screened in Synthetic Dextrose minimal medium (SD medium) [6.7 g/l yeast nitrogen base without amino acids (Difco Laboratories, MI), 20 g/l glucose] supplemented with appropriate amino acids and nucleic acids. Yeast cells were cultured under aerobic conditions in SD medium, SDC medium [SD medium containing 20 g/l casamino acids (Difco Laboratories)] supplemented with appropriate amino acids and nucleic acids, and in YPD medium [10 g/l yeast extract, 20 g/l polypeptone, 20 g/l glucose].

2.2. Lignocellulosic hydrolysate

A liquid fraction obtained by LHW (Alvira et al., 2010) of rice straw at 130–300 °C under the pressure of less than 10 MPa was purchased from Mitsubishi Heavy Industries, Ltd. (Tokyo, Japan). The hydrolysate was separated from solid cellulose-enriched fraction by filtration. The pH of the hydrolysate was adjusted to 5 using NaOH. Sugar components in the hydrolysate were analyzed using a high performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD) (Shimadzu, Kyoto, Japan) after filtration with an Amicon Ultra filter (MWCO 10 kDa, Millipore, MA). Chromatographic separation was achieved using a Unison UK-Amino column (3 μm, 4.6 mm i.d. × 250 mm, Imtakt, Kyoto, Japan); the column temperature was set at 25 °C. The mobile phase consisted of water and acetonitrile at flow rates of 1 ml/min, with gradient elution as follows: acetonitrile concentration at 0 min, 90%; 5 min, 90%; 30 min, 55%; 31 min, 30%; 36 min, 30%; 36.01 min, 90%; 48 min, 90%; v/v. The evaporator temperature for the ELSD was set at 40 °C. Nitrogen gas was used at a flow rate of 3 l/min at a gas pressure of 350 kPa. Fermentation inhibitors, such as acetate, formate, furfural, 5-hydroxymethyl-2-furfural (5-HMF), vanillin, *o*-vanillin, eugenol, *iso*-eugenol, and syringaldehyde (Almeida et al., 2007; Olsson and Hahn-Hägerdal, 1996; Palmqvist and Hahn-Hägerdal, 2000) in the hydrolysate were analyzed by gas chromatography–mass spectrometry (GC–MS) (QP2010Plus, Shimazu). Acids were measured using a DB-FFAP column (60 m × 0.25 mm i.d., 0.5 μm film thickness; Agilent, Palo Alto, CA). The flow rate of helium gas through the column was 0.94 ml/min. The column temperature was held at 100 °C for 5 min isothermally, before being raised by 10 °C/min to 230 °C where it was maintained for 6 min. Furan compounds and phenolics were measured with a CP-Sil 8-CB low Bleed/MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Varian Inc., Palo Alto, CA). The flow rate of helium gas through the column was 1.69 ml/min. The column temperature was held at 50 °C for 5 min isothermally, before being raised by 20 °C/min to 280 °C, where it was maintained for 5 min. For the phenolics, the column temperature was held at 50 °C for 1 min isothermally, before being increased by 20 °C/min to 280 °C where it was maintained for 1 min. The interface and the ion source temperatures were 250 °C and 230 °C, respectively. Ions were generated using a 70 V electron impact and ion fragments were detected in the selected ion monitoring mode.

2.3. Construction of recombinant plasmid and yeast strains

Plasmid pIHBGXylA, which was used for the cell surface display of both β-glucosidase and β-xylosidase, was constructed as follows. The *Not*I fragment containing the *S. cerevisiae TDH3* promoter, the secretion signal sequence from the *Rhizopus oryzae*

Table 1
Characteristics of strains and plasmids used in this study.

	Description	Reference
Strains		
MT8-1	*MATa ade his3 leu2 trp1 ura3*	Katahira et al. (2006)
MT8-1/X	MT8-1 (pIUX1X2XK)	Katahira et al. (2006)
MT8-1/XBX	MT8-1X (pIHBGXylA)	This study
NBRC1440ΔHUWL	*MATα his3 leu2 trp1 ura3*	Yamada et al. (2009)
NBRC1440/X	NBRC1440ΔHUWL (pIUX1X2XK)	This study
NBRC1440/XBX	NBRC1440/X (pIHBGXylA)	This study
MN8140/X	Diploid hybrid from MT8-1/X and NBRC1440/X	This study
MN8140/XBX	Diploid hybrid from MT8-1/XBX and NBRC1440/XBX	This study
MN8140/XBXX	MN8140/XBX (pδW-GPAGXynII)	This study
Plasmids		
pIUX1X2XK	*URA3*, intracellular coexpression of *XYL1*, *XYL2*, and *XKS1* genes	Katahira et al. (2006)
pIHBGXylA	*HIS3*, surface coexpression of *A. aculeatus* β-glucosidase gene (*BGL1*) and *A. oryzae* β-xylosidase gene (*XylA*)	This study
pδW-GPAGXynII	*TRP1*, surface expression of *T. reesei* β-xylanase gene (*XYNII*) by δ-integration	This study

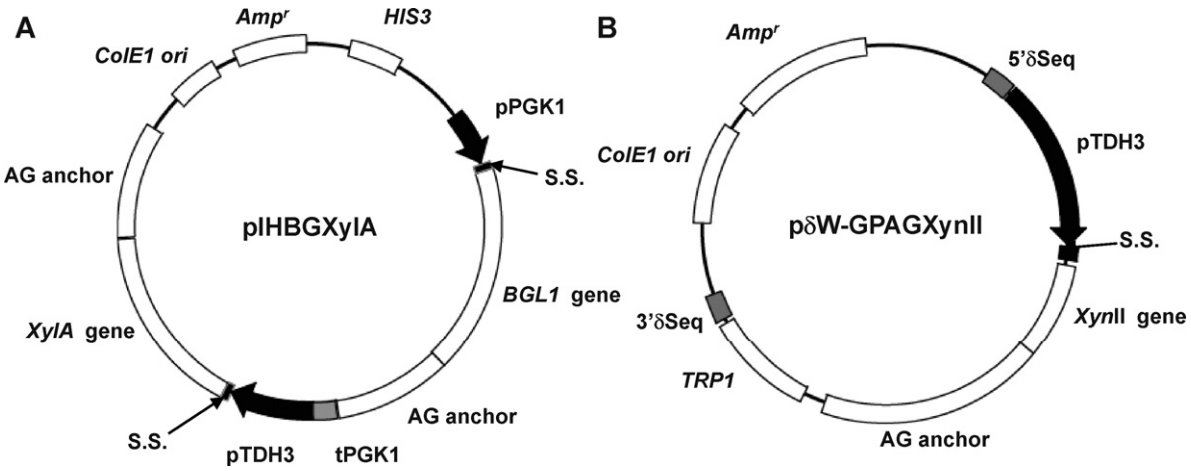

Fig. 1. Expression plasmids for codisplay of *A. aculeatus* β-glucosidase and *A. oryzae* β-xylosidase by (A) integration and (B) display of *T. reesei* β-xylanase gene by δ-integration.

α-amylase gene, the *A. oryzae* β-xylosidase gene (*XylA*), and the 3′ half of the α-agglutinin gene was amplified from pUCSxylAf (Katahira et al., 2004) by PCR using the following primers: 5′-ATAAGAATGCGGCCGCTTTGATTATGTTCTTTCTATTTGAATGAGATATG-3′ and 5′-ATGCGCGGCCGCACCAGTTCTCACACGGAACA-3′. The fragment was inserted into the *Not*I site of the *A. aculeatus* β-glucosidase gene (*BGL1*) harboring plasmid pIHAGBGL (Yanase et al., 2010) to yield pIHBGXylA. The plasmid pδW-GPAGXynII, which was used for the cell surface display of endoxylanase, was constructed as follows. The *Not*I fragment encoding the *S. cerevisiae TDH3* promoter, the secretion signal sequence, *T. reesei XYNII*, and the α-agglutinin gene, was amplified from pCAS-RGSHis6-XYNII (Fujita et al., 2002) by PCR using the following primers: 5′-ATAAGAATGCGGCCGCTTTGATTATGTTCTTTCTATTTGAATGAGATATG-3′ and 5′-ATGCGCGGCCGCACCAGTTCTCACACGGAACA-3′. The fragment was subcloned into the *Not*I site of pδW (Yamada et al., 2009) to yield pδW-GPAGXynII. The plasmids are shown in Fig. 1.

The haploid strains MT8-1/XBX, NBRC1440/X, and NBRC1440/XBX were constructed as follows. The plasmid pIUX1X2XK and pIHBGXylA were digested with *Pst*I within the *URA3* gene and *Nde*I within the *HIS3* gene for transformation into the haploid *S. cerevisiae* strains MT8-1 and NBRC1440ΔHUWL, respectively, using the lithium acetate method (Chen et al., 1992).

The diploid strains MN8140/X and MN8140/XBX were constructed by mating as described previously (Yamada et al., 2009). The MN8140/X strain was constructed using the MT8-1/X and NBRC1440/X strains; the MN8140/XBX strain was constructed using the MT8-1/XBX and NBRC1440/XBX strains. The diploid strain, MN8140/XBXX, was constructed as follows. The *TRP1* gene in the plasmid, pδW-GPAGXynII, was digested with *Asc*I so that it could be transformed into the diploid strain MN8140/XBX.

2.4. Saccharification of hemicellulosic materials

To analyze the sugar components of the hydrolysate, the polysaccharides and oligosaccharides contained in the lignocellulosic hydrolysate were hydrolyzed with 10 g/l hemicellulase (G-Amano; Amano Enzyme, Nagoya, Japan) by shaking at 25 rpm at 37 °C for 24 h. After removing proteins by filtration with Amicon Ultra, the sugar content was measured by HPLC-ELSD. The activities of endoxylanase, β-xylosidase, and β-glucosidase in the hemicellulase were 723.5, 22.8, and 71.0 U/g, respectively.

2.5. Enzyme assays

Yeast cells were cultivated in YPD medium for 72 h at 30 °C and collected by centrifugation for 5 min at 3000 × *g* at 4 °C. The cells were then washed twice with distilled water and β-glucosidase, β-xylosidase and endoxylanase activities were determined using the cells as described previously (Katahira et al., 2004). Dry cell weight of the yeast strains was estimated to be 0.15-fold that of the wet cell weight.

2.6. Fermentation

After precultivation in SD medium for 24 h, yeast cells were aerobically cultivated for 48 h at 30 °C in YPD medium. The cells were collected by centrifugation at 3000 × *g* at 4 °C for 10 min and washed twice with distilled water. The cell pellets were then inoculated into the fermentation medium [YP medium (10 g/l yeast extract, 20 g/l polypeptone), 80% (v/v) rice straw hydrolysate] and ethanol production was performed under oxygen limited conditions at 30 °C with mild agitation in 100 ml bottles equipped with an outlet for CO_2. The initial cell concentration was 100 g/l of wet cells and the concentrations of xylose, xylobiose, glycerol, and xylitol in the fermentation medium were measured using a GC–MS equipped with the CP-SIL 8 CB low bleed/MS column described above after derivatization. The injection temperature was 230 °C and the helium gas flow rate through the column was 1 ml/min. The column temperature was held at 80 °C for 2 min isothermally before being increased at 15 °C/min to 330 °C where it was maintained for 6 min. The interface and the ion source temperatures were 250 °C and 200 °C, respectively. Derivatization was performed with methoxyamine hydrochloride and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide as described previously (Pongsuwan et al., 2007). Ethanol produced during the fermentation was measured by a GC–MS equipped with the DB-FFAP column described above. The injection temperature was 250 °C and the flow rate of helium gas through the column was 1 ml/min. The column temperature was held at 40 °C for 5 min isothermally before being increased by 30 °C/min to 230 °C, and held at 330 °C for 10 min. The interface and the ion source temperatures were 250 °C and 230 °C, respectively.

3. Results

3.1. Analysis of rice straw hydrolysate

LHW treatment solubilizes the hemicellulosic component of rice straw to yield liquid-soluble fractions containing mono-, oligo-, and polysaccharides (Hendriks and Zeeman, 2009; Kim et al., 2009; Laser et al., 2002). We determined the contents of solubilized sugars in the hydrolysate obtained by LHW treatment by HPLC (Table 2). Major sugars in the hydrolysate were xylose (6.85 g/l), xylobiose

Table 2
Sugar components of rice straw hydrolysate.

Sugar [g/l][a]	Saccharification	
	–	+
Xylose	6.85	21.17
Xylobiose	2.02	N.D.
Xylotriose	1.18	N.D.
Xylotetraose	0.87	N.D.
Xylopentaose	0.79	N.D.
Xylohexaose	0.91	N.D.
Glucose	0.88	11.35
Cellobiose	0.19	N.D.
Cellotriose	0.29	N.D.
Galactose	0.48	1.61
Arabinose	2.96	3.05
Total	17.42	37.18

N.D., not detected.
[a] Values are averages of three independent experiments and relative standard deviations (RSDs) were less than 10%.

Table 3
Inhibitor concentration in rice straw hydrolysate.

Inhibitors	Concentration [g/l][a]
Weak acids	
Acetate	3.23
Formate	1.08
Furan compounds	
Furfural	1.22
5-HMF	0.18
Phenolics	
Vanillin	0.02
o-Vanillin	0.01
Eugenol	0.05
iso-Eugenol	0.01
Syringaldehyde	0.02

[a] Values are averages of three independent experiments and RSDs were less than 10%.

(2.02 g/l), xylotriose (1.18 g/l), glucose (0.88 g/l), and arabinose (2.96 g/l). A peak, considered to represent polysaccharide, was detected in the chromatogram of the HPLC and used to estimate the amount of constituent monosaccharide in the hydrolysate after the sugars were fully hydrolyzed by the addition of hemicellulase. As shown in Table 2, after 24 h hydrolysis the oligosaccharides and polysaccharide were degraded to xylose (21.17 g/l), glucose (11.35 g/l), galactose (1.61 g/l), and arabinose (3.01 g/l). The sum of the monosaccharides and oligosaccharides increased from approximately 17 g/l to 37 g/l due to the saccharification, indicating that at least 20 g/l polysaccharide was contained in the hydrolysate.

Since fermentation inhibitors generated by over-degradation of lignocelluloses from the LHW treatment were present in the hydrolysates, we elucidated the components of inhibitors such as weak acids, furan compounds and phenolics in the hydrolysates by GC–MS (Table 3). Major components were acetate (3.23 g/l), formate (1.08 g/l), and furfural (1.22 g/l), while phenolics from lignin were hardly present (<0.20 g/l). All of the identified compounds are known to inhibit cell growth and to reduce ethanol yield (Almeida et al., 2007; Casey et al., 2010; Martín et al., 2007; Olsson and Hahn-Hägerdal, 1996; Palmqvist and Hahn-Hägerdal, 2000).

3.2. Enzymatic activities on the cell surface

As shown in Table 2, sugar-component analysis revealed that the major abundant sugars in the hydrolysate were xylose and polysaccharides. While the direct conversion of hydrolysates into ethanol is considerably attractive for reducing ethanol production costs, wild-type strains of *S. cerevisiae* are not able to utilize xylose and polysaccharide directly. We therefore constructed a recombinant strain expressing XR and XDH from *P. stipitis* and endogenous xylulokinase (XK) to produce the xylose-assimilating yeast strains MT8-1X and NBRC1440X. Next, for cell surface expression, genes encoding β-xylosidase from *A. oryzae* and β-glucosidase from *A. aculeatus* were introduced into the genome of the MT8-1/X and NBRC1440/X strains using pIHBGXylA (Fig. 1) to yield MT8-1/XBX and NBRC1440/XBX. The diploid strains MN8140/X and MN8140/XBX were then constructed by mating MT8-1/X and NBRC1440/X, and MT8-1/XBX and NBRC1440/XBX, respectively. Finally genes encoding endoxylanase from *T. reesei* were introduced into the genome of MN8140/XBX using the δ-integration plasmid pδW-GAPXynII (Fig. 1) to produce the diploid strain MN8140/XBXX.

The recombinant strains, MN8140/X and MN8140/XBXX, were cultured in the YPD medium at 30 °C for 72 h. Enzymatic activities of the endoxylanase, β-xylosidase and β-glucosidase in the recombinant strains were measured using the cell pellet fractions and culture supernatants obtained by centrifugation. As shown in Table 4, endoxylanase, β-xylosidase, and β-glucosidase activities were detected in the pellet fraction of strain MN8140/XBXX (41.2, 16.8 and 48.1 U/g-dry cell weight, respectively). Conversely, no enzyme activity was detected in MN8140/X or in the culture supernatant of both strains (Table 4), indicating that endoxylanase, β-xylosidase and β-glucosidase were successfully codisplayed on the cell surface of strain MN8140XBXX. The maintenance of integrated genes in our recombinant strains was stable after 50 generations in non-selectable synthetic medium.

3.3. Ethanol production from lignocellulosic hydrolysate

Fermentation of lignocellulosic hydrolysate as a sole carbon source was conducted for the strains MN8140/X and MN8140/XBXX under oxygen limited conditions. Initial cell concentration was adjusted to 100 g of wet cells/l. As shown in Fig. 2, the codisplaying strain MN8140/XBXX produced 8.2 g/l ethanol after 72 h of fermentation, while the control strain MN8140/X produced only 3.0 g/l ethanol. In the codisplaying strain, xylobiose increased for 6 h after the initiation of fermentation and then decreased thereafter, but it remained almost constant in the control strain. These results indicate that the xylanase and xylosidase displayed on the cell surface of the recombinant strain facilitated the hydrolysis of hemicellulose. In addition, the xylose produced by

Table 4
Xylanase, β-xylosidase and β-glucosidase activities of cell surface enzymes.

Strain	Specific activity [U/g (dry weight) of cell][a]					
	Cell pellet			Culture supernatant		
	Xylanase	β-Xylosidase	β-Glucosidase	Xylanase	β-Xylosidase	β-Glucosidase
MN8140/X	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
MN8140/XBXX	41.2	16.8	48.1	N.D.	N.D.	N.D.

N.D., not detected.
[a] Values are averages of three independent experiments and RSDs were less than 10%

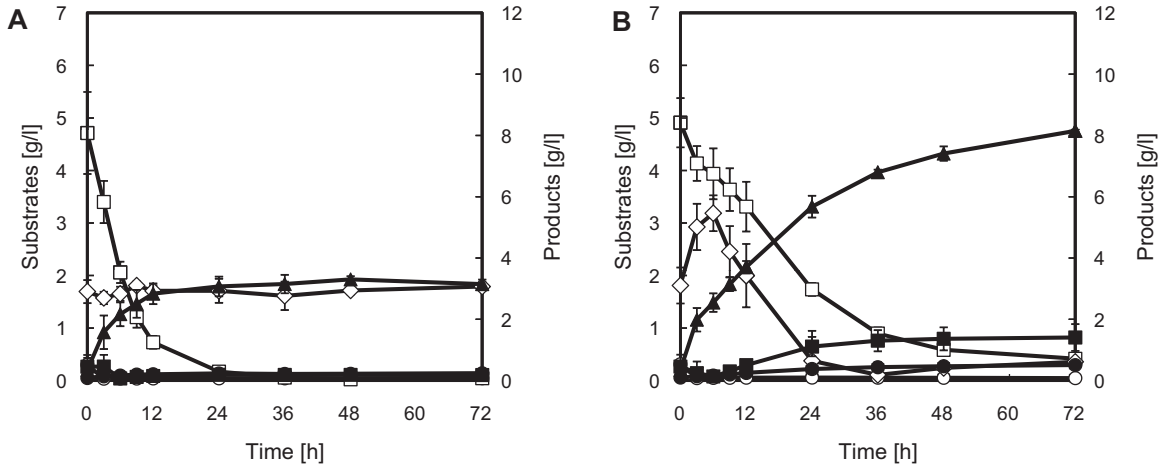

Fig. 2. Ethanol fermentation from hydrolysate without addition of hemicellulase by *S. cerevisiae* strains (A) MN8140/X and (B) MN8140/XBXX: (○) glucose, (□) xylose, (◊) xylobiose, (●) glycerol, (■) xylitol, and (▲) ethanol. Values are averages of three independent experiments, ±SD.

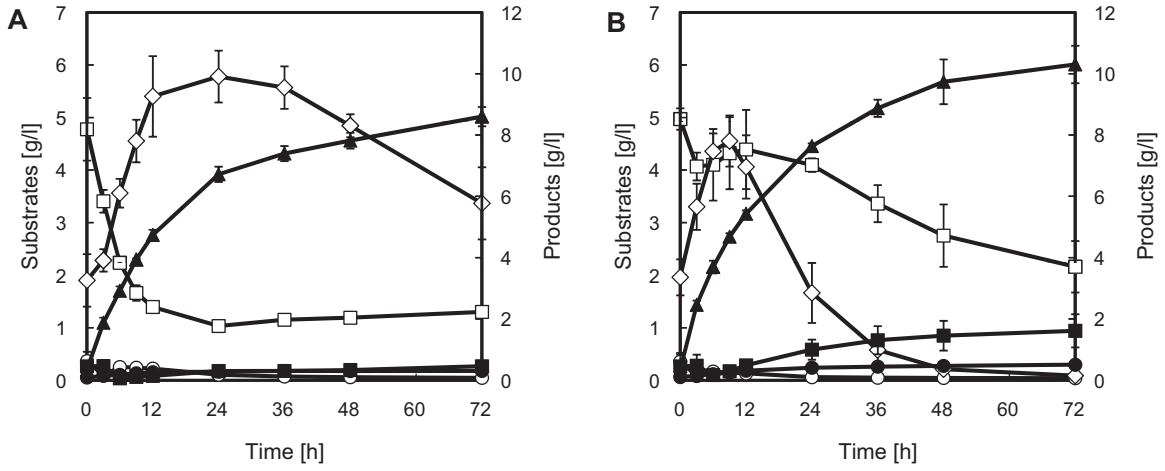

Fig. 3. Ethanol fermentation from hydrolysate with the addition of 0.2 g/l hemicellulase by *S. cerevisiae* strains (A) MN8140/X and (B) MN8140/XBXX: (○) glucose, (□) xylose, (◊) xylobiose, (●) glycerol, (■) xylitol, and (▲) ethanol. Values are averages of three independent experiments, ±SD.

these enzymes was consumed at the same time during saccharification, and glucose was rapidly consumed by both strains (Fig. 2). Galactose and arabinose were not consumed at all (data not shown).

Fig. 3 shows the fermentation of the hydrolysate in the presence of the commercial hemicellulase (0.2 g/l). The codisplaying strain produced 10.3 g/l ethanol after 72 h of fermentation, while the control strain produced 8.6 g/l ethanol. In the control strain, xylobiose increased over time due to the addition of the hemicellulase, with the MN8140XBXX strain completely utilizing all of the xylobiose after 48 h fermentation. As in the case of fermentation without hemicellulase addition, rapid consumption of glucose was observed while galactose and arabinose were not consumed.

Total sugar concentration in the fermentation medium was determined by the phenol–sulfuric acid method (DuBois et al., 1956) (Fig. 4). Sugar consumption of the MN8140XBXX strain was higher than that of the control strain with or without the addition of hemicellulase. In the absence of enzyme, sugar consumed by the codisplaying strain and the control strain after 72 h fermentation was 19.7 g/l and 8.2 g/l, respectively. In the presence of enzyme, consumed sugar was 24.4 g/l and 20.0 g/l, respectively. Sugar consumption was thus remarkably improved by the cell surface engineering.

Fermentation profiles of the recombinant strains are summarized in Table 5. The percentage of consumed sugar in total sugar was calculated based on the total amount of glucose and xylose

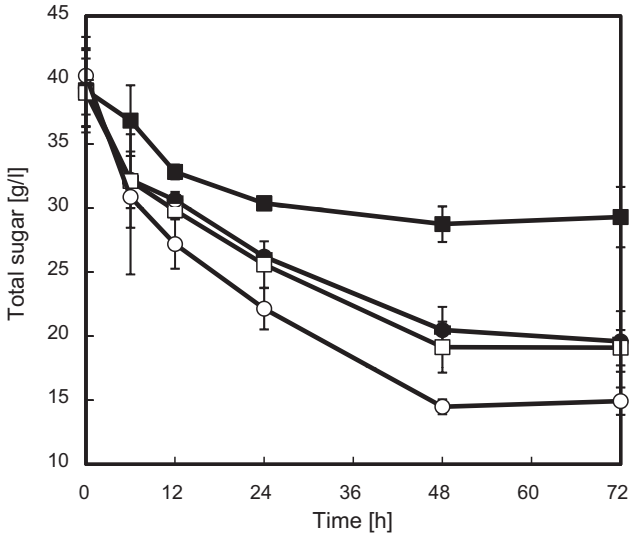

Fig. 4. Time course of total sugar concentration during fermentation from lignocellulosic hydrolysates: (■) MN8140/X, (●) MN8140/X + 0.2 g/l hemicellulase, (□) MN8140/XBXX, and (○) MN8140/XBXX + 0.2 g/l hemicellulase. Values are averages of three independent experiments, ±SD.

Table 5
Ethanol production from lignocellulosic hydrolysate.

Strain	$Sugar_{consumption}$[a] [g/l]	Ratio of sugar consumed to total sugar [% (w/w)]	Ethanol[b] [g/l]	Ethanol yield[c] [g/g]	Productivity[d] [g/l/h]
MN8140/X	8.2 ± 2.5	32 ± 9	3.0 ± 0.11	0.12 ± 0.00	0.30 ± 0.01
MN8140/X + hemicellulase	20.0 ± 1.4	77 ± 4	8.6 ± 0.22	0.33 ± 0.01	0.43 ± 0.02
MN8140/XBXX	19.7 ± 1.3	76 ± 4	8.2 ± 0.03	0.32 ± 0.00	0.37 ± 0.01
MN8140/XBXX + hemicellulase	24.4 ± 1.2	93 ± 5	10.3 ± 0.43	0.40 ± 0.02	0.56 ± 0.05

Values are averages of at least three independent experiments, ±SD.
[a] $Sugar_{consumption}$ calculated by subtracting total sugar concentration at 0 h fermentation from that at 72 h.
[b] Ethanol calculated using data obtained after 72 h fermentation.
[c] Ethanol yield calculated using data of the concentration glucose and xylose after saccharification (Table 2).
[d] Productivity calculated using data obtained after 6 h fermentation.

that were released by the hydrolysis of hemicellulosic material, although the percentage might be slightly higher than real value due to the possible end-product inhibition of enzyme saccharification. The percentage was improved from 32% to 76% by cell surface engineering (Table 5). Based on the total sugar, ethanol yield using only the cell surface-engineered yeast increased from 0.12 g/g to 0.32 g/g (Table 5). Further improvement was achieved by the addition of commercial hemicellulase, when the percentage increased from 77% to 93% and ethanol yield increased from 0.33 g/g to 0.40 g/g (Table 5). Using a combination of cell surface modification and commercial hemicellulase, ethanol productivity was improved by about 2-fold (from 0.30 g/l/h to 0.56 g/l/h) (Table 5). The capacity for ethanol conversion from hemicellulosic hydrolysate in the codisplaying strain in the absence of commercial hemicellulase was similar to that of the control strain in the presence of 0.2 g/l commercial hemicellulase. Thus, use of the codisplaying strain facilitated high ethanol conversion from hemicellulosic hydrolysate and a reduction in enzyme loads.

4. Discussion

We successfully demonstrated direct ethanol production from lignocellulosic hydrolysate obtained by LHW pretreatment using xylose assimilating yeast codisplaying xylanase, β-xylosidase, and β-glucosidase. To our knowledge, this is the first report to directly convert a mixture of sugars, such as xylan, xylooligosaccharide and cellooligosaccharide, into ethanol in the presence of fermentation inhibitors released by pretreatment of lignocellulose.

Compared to the control strain MN8140/X, the codisplaying strain MN8140/XBXX produced more than 2.5-fold more ethanol after 72 h fermentation. Sugar consumption of the codisplaying strain was 2-fold higher than that of the control strain (Table 5). As shown in Table 2, the total amount of mono- and oligosaccharides in the hydrolysate increased from 17 g/l to 37 g/l in response to the addition of enzyme. These results indicate that the improvement of ethanol production in MN8140/XBXX can be attributed to the degradation of oligo- and polysaccharides in the hydrolysate by codisplaying enzymes (Fig. 2B). In the fermentation by our codisplaying strain, xylobiose liberated from xylan by xylanase was immediately converted into xylose by xylosidase. This result indicates that synergism between xylanase and xylosidase is successfully induced on the yeast cell surface.

In the control strain, ethanol production was improved by the addition of hemicellulase (Table 5), however, ethanol (8.6 g/l) produced by the control strain in the presence of hemicellulase added is similar to ethanol (8.2 g/l) obtained using the codisplaying strain without the addition of enzyme. In general, commercially available hemicellulase is more expensive than cellulase, because hemicelluloses contain numerous kinds of polysaccharides (Girio et al., 2010; Saha, 2003). In addition, the demand for hemicellulase is considerably lower than that of cellulose, because hemicellulose can easily be decomposed by pretreatment with acids (Alvira et al., 2010; Girio et al., 2010). In the present study, our arming strategy made it possible to reduce the addition of hemicellulase by the expression of the enzyme on the yeast cell surface.

Recently, the fermentation of hemicellulosic hydrolysate obtained by diluted sulfuric acid pretreatment has been reported (Canilha et al., 2010; Silva et al., 2011). Since such a pretreatment normally hydrolyzes hemicellulose to release xylose, the fermentation of acid-pretreated hemicellulose was performed using *P. stipitis*, which is capable of utilizing xylose as a carbon source. Canilha et al. (2010) successfully improved ethanol production by detoxification of bagasse hydrolysate, which enabled ethanol yields to reach 0.30 g/g-sugar consumed. Silva et al. (2011) produced ethanol from wood chips at yields of 0.32 g-ethanol/g-sugar consumed by optimizing pretreatment conditions. However, in general, compared to *S. cerevisiae*, *P. stipitis* is less tolerant to fermentation inhibitors such as weak acids and phenolics. In this study, we successfully conferred a sugar-hydrolyzing ability to *S. cerevisiae*, which enabled us to convert hemicellulose in the rice straw hydrolysate into ethanol directly in the presence of fermentation inhibitors. Indeed, compared with previous reports (Canilha et al., 2010; Silva et al., 2011), ethanol yield and productivity were significantly improved using the cell surface-engineered *S. cerevisiae* strain.

As shown in Table 5, the codisplaying strain MN8140/XBXX utilized 19.7 g/l sugars, which corresponded to approximately 76% of total sugar (sum of glucose and xylose) in the hydrolysate without the addition of hemicellulase. This high capacity for sugar degradation by codisplaying yeast could be attributed to overexpression of xylanase by δ-integration (Yamada et al., 2009). δ-Integration makes it possible to integrate heterologous genes into the chromosomal DNA in a multi-copy manner, because more than 100 copies of the δ-sequence derived from the Ty retrotransposon is present in the genome of *S. cerevisiae*. Compared to *S. cerevisiae* displaying endoxylanase strain, MT8-1/pUCSXIIXA in a previous report (Katahira et al., 2004), endoxylanase activity can be increased by more than 2-fold (Table 3).

The hydrolysate used in the present study contained a number of fermentation inhibitors, including acetic and formic acids, and furan derivatives (Table 3). These compounds are known to inhibit ethanol fermentation from sugars (Almeida et al., 2007; Martín et al., 2007; Olsson and Hahn-Hägerdal, 1996; Palmqvist and Hahn-Hägerdal, 2000). Especially, xylose assimilation is more severely affected by such inhibitors compared to glucose assimilation (Casey et al., 2010; Hasunuma et al., 2011). However, in the present study, the codisplaying strain was capable of utilizing xylose in the hydrolysate to produce ethanol (Figs. 2 and 3) without any detoxification treatment. In general, diploid strains are more tolerant to inhibitors than haploid strains (Sonderegger et al., 2004). Since ethanol production of the polyploid strain is superior to that of the haploid strain (Yamada et al., 2009), further improvement of ethanol conversion from lignocellulosic material could be achieved by using a polyploid strain.

As shown in Figs. 2B and 3B, ethanol production was enhanced by the addition of commercial hemicellulase into the fermentation medium, although xylobiose was completely consumed by the cell surface-engineered yeast strain after 72 h fermentation. The result indicates the hydrolysis of hemicellulosic polysaccharide would be one of the limiting factors in the hydrolysate fermentation. On the other hand, 2.2 g/l xylose remained in the fermentation medium after 72 h fermentation. Accordingly, the improvement of xylose consumption rate as well as xylan hydrolysis activity is required for further production of ethanol. Also, ethanol production from the hydrolysate might be improved by the coculture of different strains displaying each enzyme, because the codisplaying enzymes for utilizing the hydrolysate on the surface of one strain might cause burden for the cell growth and metabolism. Also, the coculture system might enable to optimize hemicellulose utilization by controlling the concentration of each strain expressing single enzyme.

Recently, the impetus for developing a cost effective, bioethanol production process is strongly desired. In this regard, process consolidation through the combination of enzyme production, saccharification and fermentation into a single process would lead to a reduction in costs by decreasing the number of processing steps (Lynd et al., 2005; Olofsson et al., 2008; Xu et al., 2009; van Zyl et al., 2007). To achieve such a consolidation, the ideal microorganism would be capable of simultaneous saccharification and ethanol production from sugars by hydrolytic enzymes produced by the microorganism. However, few reports of direct fermentation from hemicellulosic material have been published to date because the yield of ethanol produced from hemicellulosic material obtained by biomass pretreatment has been low (Katahira et al., 2004; Miyazaki et al., 2008; Skory et al., 1997; Voronovsky et al., 2009). In the present study, a recombinant *S. cerevisiae* strain that not only codisplays hemicellulolytic enzymes on the cell surface but also expresses xylose-assimilating enzymes in the cell was constructed. These modifications allow for direct and efficient ethanol fermentation from lignocelluloses hydrolysate containing sugar mixture and fermentation inhibitors. Our approach employing cell surface engineering of *S. cerevisiae* is considered to have considerable potential for the future development of effective bioethanol production systems.

5. Conclusion

A recombinant, xylose-assimilating *S. cerevisiae* strain codisplaying endoxylanase, β-xylosidase, and β-glucosidase on the yeast cell surface directly produced ethanol from a rice straw hydrolysate obtained by liquid hot water pretreatment. By using the recombinant yeast strain, hemicellulosic material containing xylan, xylooligosaccharides, and cellooligosaccharide was successfully converted into ethanol without requiring any detoxification treatment or the addition of sugar-hydrolyzing enzymes. Our results demonstrate that the cell surface-engineered strain was highly effective for facilitating the process consolidation of ethanol production from hemicellulosic materials.

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