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# Construction of a Xylan-Fermenting Yeast Strain through Codisplay of Xylanolytic Enzymes on the Surface of Xylose-Utilizing *Saccharomyces cerevisiae* Cells

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**Hemicellulose is one of the major forms of biomass in lignocellulose, and its essential component is xylan. We used a cell surface engineering system based on α-agglutinin to construct a *Saccharomyces cerevisiae* yeast strain codisplaying two types of xylan-degrading enzymes, namely, xylanase II (XYNII) from *Trichoderma reesei* QM9414 and β-xylosidase (XylA) from *Aspergillus oryzae* NiaD300, on the cell surface. In a high-performance liquid chromatography analysis, xylose was detected as the main product of the yeast strain codisplaying XYNII and XylA, while xylobiose and xylotriose were detected as the main products of a yeast strain displaying XYNII on the cell surface. These results indicate that xylan is sequentially hydrolyzed to xylose by the codisplayed XYNII and XylA. In a further step toward achieving the simultaneous saccharification and fermentation of xylan, a xylan-utilizing *S. cerevisiae* strain was constructed by codisplaying XYNII and XylA and introducing genes for xylose utilization, namely, those encoding xylose reductase and xylitol dehydrogenase from *Pichia stipitis* and xylulokinase from *S. cerevisiae*. After 62 h of fermentation, 7.1 g of ethanol per liter was directly produced from birchwood xylan, and the yield in terms of grams of ethanol per gram of carbohydrate consumed was 0.30 g/g. These results demonstrate that the direct conversion of xylan to ethanol is accomplished by the xylan-utilizing *S. cerevisiae* strain.**

In recent years, lignocellulosic biomass, such as agricultural and forestry residues, waste paper, and industrial wastes, has been recognized as an ideally inexpensive and abundantly available source of sugar for fermentation into the sustainable transportation fuel ethanol. The use of fuels bioconverted from biomass offers solutions to many environmental problems.

Lignocellulose is composed of cellulose (insoluble fibers of β-1,4-glucan), hemicellulose (noncellulosic polysaccharides, including xylans, mannans, and glucans), and lignin (a complex polyphenolic structure) (1, 38). β-1,4-Xylan, a main component of hemicellulose, is a complex polysaccharide consisting of a backbone of β-1,4-linked xylopyranoside which is partially substituted with acetyl, glucuronosyl, and arabinosyl side chains. For the efficient utilization of biomass, the bioconversion of xylan as well as cellulose is required.

Xylan is hydrolyzed to xylooligosaccharides by endo-β-xylanase (EC 3.2.1.8), following which β-D-xylosidase (EC 3.2.1.37) hydrolyzes xylooligosaccharides to D-xylose (2, 23). Many bacterial and fungal species are able to utilize xylans as a carbon source (17). *Trichoderma* spp. and *Aspergillus* spp. secrete large amounts of xylan-degrading enzymes (8, 14, 16, 31, 42). *Trichoderma reesei*, which is a filamentous mesophilic fungus well known for its cellulolytic and xylanolytic activities, secretes the two major inducible endo-β-xylanases xylanase I (XYNI) and xylanase II (XYNII) (37, 40). XYNII represents more than 50% of the total xylanolytic activity of *T. reesei* cultivated on xylan (39). Meanwhile, the industrial koji mold *Aspergillus oryzae* is also known to be an efficient producer of xylan-degrading enzymes and secretes β-xylosidase (21). The gene encoding the major β-xylosidase, XylA, comprises 2,397 bp with no intron and encodes a protein consisting of 798 amino acids. The deduced amino acid sequence shows high similarity to those of *Aspergillus nidulans* XlnD, *Aspergillus niger* XlnD, and *T. reesei* BxlI, which were isolated previously.

Many researchers have studied the production of xylanolytic enzymes in the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*. The former, which is the yeast most widely used in ethanol fermentation, has been used as a host organism for the heterologous production of bacterial or fungal xylanase (3, 25, 27, 29, 32) and β-xylosidase (26, 28); attempts have been made to coproduce xylanase and β-xylosidase in this yeast as a means of converting xylan to cell mass or ethanol through simultaneous saccharification and fermentation (24). However, *S. cerevisiae* has been found to be unable to utilize xylose for growth or fermentation. While a number of researchers have developed engineered yeasts capable of xylose fermentation by introducing heterologous genes encoding xylose reductase (XYL1) and xylitol dehydrogenase (XYL2) from *P. stipitis* and xylulokinase (XKS1) from *S. cerevisiae* (10, 15, 18, 19), the direct fermentation of xylan by *S. cerevisiae* has not yet been reported.

However, xylanolytic enzymes have been heterologously expressed and produced in *P. stipitis*, which has the ability to ferment xylose (5, 6, 30). The direct production of ethanol with xylan as the sole carbon source was obtained with recombinant *P. stipitis* secreting *Cryptococcus albidus* xylanase, although the ethanol productivity and yield were not reported (30).

Recently, yeast strains displaying various active enzymes on the cell surface were developed by using genetic engineering

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TABLE 1. Characteristics of microbial strains and plasmids used

| Strain or plasmid | Relevant features | Reference or source |
|---|---|---|
| Strains | | |
| Yeasts and fungi | | |
| *S. cerevisiae* | | |
| MT8-1 | *MAT***a** *ade his3 leu2 trp1 ura3* | 35 |
| MT8-1/pMUCS | No display (control strain) | 13 |
| MT8-1/pMUCS/pWGP3 | No expression (control strain) | This work |
| MT8-1/pCAS1-RGSHis6-XYNII | Display of XYNII | 12 |
| MT8-1/pUCSxylAf | Display of XylA | This work |
| MT8-1/pUCSXIIXA | Codisplay of XYNII and XylA | This work |
| MT8-1/pUCSXIIXA/pWX1X2XK | Codisplay of XYNII and XylA in xylose-assimilating yeast strain | This work |
| *A. oryzae* NiaD300 | | 41 |
| Bacteria: *E. coli* NovaBlue | *endA1 hsdR17* ($r_{K12}^{-}$ $m_{K12}^{+}$) *supE44 thi-1 gyrA96 relA1 lac recA1/F′* [*proAB*$^{+}$ *lacI*$^{q}$*Z*ΔM15::Tn*10* (Tet$^{r}$)] | Novagen |
| Plasmids | | |
| pMUCS | *URA3* no display (control plasmid) | 13 |
| pWGP3 | *TRP1* no expression (control plasmid) | 36 |
| pCAS1-RGSHis6-XYNII | *TRP1* surface expression of *T. reesei* β-xylanase gene (XYNII) | 12 |
| pUCSxylAf | *URA3* surface expression of *A. oryzae* β-xylosidase gene (XylA) | This work |
| pUCSXIIXA | *URA3* surface coexpression of XYNII and XylA genes | This work |
| pWX1X2XK | *TRP1* coexpression of XYL1, XYL2, and XKS1 genes | This work |

techniques (22). Fujita et al. reported direct and efficient ethanol production from cellulosic materials through codisplay of cellulolytic enzymes (11, 13). In the present study, by codisplaying *T. reesei* XYNII and *A. oryzae* XylA on the cell surface of *S. cerevisiae*, we undertook the construction of a yeast strain which hydrolyzes birchwood xylan to xylose. Further, by introducing XYL1, XYL2, and XKS1 into a xylan-degrading yeast strain, we achieved simultaneous saccharification and fermentation of birchwood xylan to ethanol. To our knowledge, this is the first report of direct ethanol fermentation from xylan in a recombinant *S. cerevisiae* strain without the addition of xylanolytic enzymes.

## MATERIALS AND METHODS

**Microbial strains and media.** The characteristics of the microbial strains constructed and used in this study are summarized in Table 1. *Escherichia coli* NovaBlue (Novagen, Inc., Madison, Wis.) was used as the host strain for recombinant DNA manipulation. The yeast strain *S. cerevisiae* MT8-1 was used for cell surface display and fermentation (35).

*E. coli* was grown in Luria-Bertani medium (10 g of tryptone per liter, 5 g of yeast extract per liter, and 5 g of sodium chloride per liter) containing 100 μg of ampicillin per ml. Following precultivation, the yeast cells were aerobically cultivated at 30°C in synthetic medium (20 g of glucose per liter, 6.7 g of yeast nitrogen base without amino acids [Difco Laboratories, Detroit, Mich.] per liter, and appropriate supplements) containing 20 g of Casamino Acids (Difco) per liter (SDC medium).

**PCR amplification.** All PCRs were carried out with KOD-Plus-DNA polymerase (Toyobo Co. Ltd., Kyoto, Japan). Table 2 summarizes the primers used.

**Construction of plasmids for display of xylanolytic enzymes.** The characteristics of the plasmids constructed and used in this study are summarized in Table 1. For cell surface expression, genes of xylanolytic enzymes were fused with the 3′-end region of the α-agglutinin gene (the gene encoding the C-terminal 320 amino acid residues) (Fig. 1). Plasmid pCAS1-RGSHis6-XYNII, used for cell surface expression of the XYNII gene from *T. reesei* QM9414, was constructed as described previously (12).

Plasmid pUCSxylAf (Fig. 1A), used for cell surface expression of the XylA gene from *A. oryzae* NiaD300, was constructed as follows. Chromosomal DNA was prepared from *A. oryzae* NiaD300 by a previously described method with some modifications (41). The 2.3-kbp BglII-XhoI DNA fragment carrying the XylA gene without its native signal sequence fused to the FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) peptide tag at the C terminus was prepared by PCR with genomic DNA prepared from *A. oryzae* as the template. This DNA fragment was digested with BglII and XhoI and introduced into the BglII-XhoI site of plasmid pMUCS (13). The resulting plasmid was designated pUCSxylAf.

Plasmid pUCSXIIXA (Fig. 1B), used for cell surface coexpression of the XYNII and XylA genes, was constructed as follows. The 2.24-kbp AatII-AatII DNA fragment containing a portion of 2μm DNA was prepared by PCR with plasmid pWI3 (20) as the template. This DNA fragment was digested with AatII and introduced into the AatII section of plasmid pRS406 (Stratagene). The resulting plasmid was designated pUM. A 3.2-kbp SalI-SalI DNA fragment (fragment A)—composed of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter from *S. cerevisiae*, the secretion signal sequence of the glucoamylase gene from *Rhizopus oryzae*, the XYNII gene fused to the $RGSHis_6$ (Arg-Gly-Ser-His-His-His-His-His-His) peptide tag at the N terminus, and the 3′-end region of the α-agglutinin gene with 446 bp of the 3′-flanking region—was prepared by PCR with plasmid pCAS1-RGSHis6-XYNII as the template and digested with SalI. A 4.9-kbp NotI-NotI DNA fragment (fragment B)—composed of the GAPDH promoter, the secretion signal sequence of the glucoamylase gene from *R. oryzae*, the XylA gene fused to the FLAG peptide tag at the C terminus, and the 3′-end region of the α-agglutinin gene with 446 bp of the 3′-flanking region—was prepared by PCR with plasmid pUCSxylAf as the template and digested with NotI. These DNA fragments, A and B, were introduced into the SalI and NotI sites of plasmid pUM, respectively. The resulting plasmid was designated pUCSXIIXA.

**Construction of plasmids for xylose utilization.** Plasmid pWX1X2XK (Fig. 1C), used for intracellular coexpression of the XYL1, XYL2, and XKS1 genes, was constructed as follows. Multicopy plasmid pRS405 + 2 was constructed by introducing the blunted 2.24-kbp EcoRI-EcoRI DNA fragment of plasmid pWI3 containing a portion of 2μm DNA into the AatII site of plasmid pRS405 (Stratagene). A blunted 1.3-kbp HindIII-HindIII DNA fragment containing the

TABLE 2. PCR primers used

| Gene or fragment | Primers (restriction endonuclease) | Sequence[a] | Source of DNA sequence |
|---|---|---|---|
| *A. oryzae* XylA | XylAF (BglII)<br>XylAR (XhoI) | 5′-CCGGAGATCTCCCAAGCAAACCAAAGCTACGTCGACTACAT-3′<br>5′-TAGACTCGAGAACTTGTCATCGTCATCCTTGTAGTCCTGCGGCGCAATCAACT-3′ | AB013851[b] |
| 2μm DNA | 2μmF (AatII)<br>2μmR (AatII) | 5′-CCCGACGTCCACGGACTATAGACTATACTA-3′<br>5′-CCTGACGTCTGAACCAGTCCTAAAACGAGT-3′ | pW13 |
| XYNII expression cassette | XYNIIcF (SalI)<br>XYNIIcR (SalI) | 5′-ACGCGTCGACACCAGTTCTCACACGGAACACCACTAATGGACACAAATTC-3′<br>5′-ACGCGTCGACTTTGATTATGTTCTTTCTATTTGAATGAGATATGAGAGAG-3′ | pCAS1-RGSHis$_6$-XYNII |
| XylA expression cassette | XylAcF (NotI)<br>XylAcR (NotI) | 5′-ATAAGAATGCGGCCGCACCAGTTCTCACACGGAACACCACTAATGGACAC-3′<br>5′-ATAAGAATGCGGCCGCTTTGATTATGTTCTTTCTATTTGAATGAGATATG-3′ | pUCSxylAf |
| *P. stipitis* XYL1 | XYL1F (SacI)<br>XYL1R (BamHI) | 5′-CTATGAGCTCATGCCTTCTATTAAGTTGAACTCTGG-3′<br>5′-AGCAGGATCCTTAGACGAAGATAGTAATCTTGTCCC-3′ | X59465[b] |
| *P. stipitis* XYL2 | XYL2F (SacI)<br>XYL2R (BamHI) | 5′-ACCAGAGCTCATGACTGACTTAACTACACAAGAAGC-3′<br>5′-TTTCGGATCCTCATTCCGGGCCCTCAATGA-3′ | X55392[b] |
| *S. cerevisiae* XKS1 | XKS1F (BamHI)<br>XKS1R (SalI) | 5′-TAGTGGATCCATGTTGTGTTCAGTAATTCAGAGACAGAC-3′<br>5′-CAAAGTCGACTTAGATGAGAGTCTTTTCCAGTTCGC-3′ | X61377[b] |
| XYL1 expression cassette | XYL1cF (KpnI)<br>XYL1cR (XhoI) | 5′-CGGGGTACCACCAGTTCTCACACGGAACACCACTAATGGA-3′<br>5′-CCGCTCGAGTCAATCAATGAATCGAAAATGTCATTAAAAT-3′ | pWPXYL1 |
| XYL2 expression cassette | XYL2cF (XhoI)<br>XYL2cR (NotI) | 5′-CCGCTCGAGACCAGTTCTCACACGGAACACCACTAATGGA-3′<br>5′-ATAGTTTAGCGGCCGCTCAATCAATGAATCGAAAATGTCATTAAA-3′ | pUPXYL2 |
| XKS1 expression cassette | XKS1cF (NotI)<br>XKS1cR (SacII) | 5′-ATAAGAATGCGGCCGCACCAGTTCTCACACGGAACACCACTAATGGACAC-3′<br>5′-TCCCCGCGGTCAATCAATGAATCGAAAATGTCATTAAAAT-3′ | pLXKS1 |

[a] Restriction enzyme sites are underlined.
[b] GenBank accession number.

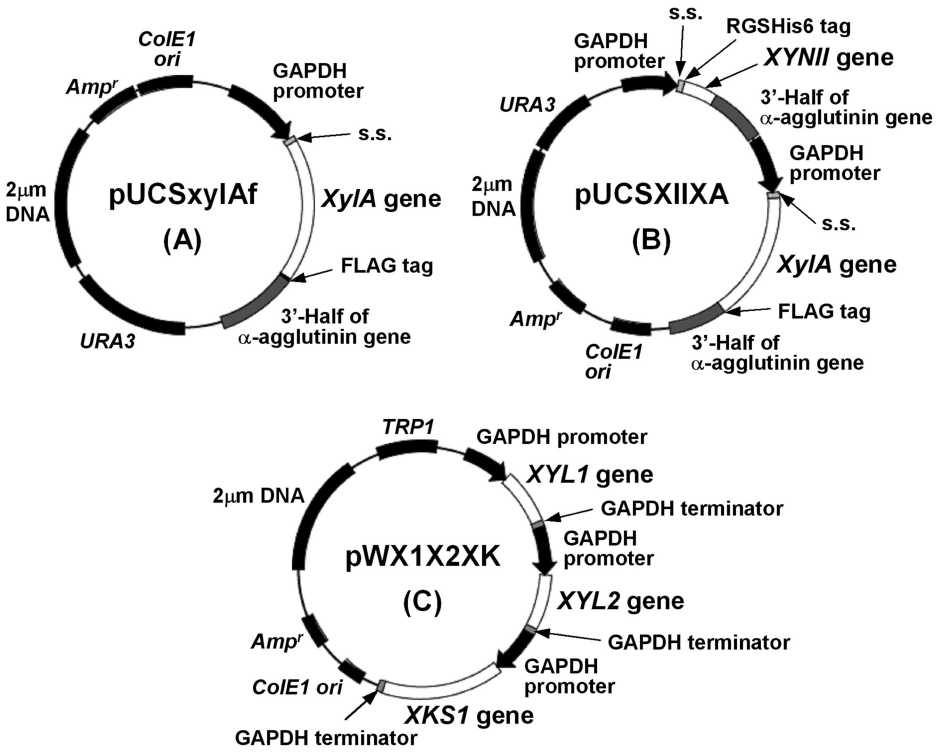


FIG. 1. Schematic maps of plasmids for yeast cell surface display of XylA–FLAG–α-agglutinin fusion protein (pUCSxylAf) (A), codisplay of $RGSHis_6$–XYNII–α-agglutinin and XylA–FLAG–α-agglutinin fusion proteins (pUCSXIIXA) (B), and intracellular coproduction of XYL1, XYL2, and XKS1(pWX1X2XK) (C). s.s., secretion signal sequence of the *R. oryzae* glucoamylase gene.

GAPDH promoter and the GAPDH terminator from plasmid pYE22m (34) was introduced into the PvuII site of plasmid pRS405 + 2, and the resulting plasmid was designated pLGP3. A 0.95-kbp SacI-BamHI DNA fragment containing the *P. stipitis* XYL1 gene, a 1.09-kbp SacI-BamHI DNA fragment containing the *P. stipitis* XYL2 gene, and a 1.8-kbp BamHI-SalI DNA fragment containing the *S. cerevisiae* XKS1 gene were prepared by PCR with genomic DNAs from *P. stipitis* and *S. cerevisiae* as the templates. These DNA fragments were introduced into the SacI-BamHI section of plasmids pWGP3 (36) and pUGP3 (36) and the BamHI-SalI section of plasmid pLGP3, respectively. The resulting plasmids were designated pWPXYL1, pUPXYL2, and pLXKS1. A 2.24-kbp AatII-Aat II DNA fragment containing a portion of 2μm DNA was prepared by PCR with plasmid pWI3 as the template and introduced into the AatII site of plasmid pRS404 (Stratagene), and the resulting plasmid was designated pWM. The following fragments were also prepared by PCR: a 2.2-kbp KpnI-XhoI DNA fragment (fragment C), composed of the GAPDH promoter, the XYL1 gene, and the GAPDH terminator, with plasmid pWPXYL1 as the template; a 2.3-kbp XhoI-NotI DNA fragment (fragment D), composed of the GAPDH promoter, the XYL2 gene, and the GAPDH terminator, with plasmid pUPXYL2 as the template; and a 3.04-kbp NotI-SacII DNA fragment (fragment E), composed of the GAPDH promoter, the XKS1 gene, and the GAPDH terminator, with plasmid pLXKS1 as the template. These DNA fragments were introduced into the KpnI-XhoI section, the XhoI-NotI section, and the NotI-SacII section of plasmid pWM, respectively, and the resulting plasmid was designated pWX1X2XK.

**Yeast transformation.** Transformation of plasmids pCAS1-RGSHis6-XYNII, pUCSxylAf, pMUCS, pUCSXIIXA, pWGP3, and pWX1X2XK into *S. cerevisiae* MT8-1 was carried out by the lithium acetate method with a YEASTMAKER yeast transformation system (Clontech Laboratories, Inc., Palo Alto, Calif.).

**Immunofluorescence microscopy.** Immunofluorescence microscopy was performed as described previously (13). As the primary antibodies, mouse immunoglobulin G (IgG) against $RGSHis_4$ (Qiagen, Valencia, Calif.) and rabbit IgG against the FLAG peptide tag (Sigma Chemical Co., St. Louis, Mo.) were used simultaneously at a dilution rate of 1:500. As the secondary antibodies, goat anti-mouse IgG (heavy and light chains) conjugated with Alexa Fluor 488 and goat anti-rabbit IgG (heavy and light chains) conjugated with Alexa Fluor 546 (Molecular Probes, Inc., Eugene, Oreg.) were used at a dilution rate of 1:300.

**Enzyme assays.** Xylanase activity was assayed by a previously described method (12). After cultivation in SDC medium for 48 h at 30°C, the cells were collected by centrifugation. The cell pellets were resuspended in a reaction mixture, and the optical density at 600 nm ($OD_{600}$) was adjusted to 5. After the reaction, the supernatants were used for measurement of the amount of reducing sugar and for high-performance liquid chromatography (HPLC) analysis of hydrolysis products as described below. The amount of reducing sugar released from the substrate was measured by determining the number of xylose equivalents by the Somogyi-Nelson method (43).

β-Xylosidase activity was measured by a previously described method (33) with 50 mM sodium acetate buffer (pH 5.0) and 10 mM *p*-nitrophenyl-β-D-xylopyranoside (Nacalai Tesque, Inc., Kyoto, Japan) as the substrate at 30°C. The cell density in the reaction medium was adjusted to an $OD_{600}$ of 0.5. After the reaction, the *p*-nitrophenol released was measured by determining the absorbance at 400 nm. All activities were expressed such that 1 U of enzyme activity was defined as the amount of enzyme that released 1 μmol of the product from the substrate per min.

**Analysis of hydrolysis products.** HPLC analysis of hydrolysis products was performed by using a refractive index detector (model RID-10A; Shimadzu, Kyoto, Japan). The column used for separation was a Shim-pack SPR-Pb column (Shimadzu). The HPLC apparatus was operated at 80°C with water at a flow rate of 0.4 ml/min as the mobile phase.

**Fermentation.** The xylan-utilizing yeast strain (MT8-1/pUCSXIIXA/pWX1X2XK) and the control strain (MT8-1/pMUCS/pWGP3) were aerobically cultivated in SDC medium for 72 h at 30°C. The cells were collected by centrifugation for 10 min at 6,000 × *g* and 4°C and washed with distilled water three times. These strains were inoculated into 50 ml of fermentation medium, containing 6.7 g of yeast nitrogen base without amino acids per liter, 20 g of Casamino Acids per liter, and birchwood xylan corresponding to 100 g of total sugar per liter as the sole carbon source. Ethanol fermentation was carried out in 100-ml closed bottles equipped with a bubbling $CO_2$ outlet. The cell density in fermentation medium was adjusted to an $OD_{600}$ of 50. All fermentations were performed at 30°C with mild agitation at 100 rpm. Ethanol and total sugar were

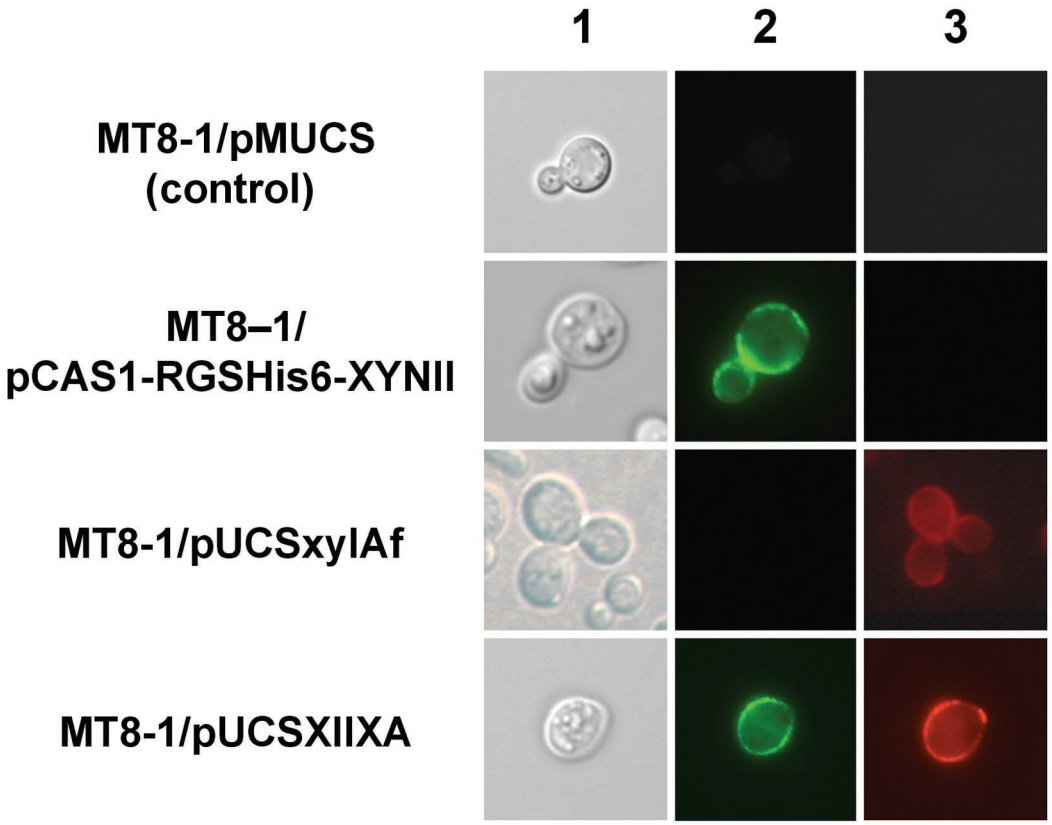


FIG. 2. Immunofluorescence labeling of transformants. Images show Nomarski differential interference micrographs (column 1) and immunofluorescence micrographs (columns 2 and 3) of *S. cerevisiae* MT8-1/pMUCS (control), MT8-1/pCAS1-RGSHis6-XYNII, MT8-1/pUCSxylAf, and MT8-1/pUCSXIIXA. Cells were labeled with mouse anti-$RGSHis_4$ antibody and goat anti-mouse IgG conjugated with Alexa Fluor 488 (column 2) and with rabbit anti-FLAG antibody and goat anti-rabbit IgG conjugated with Alexa Fluor 546 (column 3).

measured by using an Ethanol F kit (Roche, R-Biopharm GmbH, Darmstadt, Germany) and the phenol-sulfuric acid method (9).

## RESULTS

**Codisplay of XYNII and XylA.** In the recombinant yeast strains MT8-1/pCAS1-RGSHis6-XYNII, MT8-1/pUCSxylAf, and MT8-1/pUCSXIIXA, constructed by introducing plasmids pCAS1-RGSHis6-XYNII for display of XYNII, pUCSxylAf for display of XylA, and pUCSXIIXA for codisplay of XYNII and XylA, respectively (Table 1), the $RGSHis_6$–XYNII–α-agglutinin fusion gene and the XylA–FLAG–α-agglutinin fusion gene were expressed or coexpressed under the control of the GAPDH promoter.

To confirm the presence of XYNII and XylA on the yeast cell surface, immunofluorescence labeling of the cells was performed with mouse anti-$RGSHis_4$ IgG and rabbit anti-FLAG IgG as the primary antibodies and goat anti-mouse IgG conjugated with Alexa Fluor 488 and goat anti-rabbit IgG conjugated with Alexa Fluor 546 as the secondary antibodies. Cells were aerobically cultivated in SDC medium at 30°C for 72 h. As shown in Fig. 2, when strains MT8-1/pCAS1-RGSHis6-XYNII and MT8-1/pUCSXIIXA were labeled with goat anti-mouse IgG conjugated with Alexa Fluor 488, green fluorescence was observed at the cell outline; when strains MT8-1/pUCSxylAf and MT8-1/pUCSXIIXA were labeled with goat anti-rabbit IgG conjugated with Alexa Fluor 546, red fluorescence was observed at the cell outline. These results confirm that the $RGSHis_6$–XYNII–α-agglutinin and the XylA–FLAG–α-agglutinin fusion proteins were codisplayed on the cell surface of strain MT8-1/pUCSXIIXA.

**Enzyme activities.** The XYNII and XylA activities of the yeast strains displaying XYNII and XylA were examined with both culture supernatant and cell pellet fractions (Table 3). XYNII activity was determined with birchwood xylan as a substrate. As shown in Table 3, the strain displaying XYNII (MT8-1/pCAS1-RGSHis6-XYNII) and the strain codisplaying XYNII and XylA (MT8-1/pUCSXIIXA) showed similar XYNII

TABLE 3. Distribution of enzyme activities

| Strain | Activity (U/g [dry wt] of cells) of the indicated enzyme in[a]: | | | |
|---|---|---|---|---|
| | Culture supernatant | | Cell pellet | |
| | XYNII | XylA | XYNII | XylA |
| MT8-1/pMUCS | ND | ND | ND | ND |
| MT8-1/pCAS1-RGSHis6-XYNII | ND | ND | 15.4 | ND |
| MT8-1/pUCSxylAf | ND | ND | ND | 316 |
| MT8-1/pUCSXIIXA | ND | ND | 16.0 | 234 |

[a] Values are averages of three independent experiments. ND, not detected.

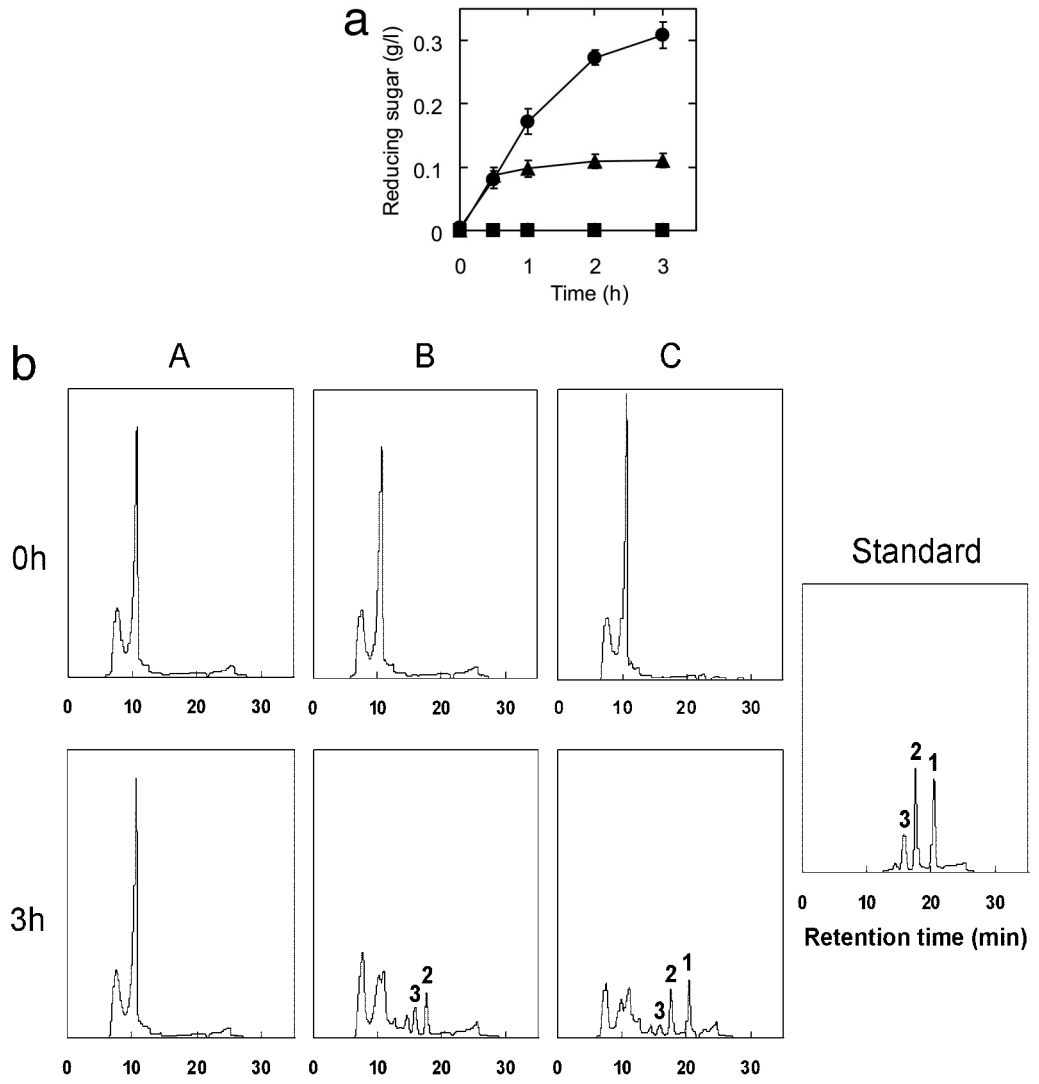


FIG. 3. (a) Time course of hydrolysis of birchwood xylan by cell surface-engineered yeast strains at 30°C. Symbols: squares, MT8-1/pMUCS (control); triangles, MT8-1/pCAS1-RGSHis6-XYNII; circles, MT8-1/pUCSXIIXA. The data points represent the means and standard deviations of three independent experiments. (b) HPLC analysis of hydrolysis products released from birchwood xylan after a 3-h reaction. A, MT8-1/pMUCS (control); B, MT8-1/pCAS1-RGSHis6-XYNII; C, MT8-1/pUCSXIIXA. 1, xylose; 2, xylobiose; 3, xylotriose.

activities (15.4 and 16.0 U per g [dry weight] of cells, respectively), while no activity was detected in strains MT8-1/pMUCS (control strain) and MT8-1/pUCSxylAf. No XYNII activity was detected in culture supernatants for any strain.

XylA activity was determined with *p*-nitrophenyl-β-D-xylopyranoside as the substrate. As shown in Table 3, the strain displaying XylA (MT8-1/pUCSxylAf) and the strain codisplaying XYNII and XylA (MT8-1/pUCSXIIXA) showed XylA activities of 316 and 234 U per g (dry weight) of cells, respectively, while no activity was detected in strains MT8-1/pMUCS and MT8-1/pCAS1-RGSHis6-XYNII. XylA activity was not detected in culture supernatants for any strain.

The above results demonstrate that active XYNII and XylA are successfully displayed on the yeast cell surface without leakage into the culture medium.

**Hydrolysis of xylan and analysis of hydrolysis products.** Figure 3a shows the time course of the hydrolysis of birchwood xylan by cell surface-engineered yeast strains at 30°C. A larger amount of reducing sugar was released by strain MT8-1/pUCSXIIXA (0.31 g per liter) than by strain MT8-1/pCAS1-RGSHis6-XYNII (0.11 g per liter) in a 3-h reaction. To analyze the hydrolysis products released by the two yeast strains, HPLC analysis was performed. As shown in Fig. 3b, xylobiose and xylotriose were detected as the main products of strain MT8-1/pCAS1-RGSHis6-XYNII, but xylose was detected as the main product of strain MT8-1/pUCSXIIXA. No hydrolysate was detected in the reaction mixture containing the control cells.

**Ethanol production from birchwood xylan.** For direct fermentation of birchwood xylan to ethanol, a xylan-utilizing yeast strain was constructed by introducing plasmid pWX1X2XK—which, for the purpose of xylose utilization, intracellularly coexpresses the XYL1, XYL2, and XKS1 genes under the control of the GAPDH promoter—into the xylan-degrading yeast

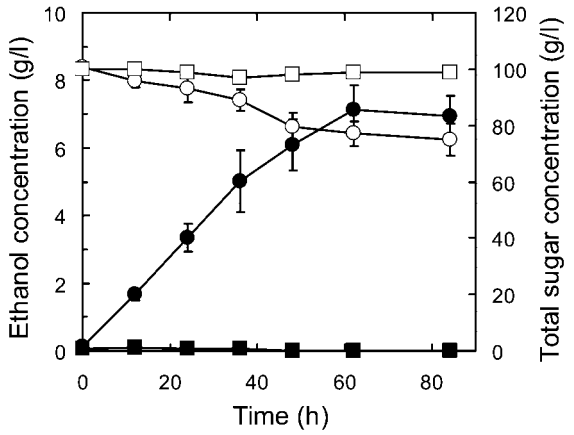


FIG. 4. Time course of production of ethanol from birchwood xylan as the sole carbon source by MT8-1/pMUCS/pWGP3 (control) (square) and MT8-1/pUCSXIIXA/pWX1X2XK (circle). Open and closed symbols represent the total sugar and ethanol concentrations, respectively. The data points represent the means and standard deviations of three independent experiments.

strain MT8-1/pUCSXIIXA. With the resulting xylan-utilizing yeast strain, MT8-1/pUCSXIIXA/pWX1X2XK, direct ethanol fermentation from xylan was performed. The ethanol concentration increased as the total sugar concentration decreased. The highest ethanol concentration was approximately 7.1 g per liter after 62 h of fermentation (Fig. 4), while ethanol was not detected in the medium of strain MT8-1/pMUCS/pWGP3, inoculated as a control. The ethanol productivity was 0.13 g per liter per h, and the ethanol yield was 0.30 g per g of sugar consumed.

## DISCUSSION

To convert xylan to ethanol through simultaneous saccharification and fermentation, it is first necessary to degrade xylan to xylose. We used a cell surface engineering system based on the C-terminal region of α-agglutinin to construct a xylan-degrading *S. cerevisiae* strain, MT8-1/pUCSXIIXA, that codisplays active XYNII and XylA on the cell surface. The activities of XYNII and XylA of strain MT8-1/pUCSXIIXA were 104 and 74% those of strains MT8-1/pCAS1-RGSHis6-XYNII and MT8-1/pUCSxylAf, respectively; hence, the sum of the numbers of XYNII and XylA molecules in the codisplay strain is larger than the sum of the numbers of XYNII or XylA molecules in a single-display strain (Table 3). These results are consistent with previous observations (11, 13) and demonstrate that active XYNII and XylA were successfully codisplayed on yeast strain MT8-1/pUCSXIIXA.

As shown in Fig. 3a, the main product of strain MT8-1/pUCSXIIXA in xylan hydrolysis experiments was xylose (Fig. 3b), indicating that XYNII and XylA codisplayed on the cell surface sequentially hydrolyzed birchwood xylan to xylose. This phenomenon is similar to that observed for the degradation of xylan by recombinant (24) and native (4, 7, 8) xylanolytic enzymes. As the enzymes were localized on the cell surface, the resulting whole-cell biocatalyst was able to directly hydrolyze a polymeric substrate such as xylan.

Since *S. cerevisiae* is not able to metabolize xylose, xylose-utilizing yeast strains have been developed by intracellularly overproducing XYL1, XYL2, and XKS1 (10, 15, 18, 19). Similarly, in the present study, we constructed a xylan-fermenting yeast strain by introducing XYL1, XYL2, and XKS1 into an *S. cerevisiae* strain codisplaying XYNII and XylA. The resulting recombinant *S. cerevisiae* strain hydrolyzed xylan to xylose and then converted the xylose to ethanol (Fig. 4). The ethanol production and consumption of xylan began without a time lag. Moreover, the ethanol yield produced by strain MT8-1/pUCSXIIXA/pWX1X2XK from birchwood xylan (0.30 g per g) was similar to that from xylose in the present study (0.29 g per g) (data not shown) and in previous studies (15, 18).

The ethanol concentration, however, reached a plateau after 62 h of fermentation (Fig. 4), while unused xylan remained. Since xylose was not observed in the medium during fermentation (data not shown), this result is most likely attributable to the inability of strain MT8-1/pUCSXIIXA/pWX1X2XK to further degrade xylan. This notion is consistent with the results shown in Fig. 3a. That is, the concentration of reducing sugar released by strain MT8-1/pUCSXIIXA was limited to approximately 30% the birchwood xylan concentration in the reaction mixture. For more complete xylan saccharification, improvement of the activities of displayed enzymes and/or codisplay of other critical xylanolytic enzymes are probably required.

The above results demonstrate that efficient direct conversion of xylan to ethanol can be accomplished by codisplaying XYNII and XylA on xylose-utilizing *S. cerevisiae* cells. The combination of a cell surface display system and additional metabolic functions is thus effective in the construction of yeast cells capable of multistep bioconversion. While further studies are required to improve the xylan-degrading and xylose-utilizing abilities of the recombinant *S. cerevisiae* strain, the findings of the present study could serve as the basis for the direct production of ethanol from hemicelluloses.

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