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Cell surface engineering of *Saccharomyces cerevisiae* combined with membrane separation technology for xylitol production from rice straw hydrolysate

Gregory Guirimand[1] · Kengo Sasaki[1] · Kentaro Inokuma[1] · Takahiro Bamba[2] · Tomohisa Hasunuma[1] · Akihiko Kondo[2,3]

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Abstract Xylitol, a value-added polyol deriving from D-xylose, is widely used in both the food and pharmaceutical industries. Despite extensive studies aiming to streamline the production of xylitol, the manufacturing cost of this product remains high while demand is constantly growing worldwide. Biotechnological production of xylitol from lignocellulosic waste may constitute an advantageous and sustainable option to address this issue. However, to date, there have been few reports of biomass conversion to xylitol. In the present study, xylitol was directly produced from rice straw hydrolysate using a recombinant *Saccharomyces cerevisiae* YPH499 strain expressing cytosolic xylose reductase (XR), along with β-glucosidase (BGL), xylosidase (XYL), and xylanase (XYN) enzymes (co-)displayed on the cell surface; xylitol production by this strain did not require addition of any commercial enzymes. All of these enzymes contributed to the consolidated bioprocessing (CBP) of the lignocellulosic hydrolysate to xylitol to produce 5.8 g/L xylitol with 79.5 % of theoretical yield from xylose contained in the biomass. Furthermore, nanofiltration of the rice straw hydrolysate provided removal of fermentation inhibitors while simultaneously increasing sugar concentrations, facilitating high concentration xylitol production (37.9 g/L) in the CBP. This study is the first report (to our knowledge) of the combination of cell surface engineering approach and membrane separation technology for xylitol production, which could be extended to further industrial applications.

Keywords Xylitol · Hemicellulose · Consolidated bioprocessing (CBP) · Fermentation · Cell surface display · Membrane filtration · Yeast

Introduction

Xylitol is a five-carbon polyol widely used as sugar substitute in food industry because of its non-cariogenic nature and low calorie content (Albuquerque et al. 2014). Xylitol also is a value-added biochemical used as the starting material for the production of many chemicals in the pharmaceutical industry (Werpy and Petersen 2004). Industrial production of xylitol from purified D-xylose involves a costly catalytic hydrogenation process requiring high-temperature (80–140 °C) and high-pressure (up to 50 atm) treatment (Chen et al. 2010). D-xylose is the secondmost abundant sugar in nature; in that context, alternative methods of production of xylitol have been extensively studied in order to both reduce the cost of production and utilize renewable biomass as feedstock (Roberto et al. 1996; Rodrigues et al. 2006; Rafiqul et al. 2015). In particular, biotechnological processes may facilitate reduced processing costs by allowing the use of agro-industrial wastes (such as rice straw) as foodstuffs for the growth of microbes capable of converting D-xylose to xylitol.

Among the microorganisms used for xylitol production, baker's yeast (*Saccharomyces cerevisiae*) is a good host for

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✉ Akihiko Kondo
akondo@kobe-u.ac.jp

[1] Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

[2] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

[3] Biomass Engineering Program, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

bioengineering, since this microbe is generally recognized as safe (GRAS), and its genome has been entirely sequenced. Although native strains of *Saccharomyces cerevisiae* cannot utilize xylose as a carbon source, it has been reported that the heterologous expression of xylose reductase (XR) in *Saccharomyces cerevisiae* enables xylose assimilation and xylitol production (Amore et al. 1991). In order to separate the different constituents contained in biomass such as cellulose and hemicellulose, several pretreatment processes have been developed, among which hydrothermal pretreatment is promising, as this process uses only hot, pressurized water (Alvira et al. 2010). However, hydrothermal pretreatment of rice straw generates mainly oligomeric forms of hemicellulosic materials (Sasaki et al. 2014; Hasunuma et al. 2014). which are not directly utilizable by *Saccharomyces cerevisiae*. Addition of costly commercial enzymes therefore is needed to degrade these oligomers of hemicellulose. Recently, cell surface engineering approaches have emerged and have been used extensively in the biorefinery industry as an advantageous way to obviate the addition of commercial enzymes (Sakamoto et al. 2012; Hasunuma and Kondo 2012; Inokuma et al. 2014; Hasunuma et al. 2014; Tanaka and Kondo 2015; Hasunuma et al. 2015). This kind of technology consists of anchoring different enzymes (e.g., those capable of degrading biomass) to the cell surface (cell wall). Cell surface engineering constitutes a cost-effective solution eliminating the need for addition of extraneous enzyme; additionally, the displayed enzymes can be re-used in repeated batch processes (Matano et al. 2013). Moreover, a consolidated bioprocessing (CBP) strategy, which integrates all biological steps such as enzyme production, saccharification, and fermentation into a single step, constitutes a promising approach to reducing costs of xylitol production (Lynd et al. 2005; Hasunuma and Kondo 2012; Yamada et al. 2013).

Industrial production of xylitol from hemicellulosic materials derived from biomass requires significant increase of the xylitol concentration after the fermentation process in order to decrease energy consumption necessitated by downstream purification processes. Therefore, a high concentration of sugars in the fermentation medium is required. However, sugars derived from hemicellulose are present at low concentration in the rice straw hydrolysate obtained after pretreatment (Qi et al. 2012). To overcome this issue, low energy consumption membrane technologies have been developed in order to remove fermentation inhibitors while simultaneously increasing sugar concentrations within the rice straw hydrolysate (He et al. 2012; Sasaki et al. 2014; Sasaki et al. 2015a, 2015b; Maiti et al. 2012; Qi et al. 2012; Weng et al. 2010).

In the present study, a xylose-utilizing strain of *Saccharomyces cerevisiae* was generated by stable integration of the *Scheffersomyces stipitis* xylose reductase (XR)–encoding gene into the genome of the YPH499 strain of *Saccharomyces cerevisiae* (yielding strain YPH499-PIU-XR). This strain then was used as a platform strain, with subsequent transformation with different combinations of integrative plasmids encoding cell surface displayed versions of *Aspergillus aculeatus* β-glucosidase 1 (BGL), *Aspergillus oryzae* β-xylosidase A (XYL), and *Trichoderma reesei* endoxylanase II (XYN). Five different cell-surface-engineered strains of *Saccharomyces cerevisiae* obtained in this study (YPH499-PIU-XR-BGL, YPH499-PIU-XR-XYL, YPH499-PIU-XR-BGL-XYL, YPH499-PIU-XR-XYL-XYN, and YPH499-PIU-XR-BGL-XYL-XYN) were able to directly degrade the hemicellulose contained in rice straw hydrolysate to produce xylitol. Performance in xylitol production was evaluated for each strain. Membrane separation of the rice straw hydrolysate permitted further improvement in xylitol production, with "filtered" hydrolysate providing another twofold increase of xylitol yield ($Y_{Xylitol/Xylose}$) compared to the results with "unfiltered" hydrolysate. Thus, the combination of cell surface engineering and membrane separation technology yielded enhanced xylitol production.

Materials and methods

Rice straw hydrolysate

Rice straw pretreated with liquid hot water (Sasaki et al., 2015a) at high temperature (160–240 °C) and high pressure (≤10 MPa) was purchased from Mitsubishi Heavy Industries, Ltd. (Tokyo, Japan). Such hydrothermal pretreatment generates both cellulosic and hemicellulosic materials. The liquid fraction (pH 4.4) was separated from the solid fraction by filtration through a mesh filter and (in some experiments) directly used as a source of hemicellulosic material; in other experiments, the liquid fraction was concentrated as previously described in Sasaki et al. (2015a). with minor modifications (see "Membrane separation" section below for additional details). The pHs of both types of liquid fractions were adjusted to 5.0 by addition of 10 N NaOH prior to fermentation.

Plasmids, strains, and media

Table 1 shows the genetic characteristics of all plasmids and strains used in this study. The host strain used for recombinant DNA manipulation was the strain NovaBlue of *Escherichia coli* (Novagen, Madison, WI, USA). Genes encoding XR, BGL, XYL, and XYN were expressed in the *Saccharomyces cerevisiae* YPH499 strain (Sikorski and Hieter 1989). *E. coli* transformants were grown in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) supplemented with 100 μg/mL of ampicillin. Yeast transformants were screened on synthetic dextrose (SD) minimal medium plates (6.7 g/L yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI, USA), 20 g/L glucose, 20 g/L agar) supplemented with appropriate amino and nucleic acids.

Table 1 Integrative plasmids and strains used in this study

Plasmids	Relevant genotype	Source/references
pRS406	*URA3* empty vector	Agilent Technologies
pδUxyl1	*URA3 TPI1 P –S. stipitis XR–TDH3 T*, δ-integration	(Kato et al. 2013)
pIBG-TA	*HIS3 TDH3 P –A. aculeatus BGL1–SAG1 A –SAG1 T*	(Inokuma et al. 2014)
pIBG-SS	*HIS3 SED1 P –A. aculeatus BGL1–SED1 A –SAG1 T*	(Inokuma et al. 2014)
pδW-GPAGXynII	*TRP1 TDH3 P –T. reesei XynII–SAG1 A –SAG1 T*, δ-integration	(Sakamoto et al. 2012)
pIU-GPXR	*URA3 TDH3 P –A. aculeatus BGL1–TDH3 T*	This study
pIHXylA-SS	*HIS3 SED1 P –A. oryzae XylA–SED1 A –SAG1 T*	This study
pILYS2-XylA-SS	*LYS2 SED1 P –A. oryzae XylA–SED1 A –SAG1 T*	This study
Strains		
Bacterial strain *E. coli* NovaBlue	*endA1 hsdR17*(*rK12* − *mK12*+) *supE44 thi-I gyrA96 relA1 lac recA1*/*F'* [*proAB*+ *lacI q* ZΔM15::Tn*10* (Tetr)]	Novagen
S. cerevisiae yeast strains		
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Life Technologies
YPH499	*MATa ura3-52 lys2-801 ade2-101 trp1- Δ63 his3-Δ200 leu2-Δ1*	(Sikorski and Hieter, 1989)
YPH499-PIU	control strain (*URA3* empty vector)	This study
YPH499-PIU-XR	XR expressing strain	This study
YPH499-PIU-XR-BGL	XR and BGL co-expressing strain	This study
YPH499-PIU-XR-XYL	XR and XYL co-expressing strain	This study
YPH499-PIU-XR-BGL-XYL	XR, BGL and XYL co-expressing strain	This study
YPH499-PIU-XR-XYL-XYN	XR, XYL and XYN co-expressing strain	This study
YPH499-PIU-XR-BGL-XYL-XYN	XR, BGL, XYL and XYN co-expressing strain	This study

Selected yeast transformants then were aerobically cultured in 5 mL of SD medium for 24 h and inoculated into 350 mL of YPD medium (10 g/L yeast extract, 20 g/L Bacto-peptone (Difco Laboratories), 20 g/L glucose) for 48–72 h prior to enzyme activity assays and xylitol fermentation experiments.

Plasmid construction and yeast transformation

PCR primers used in this study are listed in Table 2. The integrative plasmid for expression of *Scheffersomyces stipitis* XR was constructed as follows: The DNA fragment corresponding to the *TDH3* promoter region was amplified from *Saccharomyces cerevisiae* BY4741 genomic DNA by PCR using the TDH3p-F and TDH3-R primers. Similarly, the DNA fragment containing *Scheffersomyces stipitis* XR and the *TDH3* terminator region was amplified from pδUxyl1 (Kato et al. 2013) by PCR with the XR-F and TDH3t-R primers. These fragments were cloned into a *Not*I-linearized pRS406 vector by using the In-Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA), yielding the pIU-GPXR plasmid. The plasmids pIBG-TA (Inokuma et al. 2014) and pδW-GPAGXynII (Sakamoto et al. 2012) were used for cell surface display of *A. aculeatus* β-glucosidase 1 (BGL) and *T. reesei* endoxylanase II (XYN), respectively. The integrative plasmid for cell surface display of *A. oryzae* β-xylosidase A (XYL) was constructed by modifying the plasmid pIBG-SS (Inokuma et al. 2014). Inverse PCR using the pIBG-F and pIBG-R primers was performed in order to remove the coding sequence of BGL from pIBG-SS. Likewise, the DNA fragment coding for XYL was amplified from *A. oryzae* RIB40 genomic DNA by PCR using the XylA-F and XylA-R primers. These fragments were connected by the In-Fusion method, and the resulting plasmid was designated as pIHXylA-SS. Subsequently, inverse PCR using the pIXylA and pIXylA-R primers was performed in order to remove the coding sequence of *HIS3* from pIHXylA-SS. In the same way, the coding sequence of *LYS2* was amplified from *Saccharomyces cerevisiae* BY4741 genomic DNA by PCR using the LYS2-F and LYS2-R primers. These fragments were assembled together by using the In-Fusion HD Cloning Kit, and the resulting plasmid was designated pILYS2-XylA-SS. The plasmids pIU-GPXR, pIBG-TA, and pILYS2-XylA-SS were linearized by using the *Bsm*I, *Nde*I, and *Bbv*CI restriction sites (respectively) to cleave within the respective *URA3*, *HIS3*, and *LYS2* selection markers. The linearized plasmids then were transformed into *Saccharomyces cerevisiae* YPH499 by the lithium acetate method (Chen et al. 1992). resulting in integration into the respective homologous regions of chromosomal DNA by homologous recombination. The plasmid pδW-GPAGXynII was transformed into *Saccharomyces cerevisiae* YPH499 by the multi-copy integration method (δ-integration), as previously described (Yamada et al. 2010).

Table 2 Primers used in this study

Primers	Sequence
TDH3p-F	tccactagttctagagcggccgcctattttcgaggaccttgtcaccttg
TDH3p-R	gaaggcatttttttgtttgtttatgtgtgtttattc
XR-F	aacaaaaaaatgccttctattaagttgaactctg
TDH3t-R	agctccaccgcggtggcggccgctcaatcaatgaatcgaaaatgtc
XylA-F	ctcgtttctgccgcggagatctccatggctcaagcaaaccaaagctacgtcgactacaacatcg
XylA-R	ggacagttgataatttgggcccgggcccgggctgcggcgcaatcaactgctcctcc
pIBG-F	agcagttgattgcgccgcagcccgggcccgggcccaaattatcaactgtcctattatct
pIBG-R	agtcgacgtagctttggtttgcttgagccatggagatctccgcggcagaaacgagcaaagaaaagtaaga
LYS2-F	ggcgtatcacgaggccctttcgtcatgactaacgaaaaggtctggatag
LYS2-R	gctgcagctttaaataatcggcaaccttaagctgctgcg
pIXylA-F	ccgcagcagcttaaggttgccgattatttaaagctgcagcatacg
piXylA-R	ctatccagacctttttcgttagtcatgacgaaagggcctcgtgatacg

Enzyme activity assays

Yeast cells were cultured in YPD medium for 48 h at 30 °C and collected by centrifugation for 5 min at 1000×*g* and 4 °C. The cells then were washed twice with distilled water, and β-glucosidase, β-xylosidase, and endoxylanase activities were determined at 35 °C using the washed cells, as previously described by Katahira et al. (2004). Briefly, BGL and XYL activities were measured using 10 mM *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside (Nacalai Tesque, Inc., Kyoto, Japan), respectively, in 50 mM sodium acetate buffer (pH 5.0). The cell density in the reaction medium was adjusted to 12.5 g/L. After the reaction, the *p*-nitrophenol released was measured by determining the absorbance at 400 nm. One unit of these enzyme activities was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute. For the XYN activity measurement, the cell density in the reaction medium was adjusted to 50 g/L and the cells were incubated in the presence of 10 g/L xylan in 50 mM sodium acetate buffer (pH 5.0). After the reaction, the amount of reducing sugar released from the substrate was measured by determining the number of xylose equivalents by the Somogyi–Nelson method (Wood and Bhat 1988). One unit of XYN activity was defined as the amount of enzyme required to liberate 1 μmol of the reducing sugar per minute. Dry cell weight of the yeast strains was estimated to be 0.15-fold that of the wet cell weight (Inokuma et al. 2014).

Cytosolic XR activity was measured at 35 °C by spectrophotometric monitoring of NADPH oxidation at 340 nm in a reaction mixture containing 100 mM sodium phosphate buffer (pH 7.0), 200 mM xylose, and 0.24 mM NADPH. Washed cells were mixed with glass beads (0.5-mm diameter) and then disrupted by shaking at 2500 rpm and 4 °C for 5 min with a Multi-beads shocker (Yasui Kikai Corporation, Osaka Japan). Protein concentrations of crude extracts of each yeast strains were determined with the Quick Start™ Bradford protein assay kit (Bio-Rad®, Hercules, CA, USA) using BSA as a standard, according to the manufacturer's instructions. One unit of XR activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol NADPH per minute.

Sugar and fermentation inhibitor analysis by GC-MS

The concentrations of xylose, arabinose, mannose, galactose, glucose, and xylitol in the fermentation media were measured using a GC-MS (QP2010Plus, Shimadzu, Kyoto, Japan) equipped with the 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) after derivatization. Ribitol (0.1 %) was used as an internal standard; 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, at which point the temperature was maintained for 6 min. The interface and the ion source temperatures were 250 and 200 °C, respectively. Derivatization was performed by using methoxyamine hydrochloride and *N*-methyl-*N*--(trimethylsilyl) trifluoroacetamide (MSTFA), as previously described by Pongsuwan et al. (2007).

Fermentation inhibitors, such as weak acids (acetate and formate) and furan compounds [furfural and 5-hydroxymethyl-2-furfural (5-HMF)] present in the rice straw hydrolysate before and after filtration were analyzed by GC-MS (QP2010Plus, Shimadzu). Weak acid concentrations were measured by using a DB-FFAP column (60 m × 0.25 mm i.d., 0.5-μm film thickness; Agilent, Palo Alto, CA, USA). 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, at which point the temperature was maintained for 6 min. Furan compounds were quantified with a CP-Sil 8-CB Low Bleed/MS column (30 m × 0.25 mm i.d., 0.25-μm film thickness; Varian, Inc.). 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, at which point the temperature was maintained for 5 min. The interface and the ion source temperatures were 250 and 230 °C, respectively. Ions were generated using a 70-V electron impact, and ion fragments were detected in the selected ion monitoring mode.

Total hydrolysis of hemicellulosic materials for sugar composition analysis

In order to fully hydrolyze the oligomeric sugars, diluted rice straw hydrolysates were incubated with a 10-g/L hemicellulase mixture (G-Amano, Amano Enzyme, Nagoya, Japan) at 37 °C for 24 h under constant gentle agitation. The activities of endoxylanase, β-xylosidase, and β-glucosidase in the hemicellulase mixture were 737.5, 20.3, and 69.1 U/g, respectively (Sakamoto et al. 2012). Sugar composition of rice straw hydrolysates then was determined by gas chromatography–mass spectrometry (GC-MS) analysis as described in the previous section.

Membrane separation

Membrane separation was conducted as previously described by Sasaki et al. (2015a). with minor modifications. RS50 polyvinylidene fluoride ultrafiltration (UF) membrane and ESNA3 polyamide nanofiltration (NF) membrane (molecular weight cutoff, 150,000 and 150 Da, respectively) were obtained from Nitto Denko Corporation (Osaka, Japan). The membranes were cut into 7.5-cm-diameter circles. RS50 was soaked in 50 % (*v*/*v*) ethanol solution for 15 min, then in deionized water for 15 min, and finally soaked overnight in deionized water before use.

Membrane separation was carried out at 25 °C using a flat membrane test cell (diameter 104 mm, height 147 mm, working volume 380 mL; model C40-B, Nitto Denko Corporation) on the liquid fraction of hydrothermally pretreated rice straw hydrolysate. The feed solution in the cell was stirred at 400 rpm. A pressure of 0.5 (for RS50) or 2.5 MPa (for ESNA3) was applied using nitrogen gas and a pressure control valve. The membrane separation process was as follows: UF (membrane, RS50) permeation at 0.5 MPa → NF (ESNA3) concentration at 2.5 MPa → 14 times dilution → NF (ESNA3) concentration at 2.5 MPa.

Fermentation of rice straw hydrolysate by cell-surface-engineered strains

Yeast cells were precultured in SD medium for 24 h prior to aerobically culturing for 48 h at 30 °C in YPD medium. The cells then were collected by centrifugation at 1000×*g* and 4 °C for 10 min and washed twice with distilled water. The washed cells then were inoculated into the fermentation medium [YP medium (10 g/L yeast extract, 20 g/L polypeptone) containing 70 % (*v*/*v*) rice straw hydrolysate] at an initial cell concentration of 50 g/L of wet cells. The rice straw hydrolysates used in this study were derived from two different lots and were used either diluted twofold ("50 %"; Fig. 2 and Supplementary Fig. S2), or undiluted ("100 %"; Fig. 3 and Supplementary Fig. S2). Xylitol fermentation was performed at 35 °C in 100-mL bottles equipped with an outlet for CO_2, under 150 rpm constant agitation and oxygen-limited conditions, for 96 h. Yeast cell density and pH were monitored spectrophotometrically (by measuring OD_{600}) and by pH meter, respectively, during the fermentations.

Results

Construction of yeast strains

A haploid yeast strain, *Saccharomyces cerevisiae* YPH499, was used as the host strain in this study. In a first step, a xylose-utilizing strain was generated by integration of *Scheffersomyces stipitis* XR–encoding sequences into the genome of YPH499, as this enzyme is indispensable for the utilization of xylose. The resulting YPH499-XR strain was used as a platform strain for the cell surface engineering work and subsequently was transformed with different combinations of integrative plasmids, as summarized in Table 1. The integrative plasmid used for cell surface display of *A. aculeatus* BGL expressed the enzyme as a fusion protein containing the secretion signal of *Rhizopus oryzae* glucoamylase (encoded by "*SS*") and the C-terminal half of *Saccharomyces cerevisiae* α-agglutinin (SAG1) as a GPI-anchoring region (encoded by "*3′ half AG anchor*"). This hybrid gene was flanked (and regulated) by the *Saccharomyces cerevisiae TDH3* promoter and the *SAG1* terminator (Fig. 1). The integrative plasmid used for cell surface display of *A. oryzae* XYL expressed the enzyme as a fusion protein containing the secretion signal of *R. oryzae* glucoamylase and the full-length SED1 as a GPI-anchoring region (encoded by "*SED1 anchor*"). This hybrid gene was flanked (and regulated) by the *Saccharomyces cerevisiae SED1* promoter and the *SAG1* terminator (Fig. 1). These plasmids both were transduced by single-copy integration into the yeast genome, while cell surface display of *T. reesei* endoxylanase (XYN) was achieved by multi-copy δ-integration. Exhaustive enzyme activity assays were performed for each strain and for each type of enzyme, in order to simultaneously measure the XR, BGL, XYL, and XYN activities (Table 3). The activity assays all were performed at the same temperature as used in the fermentation experiments (35 °C). For each type of enzyme, we observed similar levels of activities from one strain to another. The degradation ability of hemicellulosic materials

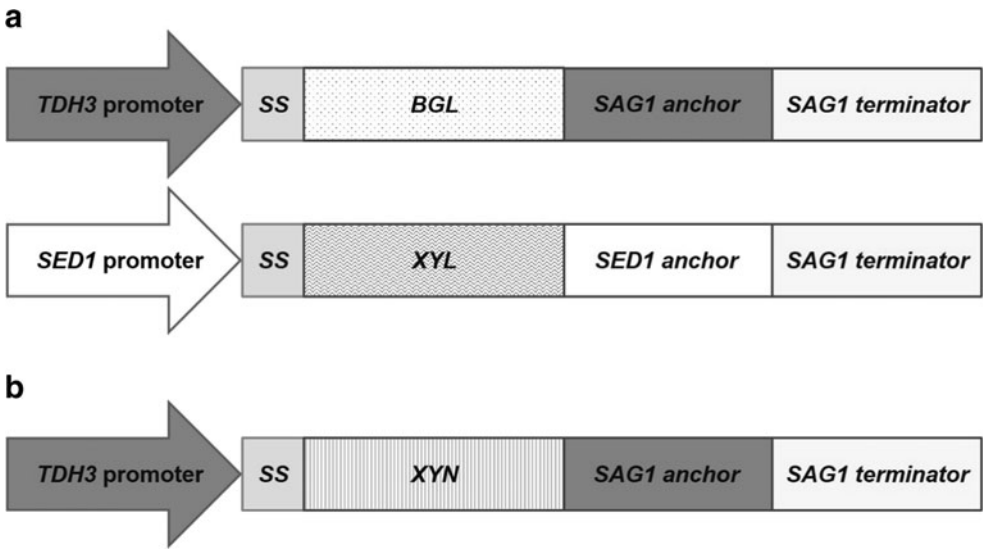

Fig. 1 Schematic view of gene cassettes used for cell surface engineering of the YPH499 strain of *S. cerevisiae*. **a** Gene cassettes cloned into integrative plasmids for stable integration of *BGL* and *XYL* expressed under the control of (respectively) the *TDH3* and *SED1* promoters, along with the *SAG1* terminator. **b** Gene cassette cloned into δ-integrative plasmid for δ-integration of *XYN* under the control of the *TDH3* promoter and the *SAG1* terminator. Cell surface display of each enzyme was ensured by the incorporation of gene sequences (*SS*) encoding the secretion signal of *R. oryzae* glucoamylase. The anchoring of BGL on the cell wall was ensured by the incorporation of gene sequences (*SAG1 anchor*) encoding the C-terminal 320 amino acids of the *S. cerevisiae* α-agglutinin. The anchoring of XYN on the cell wall was ensured by the incorporation of gene sequences (*SAG1 anchor*) encoding the complete *S. cerevisiae* SED1 protein

of the six different strains obtained in this study (Table 1) was compared to that of the control strain (YPH499-PIU) transformed by the *URA3* empty vector as negative control (Table 3 and Figs. 2 and 3 and Supplementary Figs. S1 and S2) in the following experiments.

Fermentation of rice straw hydrolysate

The amount of each constituent of the hydrothermally pretreated rice straw hydrolysate was measured by GC-MS after the hemicellulosic materials were subjected to total hydrolysis by incubating diluted samples with 10 g/L of hemicellulase mixture (G-Amano) at 37 °C for 24 h. After hydrolysis, the total sugar amount was 24.0 g/L, with concentrations of xylose and glucose achieving 12.4 and 10.1 g/L, respectively (Table 4). The rice straw hydrolysate also contained fermentation inhibitors generated by over-degradation that occurred during hydrothermal pretreatment; these fermentation inhibitors were acetate (22.35 mM), formate (20.07 mM), furfural (10.18 mM), and 5-HMF (2.16 mM). The strains constructed in this study were used for fermentations in which the hemicellulose provided by the rice straw hydrolysate was the sole source of carbon (Fig. 2). For each strain, carbohydrate consumption of xylose, glucose, and total sugar (Figs. 2a–c, respectively) was measured by GC-MS along with xylitol production (Fig. 2d).

Table 3 Enzyme activities measured at 35 °C

Enzyme activity				
Strains	Xylose Reductase (XR) (U/mg-protein)	β-D-Glucosidase (BGL) (U/dry cell (g))	β-D-Xylosidase (XYL) (U/dry cell (g))	Xylanase (XYN) (U/dry cell (g))
YPH499-PIU	ND	ND	ND	ND
YPH499-PIU-XR	1.03 ± 0.11	ND	ND	ND
YPH499-PIU-XR-BGL	1.07 ± 0.10	3.65 ± 0.13	ND	ND
YPH499-PIU-XR-XYL	0.97 ± 0.17	ND	6.13 ± 0.1	ND
YPH499-PIU-XR-BGL-XYL	1.06 ± 0.10	3.91 ± 0.11	6.41 ± 0.3	ND
YPH499-PIU-XR-XYL-XYN	1.01 ± 0.13	ND	5.99 ± 0.4	1.11 ± 0.21
YPH499-PIU-XR-BGL-XYL-XYN	0.95 ± 0.18	3.79 ± 0.10	6.22 ± 0.1	1.17 ± 0.10

Values represent averages ± standard deviation of the results from three independent experiments

ND not detected

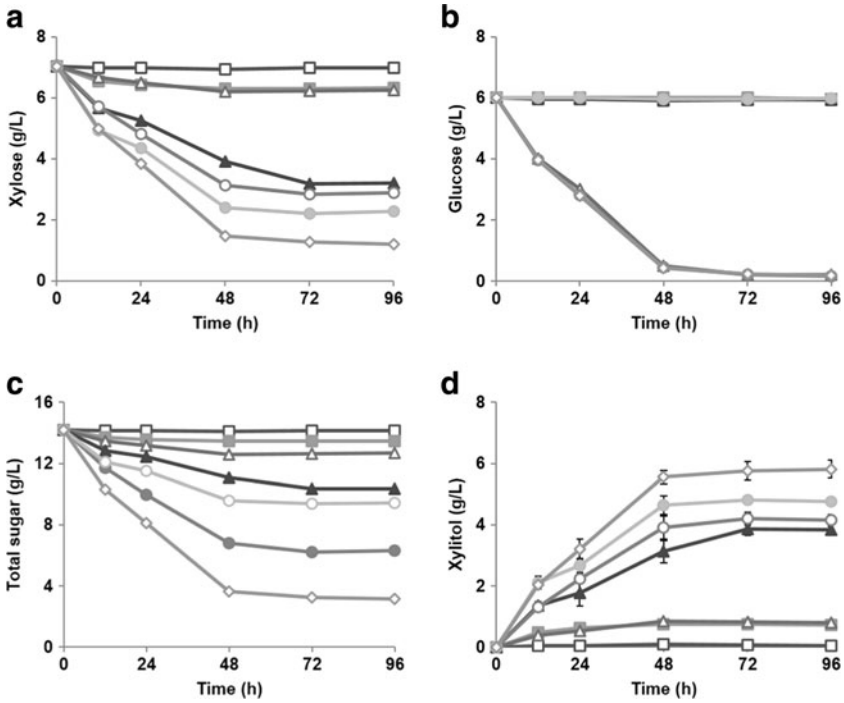

Fig. 2 Xylitol production during fermentation of 50 % rice straw hydrolysate by cell-surface-engineered derivatives of YPH499. Time course analysis of xylose, glucose, and total sugar consumption (**a**, **b**, and **c,** respectively) and xylitol production (**d**) by each strain was monitored by GC-MS analysis. *Error bars* indicate the standard deviations of three independent experiments. Symbols: *open square*, YPH499-PIU; *filled square*, YPH499-PIU-XR; *open triangle*, YPH499-PIU-XR-BGL; *filled triangle*, YPH499-PIU-XR-XYL; *open circle*, YPH499-PIU-XR-BGL-XYL; *filled circle*, YPH499-PIU-XR-XYL-XYN; *open diamond*, YPH499-PIU-XR-BGL-XYL-XYN

The YPH499-PIU strain was used as a negative control, given that YPH499-PIU (which lacks XR activity) is not able to utilize xylose. All the XR-expressing strains were able to utilize xylose. YPH499-PIU-XR and YPH499-PIU-XR-BGL produced only small amounts of xylitol and consumed only small amounts of xylose, consistent with the expected inability of these strains to degrade oligomers of xylose. YPH499-PIU-XR-XYL, YPH499-PIU-XR-BGL-XYL, YPH499-PIU-XR-XYL-XYN, and YPH499-PIU-XR-BGL-XYL-XYN efficiently degraded the hemicellulosic materials from rice straw hydrolysate. The best-performing strain was YPH499-PIU-XR-BGL-XYL-XYN, which produced 5.8 g/L of xylitol after 96 h of fermentation. Note that only the strains harboring BGL on their cell surface were able to degrade oligomers of glucose, which were almost completely consumed after 48 h, while no strain was able to completely utilize the xylose present in the medium, even after 96 h. Arabinose and galactose were not utilized by any of these strains (data not shown). The yeast cell concentrations and pHs remained essentially constant during all of the fermentations, regardless of the strain (data not shown). A comparative study of xylitol yields ($Y_{Xylitol/Xylose}$) after 96 h of fermentation (Supplementary Fig. S2a) clearly showed the benefit of multi-enzyme cell surface display for hemicellulose degradation ability of engineered strains, with a maximum $Y_{Xylitol/Xylose}$ = 0.79 for the strain YPH499-PIU-XR-BGL-XYL-XYN.

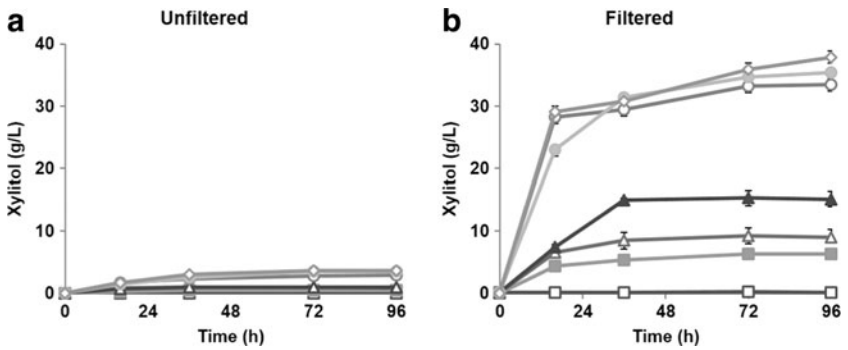

Fig. 3 Time course analysis of xylitol production (as monitored by GC-MS analysis) by each cell-surface-engineered derivative of YPH499 during fermentation of original ("unfiltered"; **a**) or membrane-separated ("filtered"; **b**) 100 % rice straw hydrolysate. *Error bars* indicate the standard deviations of three independent experiments. Symbols: *open square*, YPH499-PIU; *filled square*, YPH499-PIU-XR; *open triangle*, YPH499-PIU-XR-BGL; *filled triangle*, YPH499-PIU-XR-XYL; *open circle*, YPH499-PIU-XR-BGL-XYL; *filled circle*, YPH499-PIU-XR-XYL-XYN; *open diamond*, YPH499-PIU-XR-BGL-XYL-XYN

Table 4 Sugars and fermentation inhibitor concentrations before and after membrane separation (UF/NF)

	Sugars concentration (g/L)						Fermentation inhibitors concentration (mM)			
	Xylose	Arabinose	Mannose	Galactose	Glucose	Total sugars	Furfural	5-HMF	Formate	Acetate
Before UF/NF	12.4	1.0	ND	0.5	10.1	24.0	10.18	2.16	20.07	22.35
After UF/NF	59.3	4.9	ND	1.1	48.7	114.0	0.23	0.11	1.17	7.23

Values are averages of three independent experiments and relative standard deviations were less than 10 %

ND not detected

Concentration of sugars and removal of fermentation inhibitors by membrane separation

The liquid fraction of the hydrothermally pretreated rice straw contained fermentation inhibitors, potentially decreasing xylitol production by the engineered *Saccharomyces cerevisiae* strains (Table 4). In order to increase xylitol production during fermentation, membrane separation processes, including UF permeation and NF concentration, were performed on rice straw hydrolysate, with the intent of simultaneously removing fermentation inhibitors and concentrating sugars (Weng et al. 2010). As described previously (Sasaki et al., 2014; 2015a) we successfully increased concentrations of xylose (59.3 g/L) and glucose (48.7 g/L) and decreased concentrations of acetate (7.23 mM), formate (1.17 mM), furfural (0.23 mM), and 5-HMF (0.11 mM). The membrane-separated (filtered) rice straw hydrolysate then was used as the substrate for xylose fermentation experiments (Fig. 3 and Supplementary Figs. S1 and S2) to evaluate further improvement of the hemicellulose degradation by cell-surface-engineered strains.

Fermentation of membrane-separated rice straw hydrolysate

For this part of our study, the engineered strains were used for the fermentation of "neat" rice straw hydrolysate (without supplementation with exogenous components); in these experiments, hydrolysate-provided hemicellulose served as the sole source of carbon, permitting us to evaluate the effect of membrane separation on xylitol production (Fig. 3 and Supplementary Fig. S1). As in the earlier experiments, xylitol production (Fig. 3) and consumption of xylose, glucose, and total sugar (Supplementary Fig. S1a, b; c, d; e, f, respectively) were measured by GC-MS for each strain. The fermentation medium consisted of the original (unfiltered; Fig. 3a and Supplementary Fig. S1a, c, e) or membrane-separated (filtered; Fig. 3bB and Supplementary Fig. S1b, d, f) rice straw hydrolysate. We observed a pattern similar to that seen in Fig. 2: Strain YPH499-PIU-XR-BGL-XYN-XYN exhibited the best performance, producing 37.9 g/L of xylitol after 96 h of fermentation of membrane-separated rice straw hydrolysate, corresponding to a maximum $Y_{Xylitol/Xylose} = 0.63$ (Fig. 3b and Supplementary Fig. S2b). Here again we observed that only the strains harboring XYL and/or XYN on their cell surface efficiently consumed hemicellulosic materials from rice straw hydrolysate (Supplementary Fig. S1a, b). Similarly, only strains displaying BGL on their cell surface consumed oligomers of glucose. Again, as seen in the earlier experiments (Fig. 2), no strain exhausted the xylose present in the medium, even after 96 h. Arabinose and galactose were not utilized by any of our strains (data not shown), and both the yeast cell densities and pHs remained essentially constant during the fermentation process, regardless of the strain (data not shown). A comparative study of xylitol yields ($Y_{Xylitol/Xylose}$) after 96 h of fermentation (Supplementary Fig. S2b) showed the benefit of multi-enzyme cell surface display for hemicellulose degradation ability of engineered strains; a twofold increase in xylitol yield was detected when using the membrane-separated hydrolysate as substrate (compared to unfiltered hydrolysate).

Discussion

In the course of our cell surface engineering of the YPH499 strain of *Saccharomyces cerevisiae*, we generated six recombinant strains capable of converting xylose into xylitol. Five of these six recombinant strains were capable of directly degrading hemicellulosic materials contained in rice straw hydrolysate without supplementation with exogenous enzymes. Given the high cost of commercial enzymes, this distinction is expected to be of great value for further industrial applications.

The considerable amount of glucose contained in the hemicellulose of rice straw hydrolysate led us to evaluate the effect of displaying BGL along with XYL and XYN on the cell surface of recombinant yeast cells.

Our engineering approach consisted of stable integration (into the yeast genome) of the XR-, BGL-, and XYL-encoding sequences as single-copy genes. The resulting strains permitted us to directly evaluate the effect of each enzyme on xylitol production from medium in which rice straw hydrolysate served as the sole source of carbon. In the case of XYN-encoding sequence, we used a multi-copy integration method, thereby compensating for the relatively low level of activity of this enzyme when displayed on the cell

surface (Yamada et al. 2010). In total, we successfully achieved the co-display of three different active enzymes at the cell surface of a given strain (Table 3). For each type of enzyme, we observed similar levels of activity from one strain to another, enabling us to directly compare enzymatic performance for the degradation of biomass in real time during the fermentation process.

We clearly observed increased xylitol production from rice straw hydrolysate when the hydrolysate served as the sole source of carbon for our cell-surface-engineered strains of *Saccharomyces cerevisiae*, demonstrating the benefit of a multi-enzyme-displaying cell surface (Supplementary Fig. S2). The accumulation of xylitol during fermentation by each strain presumably was facilitated by the low level of xylitol dehydrogenase (XDH) activity in this strain background (Kim et al. 2012). since XDH would have resulted in the degradation of xylitol.

Among the strains generated in this study, the best-performing construct was the strain co-displaying three different hydrolases on the cell surface (YPH499-PIU-XR-BGL-XYL-XYN). The superior performance of this strain is consistent with synergistic effects due to the short distances between individual enzyme molecules, as previously described by Bae et al (2015).

In addition to our cell surface engineering approach, we performed membrane separation of our substrate (rice straw hydrolysate). This process removed a large fraction of known inhibitors of fermentation, while simultaneously increasing sugar concentrations. However, it is noteworthy that total consumption of xylose was not achieved by any strain [using either original (unfiltered) or membrane-separated (filtered) rice straw hydrolysate]. In the case of the unfiltered hydrolysate (Figs. 2 and 3a), this limited consumption of xylose presumably reflected the presence of fermentation inhibitors in the medium (Table 4). Indeed, we observed a more rapid degradation pattern during fermentation of the filtered hydrolysate compared to that observed during fermentation of the unfiltered hydrolysate (Supplementary Fig. S1b, d, f). Nonetheless, xylose levels still were not exhausted during fermentation of the filtered hydrolysate. We postulate that limits on the consumption of xylose may reflect a saturating effect of the large amount of sugars available in the medium, even in the absence of known inhibitors.

Interestingly, while the membrane separation process led to a nearly fivefold increase of the xylose concentration in the medium, we observed a tenfold increase in xylitol production in the resulting fermentation (Fig. 3), corresponding to a twofold increase (filtered vs. unfiltered) of the xylitol yields ($Y_{Xylitol/Xylose}$) after 96 h of fermentation (Supplementary Fig. S2b). This increase of xylitol yield is directly correlated with the removal of fermentation inhibitors and shows the benefit of using membrane separation technology in combination with multi-enzyme cell surface display to enhance xylitol production in the biorefinery industry. Studies on xylitol production from biomass by *Saccharomyces cerevisiae* are scarce compared to studies using bacteria or non-conventional yeast like *Candida* sp. or *Kluyveromyces* sp., although *Saccharomyces cerevisiae* remains a promising tool for xylitol production due to the ease of handling and modification. To date, a maximum xylitol production of 100.1 g/L ($Y_{Xylitol/Xylose} = 0.81$) was reported (after 102 h of fermentation) by researchers using vegetable waste and *Candida athensensis* SB18 as a biocatalyst (Zhang et al. 2012). The present study is nevertheless the first report (to our knowledge) of xylitol production by direct fermentation of rice straw hydrolysate using cell-surface-engineered strains of *Saccharomyces cerevisiae*, and constitutes a promising proof-of-concept for our approach.

On the economic point of view, it is quite difficult to evaluate the cost of production of unit amount of xylitol as many parameters are to be taken into account, and also as many data are not publically disclosed by industrial companies involved in xylitol production. Moreover, studies that focus on economic evaluation of biobased xylitol production, as well as studies comparing its costs to the chemical production, are not found in the literature. The integrated process of bioconversion of rye straw into ethanol and xylitol has been economically evaluated (Franceschin et al. 2011). This study showed that approximately 25.9 and 29.0 % of the costs of production correspond to the costs of the fermentation and the recovery of xylitol, respectively. Noteworthily, the authors assessed that the cost of the addition of purified enzymes represents 6.5 % of the total process costs in the case of hemicellulase cost contribution of 59.2 €/ton of xylose processed.

In that sense, the present study presents a double advantage in terms of cost savings as our cell-surface-engineered strains of *Saccharomyces cerevisiae* did not require addition of any commercial enzymes and as membrane separation technologies are cost-effective with very limited energy consumption. Moreover, these cost savings will significantly increase in the case of repeated batch processes as the cell surface displayed enzymes will be re-used several times (Matano et al. 2013). Further enhancement in the xylose uptake by yeast cells, as well as optimization of the activity of hemicellulolytic enzymes displayed on the yeast cell surface, may also provide decreases in the costs of xylitol production.

One of the serious challenges that the current CBP system might encounter when the titer and yield of xylitol are pushed to the limit concerns the increased intracellular xylitol which leads to xylulose formation. While no cytotoxic effect of xylitol has been reported so far, high intracellular concentration of xylitol and xylulose may have inhibitory effect on the biosynthetic enzymes and/or genes expression in the pathway. In that sense, substantial efflux of xylitol may help to decrease such product feedback inhibitory effect and by this way to enhance the xylitol bioproduction (Su et al. 2013). Although no studies about the secretion of xylitol have been reported

yet, the elucidation and engineering of xylitol export systems may be a useful tool to further improve xylitol bioproduction by recombinant strains of *Saccharomyces cerevisiae*.

Another challenge that our system might encounter will concern the recovery and purification of xylitol after fermentation. Indeed, in many fermentation processes, these final steps are rather complicated, as they are depending on both the composition of the fermentation medium and the nature of the compounds produced. To date, very few data about recovering xylitol from fermented broths is available (Misra et al. 2011). As recovery process accounts for almost one third (29 %) of the costs of xylitol production (Franceschin et al. 2011). enhanced recovery and purification protocols would be of great interest for industrial bioproduction of xylitol.

Altogether, our results demonstrate that multi-enzyme co-display at the cell surface of *Saccharomyces cerevisiae* was highly effective for facilitating the consolidation of bioprocessing of hemicellulosic materials to xylitol. Such a combined approach of cell surface engineering of yeast cells and membrane separation of rice straw hydrolysate is promising for the biorefinery industry and could be extended to the production of many other valuable biochemicals or biofuels.

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Compliance with ethical standards

Ethical approval This study does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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