Engineering of recombinant Saccharomyces cerevisiae for bioethanol production from renewable biomass

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ABSTRACT

For sustainable development, many research groups have made an effort to change the currently available crude oil to the renewable biomass as resource. To overcome the general problems of corn- and sugar-based biomass, non-food biomass such as cellulosic biomass (tree, straw, agricultural residue et al.) has been concerned as alternative biomass for production of bioethanol, a promising biofuel. By decomposition of cellulosic biomass, various mono-sugars are released and most abundant sugars of glucose and xylose should be converted to bioethanol for gaining its price competitiveness against gasoline. To develop a commercially available bioprocess, meanwhile, engineering of genetic and microbial systems should be complementary to bioprocess engineering by feed-forward and feed-back cycles. Microbial Factory Technology (MFT) armed with genetic, microbial and metabolic engineering, –omic technology and fermentation optimization is a promising technology able to meet the system and process complementation, and applicable for rapid development of bioethanol production process. In this presentation, an alcohol yeast of Saccharomyces cerevisiae was engineered by MFT for mass production of bioethanol from renewable biomass. The engineered S. cerevisiae could produce bioethanol from glucose and xylose in cellulosic biomass with a high conversion yield and production rate. And a high titer of bioethanol was able to be obtained by fermentation optimization.

Keywords**:** Ethanol, Xylose, *Saccharomyces cerevisiae*, NADH-preferable xylose reductase

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INTRODUCTION

Ethanol is a promising biochemical to be used as a biofuel itself and gasoline additive. As a resource for ethanol production, lignocellulosic biomass has been intensively studied by several research groups because it is the most abundant and non-food oriented resource and is autotrophically renewed by solar energy and carbon dioxide fixation (Bak et al., 2009; Hahn-Hägerdal et al., 2007; Matsushika et al., 2009; Saha, 2003). Lignocellulosic biomass consists of cellulose, hemicelluloses, lignin and small ashes. By physical, chemical and/or biological treatment, the biomass is decomposed mainly into several monosaccharides such as glucose, xylose, arabinose, mannose and byproducts (Bak et al., 2009; Saha, 2003). As xylose, the second abundant mono-saccharide, is a representative five-carbon sugar and should be utilized as a carbon source in order to realize the commercial application of cellulosic ethanol (Jin et al., 2000). Several research groups have developed wild and/or recombinant microbial systems to produce ethanol from xylose; *Escherichia coli*, *Zymomonas mobilis*, *Pichia stipitis* and *Saccharomyces cerevisiae* (Asghari et al., 1996; Jin et al., 2000; Lee et al., 2000; Zhang et al., 1995). Among them, *S. cerevisiae* has been used traditionally for ethanol production from starch- and sugar-based feedstocks. But it does not have catabolic pathways for the utilization of pentoses (xylose and arabinose) (Jin et al., 2000). Some research groups have engineered *S. cerevisiae* able to metabolize xylose and produce ethanol (Ha et al., 2011; Hahn-Hägerdal et al., 2007; Ho et al., 1998; Jin et al., 2000). Key metabolic enzymes for xylose utilization in yeast but, absent in *S. cerevisiae*, are two cofactor-dependent enzymes of xylose reductase (XR, EC 1.1.1.21) and xylitol dehydrogenase (XDH, EC 1.1.1.9).

In yeast, XR converts xylose into xylitol and then XDH catalyzes xylitol oxidation for xylulose production (Fig. 1). Xylose is converted by the two enzymes to xylulose, an intermediate in the endogenous pentose phosphate (PP) pathway, and then xylulose is metabolized into ethanol via the PP pathway and glycolysis (Fig. 1) (Matsushika et al., 2009). Among them, cofactor-utilizing systems of XR and XDH have been studied thoroughly. Mainly, *S. cerevisiae* was engineered to express XR and XDH from *P. stipitis*, a xylose-utilizing yeast (Ha et al., 2011; Ho et al., 1998; Jin et al., 2000; Jin et al., 2003; Katahira et al., 2008). One of the problems with efficient production of ethanol from xylose is accumulation of xylitol due to cofactor imbalance between XR and XDH. As a cofactor, typical XR utilizes NADPH mainly and XDH does NAD^+ solely (Watanabe et al., 2007). Because XR and XDH demand different reducing powers, recombinant *S. cerevisiae* undergoes the redox-imbalance and hence produces xylitol considerably as a major byproduct in xylose metabolism. To minimize xylitol accumulation and improve ethanol yield, many research strategies were carried out such as improvement of cofactor supply and modulation of the PP pathway and glycolysis (Hahn-Hägerdal et al., 2007; Matsushika et al., 2009). Recently, protein engineering for the manipulation of the cofactor-preference and/or dependency of XR and XDH was undertaken (Matsushika et al., 2008; Petschacher and Nidetzky, 2008; Watanabe et al., 2007). NAD^+-dependent XDH mutants from *P. stipitis* were developed by introduction of a structural zinc binding site using site-directed mutagenesis (Matsushika et al., 2008). Recently, the cofactor-preference of XR was changed from NADPH to NADH by point mutation of arginine to histidine at the 276 amino acid position (R276H) (Watanabe et al., 2007). By this mutation, xylitol production by cofactor-imbalance between XR and XDH was reduced and ethanol production was enhanced.

In this study, recombinant *S. cerevisiae* strains were developed to optimize the expression of a NADH-preferable XR mutant (XR^{R276H} equal to XR^{MUT}) (Watanabe et al., 2007) and NAD^+-dependent XDH wild type, which were integrated into the chromosome of *S. cerevisiae* for their stable expression. *S. cerevisiae* xylulokinase (XK, EC 2.7.1.17) catalyzing the ATP-consuming phosphorylation of xylulose in the PP pathway (Fig. 1) was coexpressed in order to trigger xylose consumption. Moreover, transaldolase (Tal, EC 2.2.1.2), a key enzyme in the PP pathway, was also overexpressed in xylose-metabolizing *S. cerevisiae*, of which role in xylose metabolism was already specified (Jin et al., 2005; Walfridsson et al., 1995). Aldehyde dehydrogenase 6 (ALD6, EC 1.2.1.3), catalyzing acetic acid production from acetaldehyde which is a substrate for ethanol production (Fig. 1), was disrupted for more improvement of xylose conversion to ethanol. Effects of the cofactor-balanced xylose metabolism and modulation of the ethanol metabolism on production of xylitol and ethanol were investigated in batch fermentations using xylose and/or glucose in aerobic, microaerobic and oxygen-limited conditions.

MATERIALS AND METHODS

Strains and plasmids

E. coli TOP10 (Invitrogen, Carlsbad, CA, USA) and *S. cerevisiae* D452-2 (Table 1) were used for genetic manipulation and ethanol production, respectively. Plasmids YEpM4-XR^{WT} and YEpM4-XR^{MUT} containing the wild type and mutant XR (R276H) of the *P. stipitis XYL1* gene, and plasmid pPGK-XDH harboring the *P. stipitis XYL2* gene were constructed previously (Watanabe et al., 2007). The *XYL1* and *XYL2* genes coding for XR and XDH, respectively, and the *PsTAL1* gene from *P. stipitis* encoding Tal were cloned to be expressed constitutively under the control of the phosphoglycerate kinase (PGK) promoter and the glyceraldehydes-3-phosphate dehydrogenase (GPD) promoter, respectively. Plasmid δISXK contains the *S. cerevisiae XKS1* gene coding for *S. cerevisiae* xylulokinase between the *GPD* promoter and terminator. The G418 resistance gene in plasmid δISXK was used for chromosomal integration and stable overexpression of the *XKS1* gene (Lee et al., 2003). The strains and plasmids used in this study are listed in Table 1.

Genetic manipulation

For stable expression of the *XYL1* and *XYL2* genes in *S. cerevisiae*, their expression cassettes from the *PGK* promoter to the terminator were subcloned into plasmid YIp5, a chromosomal-integration vector. To amplify each expression cassette, DNA oligomers of F_XR_NheI (5'-ctagctagcaaagatgccgatttgggcgcgaatc-3') and R_XR_SphI (5'-acatgcatgcgtcgaccagctttaacgaacgcaga-3') for the wild and mutant types of *XYL1*, and F_XDH_HindIII (5'-cccaagcttaaagatgccgatttgggcgcgaatc-3') and R_XDH_NheI (5'-ctagctagcgtcgaccagctttaacgaacgcaga-3') for *XYL2* were designed. Each primer contains the recognition site of the restriction enzyme described in its name. After three PCR products were obtained from the PCR templates of YEpM4-XR^{WT}, YEpM4-XR^{MUT} and pPGK-XDH vectors, respectively, they were cut with *Sph*I and *Nhe*I digestion enzymes for *XYL1*, and *Nhe*I and *Hind*III for *XYL2*. After digestion of plasmid YIp5 with *Sph*I and *Hind*III, two digested PCR products containing *XYL1* wild type or mutant, and *XYL2* were ligated with YIp vector. Finally, plasmids YIpXR^{WT}-XDH and YIpXR^{MUT}-XDH were constructed to

contain the expression cassettes of wild or mutant *XYL1*, and *XYL2,* respectively. In all the YIp5 vector-driven plasmids, the *XYL2* expression cassette was located behind the *XYL1* expression cassette.

The *PsTAL1* gene was amplified from the chromosome of *P. stipitis* using a forward primer of F_PsTAL1_SpeI (5'-ggactagtatgtcctccaactcccttga-3') and a backward primer of R_PsTAL1_XhoI (5'-ccgctcgagttagaatctggcttccaattgtt-3'). The amplified DNA fragments were digested with the restriction enzymes of *Spe*I and *Xho*I, ligated with p423GPD plasmid cut by the same enzymes. The resulting vector for *PsTAL1* expression was called p423PsTAL.

The truncated *ALD6* gene was obtained by PCR-amplification from the *S. cerevisiae* chromosome. Two PCR DNA oligomers were designed as follows: F_d_ALD6_SphI, 5'-acatgcatgcgccttagcccgtggggatgttacc-3'; R_d_ALD6_KpnI, 5'-cggggtaccctgatgaagtaacccttgtcaccaac-3'. The 800 bp-size DNA fragment was cut with *Sph*I and *Kpn*I digestion enzymes, and combined with pAUR101 plasmid with the aureobasidin resistance gene, resulting in plasmid pAUR_d_ALD6 construction.

Plasmids were transformed into *S. cerevisiae* by the alkali-cation method and a MicroPulser^{TM} Electroporation Apparatus (Bio-Rad, Richimond, U.S.A.) with some modification. To select the transformants containing YEpM4-XR^{WT} or YEpM4-XR^{MUT} and pPGK-XDH vectors, SC solid medium without both uracil and leucine was used. For the integration of each *XYL1-XYL2* expression cassette into the chromosomal *URA3* gene in *S. cerevisiae*, YIp5-oriented vectors were cut with *Ava*I and the resulting DNA fragments were transformed into *S. cerevisiae* D452-2. The transformants were selected on SC solid medium without uracil. Introduction of the *XKS1* gene in plasmid δ ISXK into the chromosome of *S. cerevisiae* followed the previous report (Lee et al., 2003) and its transformants were able to grow on SC solid medium with 15 g l^{-1} G418 (BIO101, Vista, CA, U.S.A.). The transformants with p423PsTAL were selected on the solid medium without histidine. For the disruption of the chromosomal *ALD6* gene by homologous recombination mechanism, plasmid pAUR_d_ALD6 cut with *Sal*I was introduced into *S. cerevisiae* and the transformants were selected able to grow against aureobasidin.

Culture conditions

LB medium (0.5 g l^{-1} yeast extract, 1 g l^{-1} tryptone and 1 g l^{-1} NaCl) was used for *E. coli* culture. A defined SC medium (6.7 g l^{-1} yeast nitrogen without amino acids (Sigma, St. Louis, MO, U.S.A.) and 1.92 g l^{-1} synthetic complete supplement mixture (Sigma, U.S.A.)) with 20 g l^{-1} glucose and appropriate amino acids was used for maintenance and pre-culture of *S. cerevisiae* wild type and recombinant strains.

For batch culture using a mixture of glucose and xylose as carbon sources, an 1.0 l-scale B. Braun multi-fermentor (Biostat-Q, Germany) with 0.5 l of YP medium (10 g l^{-1} yeast extract, 20 g l^{-1} bacto-peptone) with 50 g l^{-1} xylose and 20 g l^{-1} glucose was used. Medium acidity was controlled at pH 5.5 by addition of 2 N NaOH and temperature was maintained at 30^{o}C during the cultivation. Agitation and aeration speed were set at 500 rpm and 1.0 vvm for aerobic culture, and 300 rpm and 0.1 vvm for micro-aerobic culture, respectively. Oxygen-limited batch fermentation was carried in the bioreactor with 0.5 l of YP medium initially containing 89 g l^{-1} xylose and 17 g l^{-1} glucose. After depletion of the glucose initially added in a microaerobic condition, 600 g l^{-1} glucose solution was fed at 3 ml h^{-1} of feed rate by a peristaltic pump (Masterflex 7523-57, Cole-Parmer Instrument Co., Vernon Hills, IL, USA). After 19 h feeding, agitation and aeration rates were changed to oxygen-limited condition (200 rpm and 0.06 vvm, respectively). Except for the aeration and agitation, all conditions followed the microaerobic fermentation.

For batch fermentation with xylose as a sole carbon source, the recombinant cells pre-cultured in YP medium with 20 g l^{-1} glucose were harvested by centrifugation at 5,000 rpm for 5 min and washed with 50 ml deionized water. The cells were inoculated into a 500 ml baffled flask containing 100 ml SC medium (without uracil) containing 10.21 g l^{-1} potassium hydrogen phthalate and 53 ~ 61 g l^{-1} xylose. The initial dry cell mass was adjusted to be 2.5~3.3. Culture temperature and agitation speed were maintained at 30^{o}C and 90 rpm, respectively, in a shaking incubator (Vision, Korea).

Activity assay

For determination of XR, XDH and XK activities, *S. cerevisiae* cells were collected by centrifugation at 6,000 rpm and room temperature, and their pellets were resuspended in 50 mM phosphate buffer (pH 7.0). After adding 0.2 g glass bead (I.D. 0.5 mm, Biospec, Bartlesville, OK, U.S.A.) into the cell suspension, the mixture was vortexed vigorously for 1 min and cooled for 4 min, which was repeated three times. After centrifugation for 3 min at 4^{o}C and 12,000 rpm, the supernatant containing the protein crude extract was used for the enzyme assay. The reaction solution for XR was composed of 50 mM potassium phosphate buffer (pH 6.0), 0.4 mM NADPH or NADH, 100 mM xylose. For XDH, a solution containing 50 mM Tri-HCl buffer (pH 8.5), 50 mM $MgCl_2$, 2 mM NAD^+ and 300 mM xylitol was formulated. Based on the previous report (Lee et al., 2003), XK was assayed with a reaction solution with 20 mM glycylglycine buffer (pH 7.4), 0.2 mM NADH, 1.1 mM ATP, 5 mM $MgSO_4$, 2.3 mM

phosphoenolpyruvate, 8.5 mM xylulose, 2 U pyruvate kinase and 2 U lactate dehydrogenase. In all the cases, absorbance at 340 nm was monitored with a 96-well microplate reader (Molecular Devices Col, Menlo Park, CA, U.S.A.) after addition of the crude enzyme solution. Reactions were carried out at 30°C in triplicate. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of NADPH or NADH, or reducing NAD^+ per minute in the reaction condition as described above.

Analysis

Dry cell mass was measured with a spectrophotometer (Ultrospec 2000, Amersham Phamacia Biotech, Uppsala, Sweden) at 600 nm. Concentrations of sugars, alcohols and acids in culture broth were determined by a HPLC (1100LC, Agilent, Santa Clara, CA, U.S.A) equipped with a RI detector. The samples were separated by the Carbohydrate Analysis column (Phenomenex, Torrance, CA, U.S.A.) heated at 60°C. The mobile phase composed of 5 mM H_2SO_4 was flowed at 0.6 ml min^{-1}. Protein concentration was determined by a protein assay kit (Bio-rad Laboratories, Hercules, CA, U.S.A.).

RESULTS

System construction

To produce efficiently ethanol from xylose while minimizing xylitol accumulation, recombinant *S. cerevisiae* was constructed to express NADH-preferable XR^{MUT} (equal to XR^{R276H}) and NAD^+-dependent XDH originated from *P. stipitis* and *S. cerevisiae* XK. For stable expression of the xylose metabolic genes, the expression cassette with the XR or XR^{MUT}, and XDH genes were integrated into the chromosomal *URA3* gene in *S. cerevisiae* D452-2, of which recombinant strains were designated as $SX2^{WT}$ and $SX2^{MUT}$, respectively. The *S. cerevisiae* XK gene was additionally introduced into the chromosomal δ–sequence in $SX2^{WT}$ and $SX2^{MUT}$, resulting in the construction of $SX3^{WT}$ and $SX3^{MUT}$, respectively. $SX3^{MUT}$ was transformed with plasmid YEpM4-$XR^{MUT,}$ and p423PsTAL for more expression of XR^{MUT} and Tal, resulting in construction of $SX5^{MUT}$. $SX6^{MUT}$ was deficient in the *ALD6* gene by transforming a linearized pAUR_d_ALD6 plasmid into $SX5^{MUT}$. The recombinant *S. cerevisiae* strains constructed in this study were listed in Table 1.

Activity assay of the xylose metabolic enzymes

To confirm the successful expression of the xylose metabolic enzymes, the recombinant *S. cerevisiae* cells were cultured in YP medium with 20 g l^{-1} glucose and 50 g l^{-1} xylose, and collected after 13 h of cultivation when glucose was depleted. After preparation of their crude extracts, specific activities of XR, XDH and XK were determined. As shown in Fig. 2(A), the parental strain (lane 1) showed only NADPH-dependent activity of xylose reductase. As the positive control, recombinant *S. cerevisiae* D452-2 expressing XR^{WT} and XDH possessed the highest XR activity toward NADPH, of which 60% value was measured when using a cofactor NADH (lane 2). Meanwhile, XR^{MUT} expressed in *S. cerevisiae* D452-2/YEpM4-XR^{MUT}+pPGK-XDH (lane 3), $SX2^{MUT}$ (lane 4) and $SX6^{MUT}$ (lane 5) was preferable to NADH by about a 2.5(±0.6)-fold, relative to NADPH. A sum of specific XR activity of the three XR^{MUT}-expressing *S. cerevisiae* was 3.7(±1.7) times lower than that of the control *S. cerevisiae* D452-2/YEpM4-XR^{WT}+pPGK-XDH. For XDH expression, recombinant *S. cerevisiae* strains expressing *P. stipitis* XDH showed 2.8~5.0 times higher specific XDH activity than the wild type strain, where NAD^+ was used as a cofactor (Fig. 2(D)). As depicted in Fig. 2(C), more introduction of the *XKS1* gene into the chromosomal δ–sequence increased specific XK activity in $SX3^{MUT}$ by a 1.8-fold in comparison to $SX2^{MUT}$. $SX3^{MUT}$ and $SX5^{MUT}$ showed the same XR activity as $SX2^{MUT}$ and $SX6^{MUT}$. XDH activities of $SX3^{MUT}$, $SX5^{MUT}$ and $SX6^{MUT}$ were similar to those of $SX2^{MUT}$. $SX5^{MUT}$ and $SX6^{MUT}$ had the same XK activity as $SX3^{MUT}$ (data not shown).

Effects of XR^{MUT} expression on xylitol production

To investigate the effects of the cofactor-preference change, batch fermentations using a mixture of glucose and xylose as carbon sources were carried out in an aerobic condition. As shown in Fig. 3(A), the control strain of recombinant *S. cerevisiae* expressing the wild types of XR and XDH started to consume xylose after the exhaustion of glucose. Xylose was utilized together with ethanol produced from the glucose metabolism. In 70 hr of batch culture, 10.6 g l^{-1} xylitol accumulated in culture broth with a xylitol yield of 0.33 g g^{-1}. $SX2^{MUT}$ containing the expression cassette with the XR^{MUT} and XDH genes in its chromosome was cultivated in the same manner as the control experiment (Fig. 3(B)). Glucose consumption and concomitant ethanol production from glucose was not affected by the change of the xylose metabolic enzymes. Contrary to the control system, however, $SX2^{MUT}$ produced a small amount of xylitol (0.24 g l^{-1}) from 22.9 g l^{-1} of consumed xylose. Additionally, $SX2^{MUT}$ consumed ethanol

produced from glucose more slowly than the control strain. $SM2^{MUT}$ used 62% ethanol whereas the control strain utilized 91% ethanol for cell growth and xylitol production in an aerobic condition.

Effects of xylulokinase overexpression on ethanol production

In previous reports, overexpression of xylulokinase, catalyzing the conversion of xylulose to xylulose phosphate (Fig. 1), facilitated the consumption of xylulose (an intermediate in the xylose metabolism) and increased ethanol production (Jin et al., 2003; Lee et al., 2003; Toivari et al., 2001). To implement this positive effect of xylulokinase overexpression in the cofactor-engineered *S. cerevisiae* system, the *S. cerevisiae* XK gene was integrated into the none-functional and multiple chromosomal δ-sequence (Lee et al., 2003). Batch fermentation using xylose and glucose was carried out in a micro-aerobic condition where oxygen content in the culture broth was monitored under 2% saturation (data not shown). As shown in Fig. 3(C) and 3(D), $SX2^{MUT}$ and $SX3^{MUT}$ showed the similar performances of glucose consumption and glucose-oriented ethanol production. For xylose utilization, however, $SX3^{MUT}$ consumed 29.5 g l^{-1} xylose whereas $SX2^{MUT}$ did 12.1 g l^{-1} only. About 26% of xylose metabolized by $SX2^{MUT}$ was converted into xylitol and ethanol concentration was not increased along with xylose consumption (Fig. 3(C)). Contrary to $SX2^{MUT}$, $SX3^{MUT}$ overexpressing xylulokinase showed impressive performances of xylose conversion to ethanol. Batch culture of $SX3^{MUT}$ in a microaerobic condition resulted in 5 g l^{-1} of ethanol production from xylose, 17.3% ethanol yield based on the xylose consumed without production of xylitol (Fig. 3(D)). Interestingly, acetate accumulated as one of the major by-products. Both $SX2^{MUT}$ and $SX3^{MUT}$ produced acetate from the xylose metabolizm at 0.12 g g^{-1} and 0.085 g g^{-1} yields, respectively. Final glycerol concentration was measured under 0.5 g l^{-1} in both cases.

Ethanol production from xylose as a sole carbon source

Batch fermentation with glucose and xylose exhibited that the expression of XR^{MUT} and XDH, and overexpression of intrinsic XK allowed ethanol production from xylose in a microaerobic condition. To clearly explore the conversion of xylose to ethanol, microaerobic batch fermentations of recombinant *S. cerevisiae* $SX2^{WT}$, $SX2^{MUT}$, $SX3^{WT}$ and $SX3^{MUT}$ were carried out using a defined medium containing xylose as a sole carbon source (Fig. 4). As a control, $SX2^{WT}$ consumed a small amount of xylose used mainly for cell growth and xylitol production. Overexpression of XK in $SX3^{WT}$ increased cell growth and reduced xylitol accumulation, but did not affect xylose consumption and ethanol production in a microaerobic condition. In the case of XR^{MUT} expression, the cell growth of $SX2^{MUT}$ was higher than the corresponding control, $SX2^{WT}$. But an increase in xylose consumption triggered xylitol accumulation instead of ethanol production. Then, XR^{MUT} expression accompanied by XK overexpression was expected to increase xylose consumption and thereby ethanol production as shown in the glucose and xylose co-fermentation (Fig. 3(D)). In batch fermentation of $SX3^{MUT}$, a half of the xylose added was consumed to produce ethanol and cell mass. Xylitol was obtained at a basal level. Primarily, 1.0g l^{-1} glycerol and 1.2 g l^{-1} acetate accumulated.

More expression of XR^{MUT} and *Tal* in $SX3^{MUT}$

As shown in Fig 2(A), specific activity of XR^{MUT} in $SX2^{MUT}$ (integration system, similar to $SX3^{MUT}$) was 2 times lower than that of the episomal XR^{MUT} expression system (lane 3). More expression of XR^{MUT} was achieved by the transformation of a YEpM4-XR^{MUT} vector into $SX3^{MUT}$. An important enzyme in the PP pathway, Tal was also expressed by p423PsTAL introduction. Finally, $SX5^{MUT}$ was cultivated in a bioreactor with YP medium with 20 g l^{-1} glucose and 50 g l^{-1} in a microaerobic condition for its validation. During 70 h culture, about 90% of added xylose was consumed and 18.3 g l^{-1} ethanol was produced from total sugar (70 g l^{-1}). These genetic modifications did not influence the glucose metabolism.

Effects of *ALD6* disruption on xylose and ethanol metabolism

Acetate, a by-product in the xylose metabolism, was produced at about 2 g l^{-1} in all microaerobic fermentations (Fig 3(C), 3(D), Fig 4 and Fig 5(A)). To investigate the effect of acetate on xylose metabolism, microaerobic batch fermentation of $SX3^{MUT}$ was carried out in SC medium with 60 g l^{-1} xylose and 2 g l^{-1} acetate (Supplement 1). Without acetate addition, xylose was metabolized into ethanol by $SX3^{MUT}$. However, addition of acetate strongly inhibited cell growth and xylose metabolism. Accordingly, to minimize the negative effects of acetate and to improve xylose metabolic performance, the *ald6* gene coding for aldehyde dehydrogenase catalyzing the conversion of acetaldehyde to acetate (Fig. 1) was disrupted in $SX5^{MUT}$ by homologous recombination to construct $SX6^{MUT}$. Acetate inhibition on the xylose metabolism was verified in a microaerobic batch fermentation of $SX6^{MUT}$ (Fig. 5(B)). As expected, xylose consumption and cell growth was accelerated dramatically by the *ALD6* disruption.

Finally, all sugars added initially were metabolized into 20.7 g l^{-1} ethanol with 30 % yield and 0.30 g l^{-1} h^{-1} productivity.

For more investigation of ethanol production from xylose, oxygen-limited fermentation of $SX6^{MUT}$ was carried out in YP medium initially containing 89 g l^{-1} xylose and 17 g l^{-1} glucose. To increase the cell mass of $SX6^{MUT}$, a concentrated glucose solution was fed constantly. After stopping the glucose feeding, a microaerobic condition was changed to an oxygen-limited condition where ethanol production is favorable. Xylose consumption started after glucose depletion and was maintained while glucose concentration was kept at a basal level. In the oxygen-limited condition, xylose was consumed continuously to produce ethanol. Finally, 50 g l^{-1} ethanol and 1.6 g l^{-1} xylitol was produced from 51.5 g l^{-1} glucose (34 g l^{-1} from the glucose feeding) and 68.5 g l^{-1} xylose, and 41.7% ethanol yield and 0.50 g l^{-1} h^{-1} productivity, and 2.3% xylitol yield were achieved in 68 h of the oxygen-limited batch fermentation of $SX6^{MUT}$.

DISCUSSION

Among several obstacles to overcome for commercialization of lignocellulosic ethanol with respect to microbial engineering, robust microorganisms should be developed able to utilize efficiently most fermentable sugars in lignocelluose and have high-tolerance to ethanol and growth inhibitors present in biomass (Hahn-Hägerdal et al., 2007). *S. cerevisiae* is a well-characterized workhorse in ethanol fermentation but it is unable to utilize xylose, the second abundant monosaccharide present in lignocellulosic biomass. Even though *S. cerevisiae* was engineered to express xylose metabolic enzymes, about 30 ~ 40% of xylose was typically deposed into xylitol, a major by-product of xylose, because of cofactor-imbalance (Hahn-Hägerdal et al., 2007; Jin et al., 2003; Matsushika et al., 2009; Watanabe et al., 2007). Cofactor-imbalance is caused by the mismatch of cofactor utilization such as NADPH for the XR-catalyzed reaction and NAD^+ for the XDH reaction. In this study, a line-up of cofactor utilization was accomplished by introduction of NADH-preferring XR^{MUT} and NAD^+-dependent XDH into *S. cerevisiae*. It was expected to minimize xylitol accumulation and hence improve ethanol production from xylose.

P. stipitis XYL1 and *XYL2* have been used for transformation of xylose-fermenting properties into *S. cerevisiae* (Jin et al., 2000; Jin et al., 2003). Chromosomal integrative or episomal expression of *XYL1* (wild and mutant) and *XYL2* in *S. cerevisiae* D452-2 was confirmed by *in vitro* enzymatic assay of the crude enzyme extract. The control strain without both XR and XDH showed a xylose-reducing activity, which is well known to be ascribed to the presence of a non-specific aldose reductase, GRE3 (Kim et al., 2002). Irrespective of gene expression systems, XR and XDH were actively expressed in *S. cerevisiae* D452-2. As shown in Fig. 2, point mutation of XR conferred the cofactor-specificity change from NADPH to NADH even though total reducing activity diminished.

As already mentioned, accumulation of xylitol is a main problem in ethanol production from xylose in recombinant *S. cerevisiae* and other yeasts. Once xylitol was secreted into the culture broth, it is unable to be metabolized by the cells (Jin et al., 2000). Many researches were performed to minimize xylitol production and hence improve ethanol production. For examples, two copies of the XR mutant with a higher K_m value toward NADPH was expressed in recombinant *S. cerevisiae* expressing XDH and XK, leading to 9% xylitol yield in an oxygen-limited batch cultivation (Jeppsson et al., 2006). Disruption of para-nitophenyl phosphatase, an enzyme dephosphorylating a phosphorylated protein, was designed to manipulate xylose metabolism. Xylitol content increased slightly but cell growth and ethanol production were improved by more than a 2-fold (Van Vleet et al., 2008). Expression of xylose isomerases from *Piromyces* and *Orpinomyces* sp. reduced xylitol accumulation but overall xylose consumption and ethanol production were much lower than those of the XR-XDH expression systems (Karhumaa et al., 2007; Madhavan et al., 2009). In this study, XR^{MUT} with NADH-preference was replaced with the XR wild type in recombinant *S. cerevisiae* expressing XDH and XK. As shown in an aerobic fermentation using xylose and glucose (Fig. 3), XR^{MUT} expression ($SX2^{MUT}$) reduced xylitol production under 1% xylitol yield. Replacement of the wild XR with XR^{MUT} increased xylose consumption but was insufficient to drive the xylose flux into ethanol in a micro-aerobic condition (Fig. 3(C) and Fig. 4).

To evaluate the xylose-fermenting abilities of the recombinant *S. cerevisiae* strains expressing XR^{MUT}, kinetic parameters of all microaerobic and oxygen-limited batch fermentations using a mixture of xylose and glucose (Fig. 3, Fig. 5 and Fig. 6) were estimated on the basis of the xylose consumed and summarized in Table 2. A 2-fold increase in XK activity in $SX3^{MUT}$ allowed a significant enhancement in xylose consumption and ethanol production and a decrease in xylitol production, compared with $SX2^{MUT}$. These synergistic effects of the modulation of cofactor preference and XK overexpression on ethanol production were observed previously, in which the $NADP^+$-preferable XDH mutant replaced NAD^+-dependent XDH, so cofactor in xylose metabolism was balanced by a line-up of NADPH/$NADP^+$ (Matsushika et al., 2008). A role of XK in the xylose metabolism was already explored by several research groups (Hahn-Hägerdal et al., 2007; Lee et al., 2003; Matsushika et al., 2009), and XK

expression level should be controlled not to affect host cell's viability (Toivari et al., 2001) and to maximize ethanol production from xylose (Jin et al., 2003). Two times more expression of XR^{MUT} and Tal expression in $SX5^{MUT}$ increased the ethanol production from xylose. Especially, 3.2- and 1.5-fold increases in ethanol productivity and yield were achieved relative to $SX3^{MUT}$. As already mentioned, acetate strongly inhibits cell growth in xylose (Supplement 1). Minimization of acetate inhibition by *ALD6* knock-out in $SX6^{MUT}$ triggered xylose consumption rate and ethanol production rate by a 1.2-fold in comparison to $SX5^{MUT}$. The same positive effect of Tal overexpression and *ALD6* deletion was realized in a batch fermentation of *S. cerevisiae* expressing the wild types of XR and XDH from *P. stipitis* (Sonderegger et al., 2004; Walfridsson et al., 1995). With respect to xylose utilization as shown in Fig. 5(B), 47 g/L xylose was completely used to produce cell mass and ethanol at the apparent yields of 21% and 26%, respectively. When comparing to those of cell mass (15%) and ethanol (41%) to glucose as shown in Fig. 5(B) and other figures, low yield of ethanol to xylose might be owing to higher yield of cell mass to xylose than glucose in the microaerobic condition. More reduction of oxygen content in culture broth boosts ethanol production with a higher yield. Oxygen-limited fermentation of $SX6^{MUT}$ resulted in 1.4-, 1.2- and 1.5-fold enhancement in ethanol concentration, productivity and yield, respectively, when comparing with microaerobic fermentation of the same strain. It is interesting to note that the disruption of the *ALD6* gene did not affect the growth rate of $SM6^{MUT}$ and rather improved ethanol production from xylose by reducing acetate formation.

CONCLUSION

Expression of XR^{MUT} with NADH-preference and NAD^+-dependent XDH wild type, and overexpression of endogenous XK in recombinant *S. cerevisiae* D452-2 lead to a remarkable reduction of xylitol production. More expression of XR^{MUT}, Tal overexpression and *ALD6* deletion exploded xylose consumption and ethanol production with 39% ethanol yield and 0.25 g l^{-1} h^{-1} productivity. To increase the rates of xylose consumption and ethanol production, activities of xylose metabolic enzymes should be balanced by more expression of XR^{MUT}. On the basis of cofactor-balanced *S. cerevisiae* systems, more researches are in progress to modulate enzyme expression levels in the xylose metabolism and PP pathway.

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Table 1. *S. cerevisiae* strains and plasmids used in this study.

Name	Characteristics	Source
S. cerevisiae		
D452-2	*Matα, leu2, his3, ura3, can1*	Watanabe 2007
$SX2^{WT}$	D452-2 transformed with $YIpXR^{WT}$-XDH, *URA3*	This study
$SX2^{MUT}$	D452-2 transformed with $YIpXR^{MUT}$-XDH, *URA3*	This study
$SX3^{WT}$	$SX2^{WT}$ transformed with δISXK, *URA3*	This study
$SX3^{MUT}$	$SX2^{MUT}$ transformed with δISXK, *URA3*	This study
$SX5^{MUT}$	$SX3^{MUT}$ transformed with YEpM4-XR^{MUT} and p423PsTAL	This study
$SX6^{MUT}$	$SX5^{MUT}$ Δ*ALD6*	This study
Plasmid		
YIp5	*URA3*, no *S. cerevisiae* ori	Lee 200
p423GPD	P_{GPD}-MCS-T_{CY1}, *HIS3*, 2 micron ori	ATCC
pAUR101	*AUR1-C*	TAKARA Co.
YEpM4-XR^{WT}	P_{PGK}-*XYL1*^{WT}-T_{PGK}, *LEU2*, 2 micron ori	Watanabe 2007
YEpM4-XR^{MUT}	P_{PGK}-*XYL1*^{R276H}-T_{PGK}, *LEU2*, 2 micron ori	Watanabe 2007
pPGK-XDH	P_{PGK}-*XYL2*^{WT}-T_{PGK}, *URA3*, 2 micron ori	Watanabe 2007
δISXK	P_{GPD}-*XYL3*^{WT}-T_{GPD}, *neo*^{R}, δ-chromosomal integration, no *S. cerevisiae* ori	Lee 2003
$YIpXR^{WT}$-XDH	YIp5, P_{PGK}-*XYL1*^{WT}-T_{PGK}- P_{PGK}-*XYL2*^{WT}-T_{PGK}, *URA3*	This study
$YIpXR^{MUT}$-XDH	YIp5, P_{PGK}-*XYL1*^{R276H}-T_{PGK}- P_{PGK}-*XYL2*^{WT}-T_{PGK}, *URA3*	This study
p423PsTAL	p423GPD, P_{GPD}- *PsTAL*-T_{CY1}, *HIS3*, 2 micron ori	This study
pAUR_d_ALD6	pAUR101 with 800 bp of the truncated *S. cerevisiae ALD6* gene	This study

Table 2. Summarized results of microaerobic and oxygen-limited batch fermentations of recombinant *S. cerevisiae* strains using xylose and glucose at 30°C and pH 5.5.

S. cerevisiae strain	Culture condition	Consumed xylose ($g\ l^{-1}$)	Produced ethanol ($g\ l^{-1}$) [a]	Xylose consumption rate ($g\ l^{-1}\ h^{-1}$)	ethanol production rate ($g\ l^{-1}\ h^{-1}$)
$SX2^{MUT}$	microaerobic	11.3	0.21	0.14	0.003
$SX3^{MUT}$	microaerobic	28.9	5.05	0.35	0.06
$SX5^{MUT}$	microaerobic	39.5	10.6	0.71	0.19
$SX6^{MUT}$	microaerobic	47.5	12.4	0.85	0.22
	oxygen-limited [b]	43.2	16.8	0.64	0.25

S. cerevisiae strain	Culture condition	Yield ($g\ g^{-1}$, %) [a]			
		Ethanol	Xylitol	Acetate	Glycerol
$SX2^{MUT}$	microaerobic	1.9	27.8	13.0	1.5
$SX3^{MUT}$	microaerobic	17.5	0.0	8.7	0.8
$SX5^{MUT}$	microaerobic	26.9	3.8	4.7	2.1
$SX6^{MUT}$	microaerobic	26.0	5.9	0.0	2.3
	oxygen-limited [b]	38.9	3.7	1.0	2.5

All values were obtained after glucose depletion.

[a] The values were calculated on the basis of consumed xylose.

[b] The values in oxygen-limited fermentation were gained after stopping glucose feeding and changing the conditions to the oxygen-limited environment.

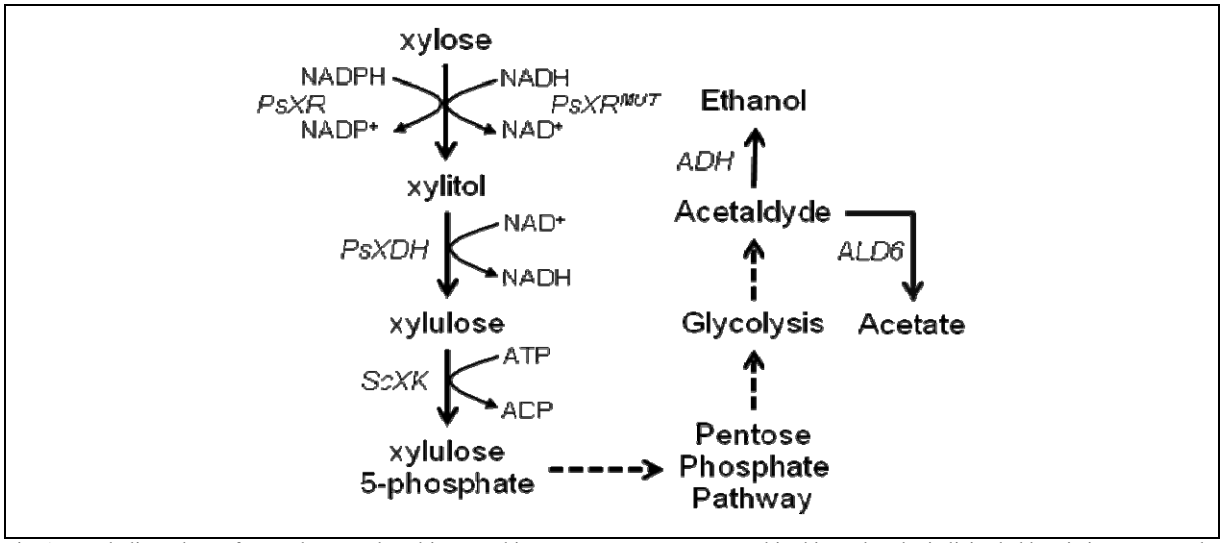


Fig. 1. Metabolic pathway from xylose to ethanol in recombinant *S. cerevisiae* constructed in this study. The italicized abbreviations present the xylose metabolic enzymes as follows: *PsXR*, NADPH-dependent wild type of *P. stipitis* xylose reductase; *PsXR*^{MUT}, NADH-preferable mutant of *P. stipitis* xylose reductase; *PsXDH*, NAD$^+$-dependent wild type of *P. stipitis* xylitol dehydrogenase; *ScXK*, *S. cerevisiae* xylulokinase; *ADH*, alcohol dehydrogenase; *ALD6*, aldehyde dehydrogenase 6..

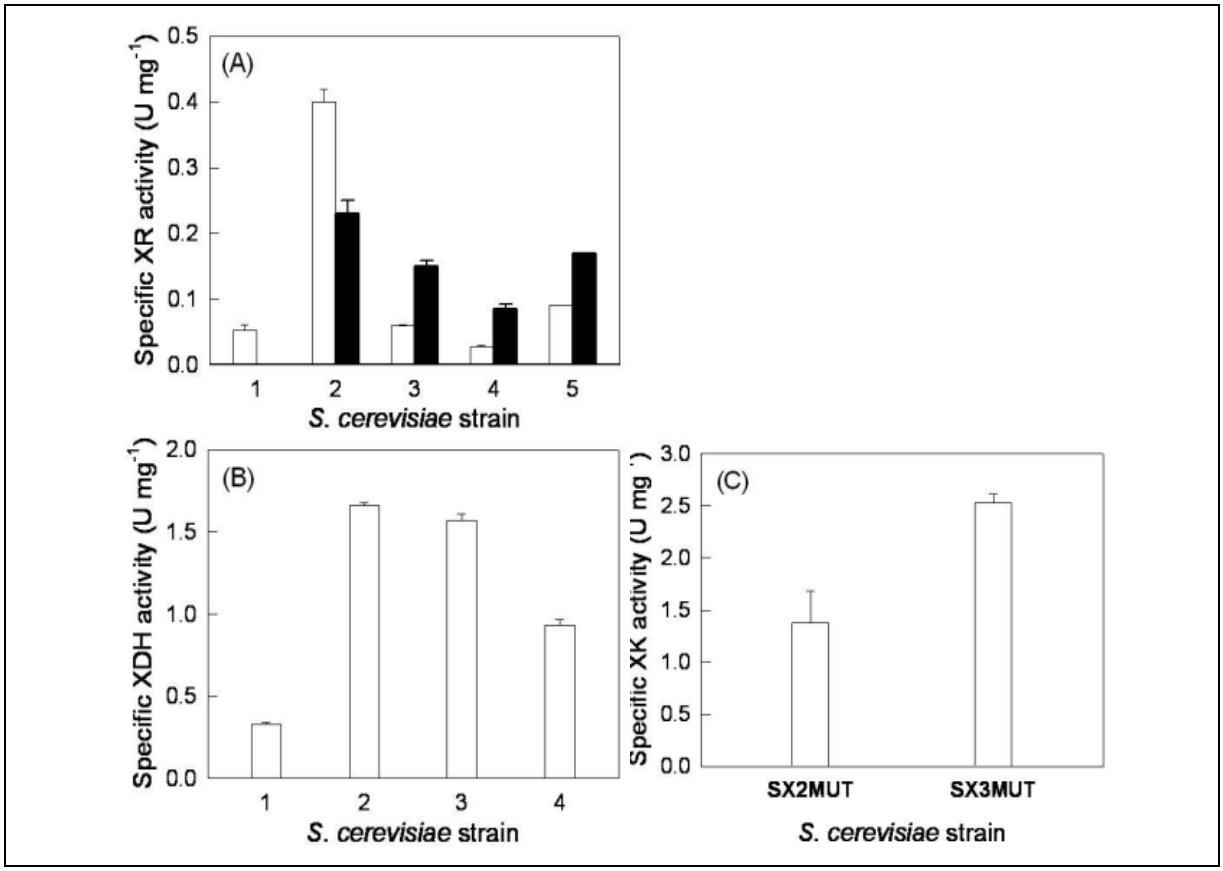


Fig. 2. Specific enzyme activities of XR (A), XDH (B) and XK (C) in recombinant *S. cerevisiae* strains containing both YEpM4 and pPGK (lane 1), YEpM4-XR^{WT} and pPGK-XDH (lane 2), YEpM4-XR^{MUT} and pPGK-XDH (lane 3), SX2^{MUT} (lane 4) and SX6^{MUT} (lane 5). Specific activity was obtained on the basis of intracellular protein concentration. For evaluation of cofactor preference, NADPH (white bar) and NADH (black bar) were used individually in panel (A) and NAD$^+$ was used only in panel (B). The experiments were repeated in triplicate.

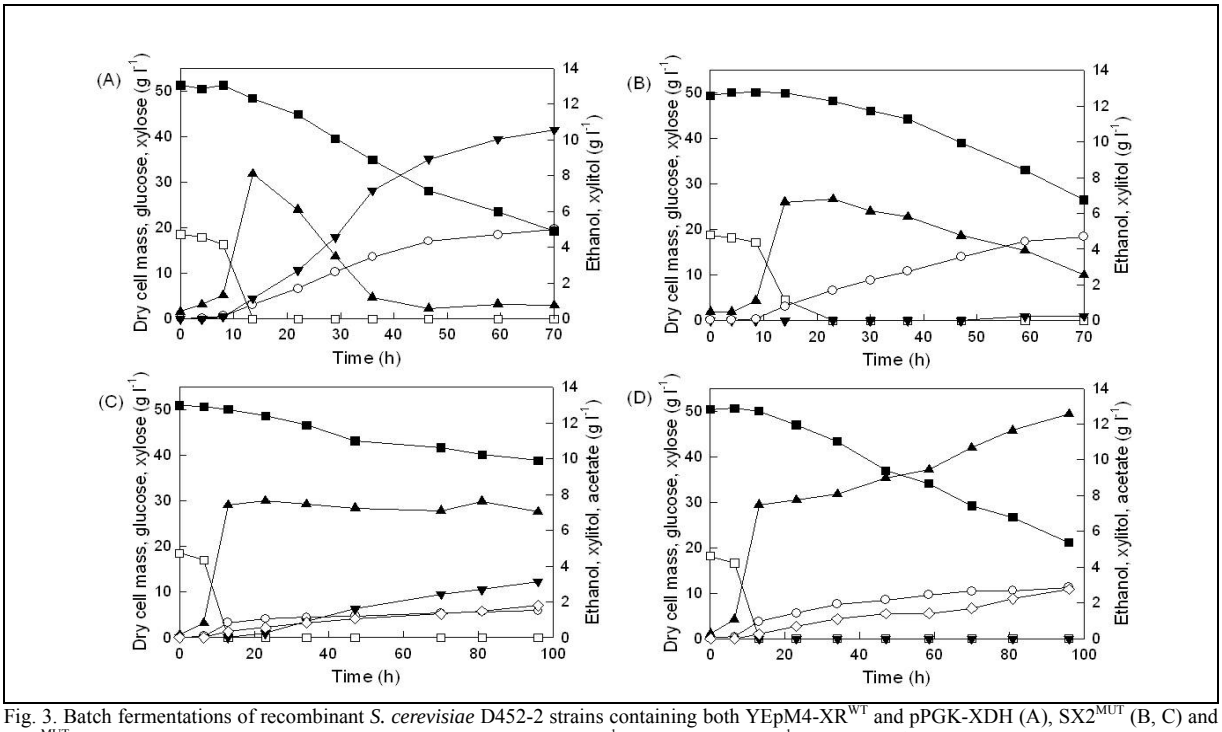


Fig. 3. Batch fermentations of recombinant *S. cerevisiae* D452-2 strains containing both YEpM4-XR^{WT} and pPGK-XDH (A), SX2^{MUT} (B, C) and SX3^{MUT} (D) in a bioreactor containing 0.5 l of YP medium with 20 g l^{-1} glucose and 50 g l^{-1} xylose at 30°C and pH5.5. Agitation and aeration rates were fixed at 500 rpm and 1.0 vvm for aerobic condition (A, B), and at 300 rpm and 0.1 vvm for microaerobic condition (C, D). Symbols denote as follows; ○, dry cell mass; □, glucose concentration; ■, xylose concentration; ▲, ethanol concentration; ▼, xylitol concentration.

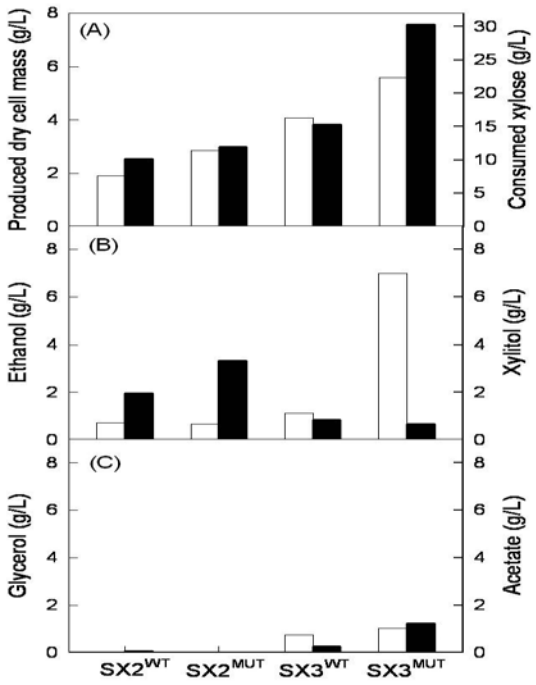


Fig. 4. Results of microaerobic fermentations of recombinant *S. cerevisiae* SX2^{WT}, SX2^{MUT}, SX3^{WT} and SX3^{MUT} strains in a 500 ml-scale flask containing 100 ml SC medium containing 53~61 g l^{-1} xylose as a sole carbon source. Initial optical density was adjusted at around 10~15. Environmental conditions were fixed at 30°C and 90 rpm. After 80 hr cultivation, the culture broths were subjected to the instrumental analysis. The white and black bars indicate the values on the left and right y-axises, respectively.

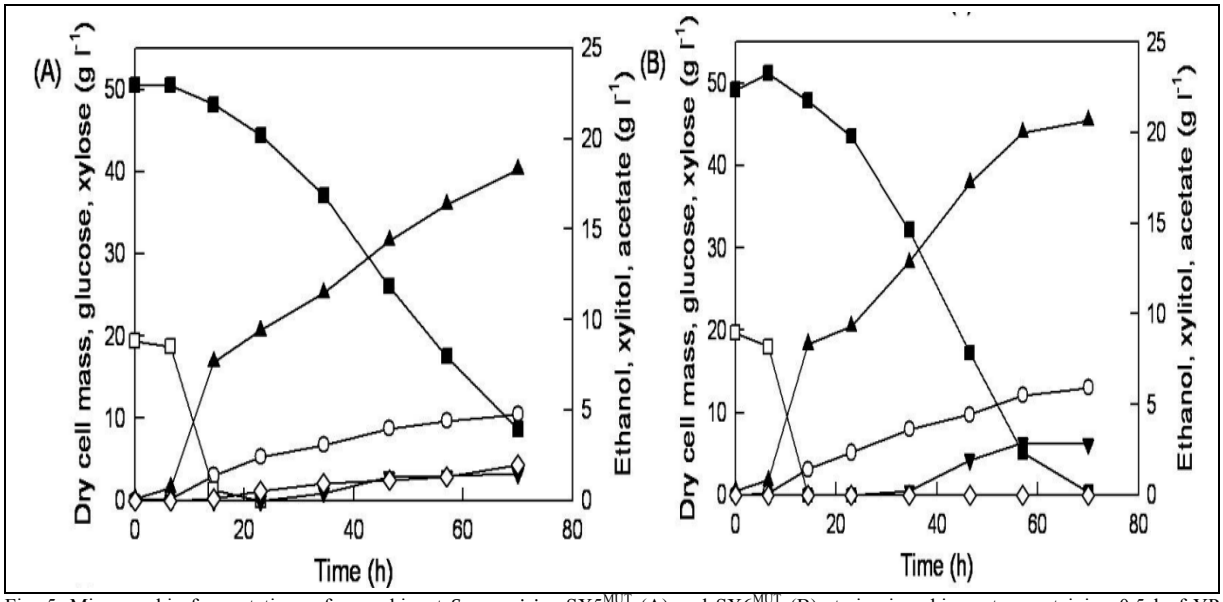


Fig. 5. Microaerobic fermentations of recombinant *S. cerevisiae* $SX5^{MUT}$ (A) and $SX6^{MUT}$ (B) strains in a bioreactor containing 0.5 l of YP medium with 20 g l^{-1} glucose and 50 g l^{-1} xylose at 300 rpm and 0.1 vvm. Symbols denote as follows; ○, dry cell mass; □, glucose concentration; ■, xylose concentration; ▲, ethanol concentration; ▼, xylitol concentration; ◇, acetate concentration..

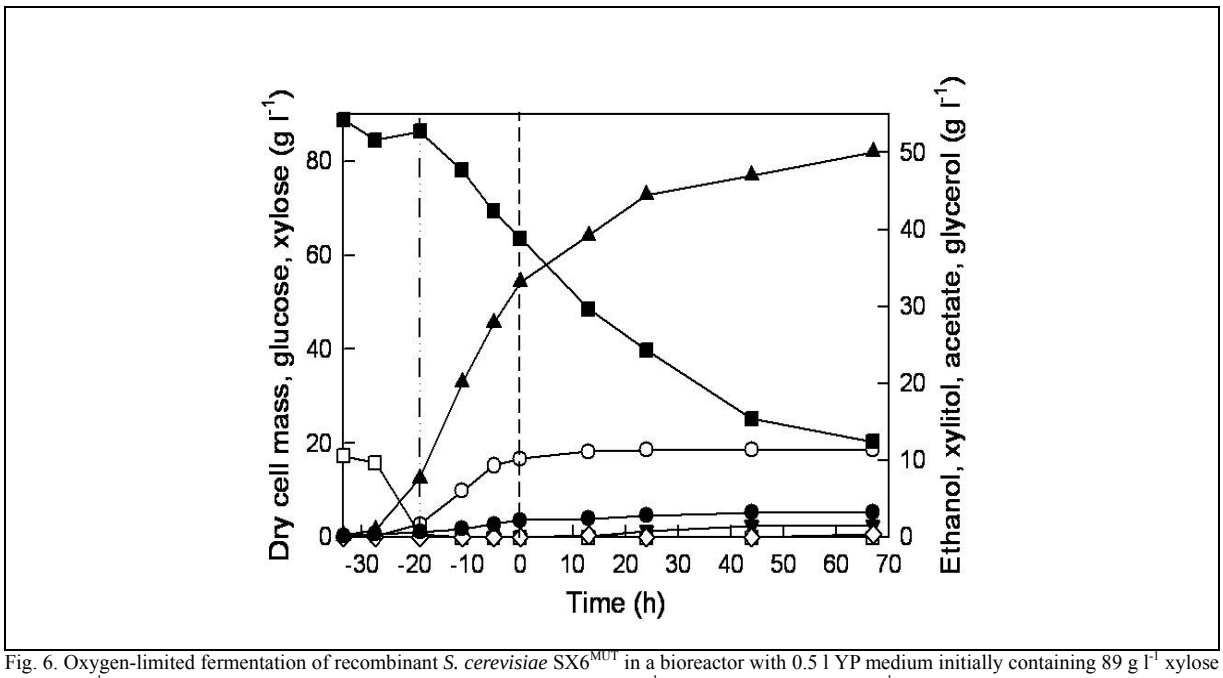
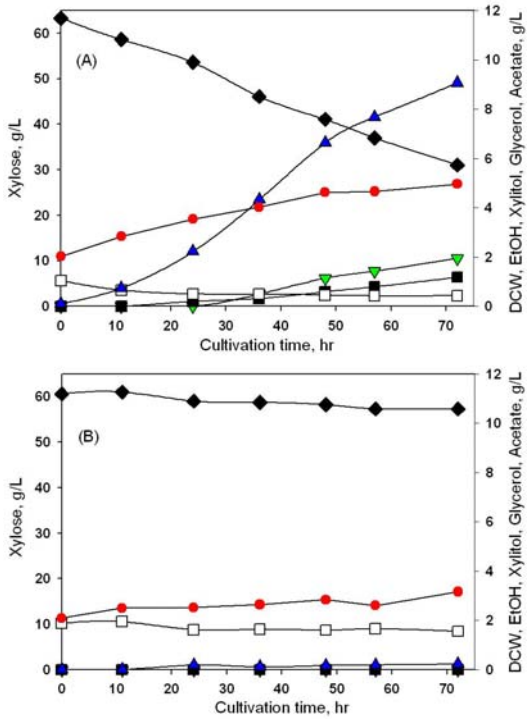


Fig. 6. Oxygen-limited fermentation of recombinant *S. cerevisiae* $SX6^{MUT}$ in a bioreactor with 0.5 l YP medium initially containing 89 g l^{-1} xylose and 17 g l^{-1} glucose. The two-dotted line indicates the start point of 600 g l^{-1} glucose feeding at 3 ml h^{-1} of feed rate. The dashed line presents feeding stop and the change of agitation and aeration rate to 200 rpm and 0.06 vvm, respectively. Symbols denote as follows; ○, dry cell mass; □, glucose concentration; ■, xylose concentration; ▲, ethanol concentration; ▼, xylitol concentration; ◇, acetate concentration; ●, glycerol concentration.



Supplement 1. Micro-aerobic fermentations of recombinant *S. cerevisiae* $SX3^{MUT}$ in 100 ml SC medium containing 60 g l^{-1} xylose without (A) or with (B) 2 g l^{-1} acetic acid. Initial optical density was adjusted at around 10. Environmental conditions were fixed at 30°C and 90 rpm. Symbols denote as follows; ●, dry cell mass; ◆, xylose; ▲, ethanol; ▼, xylitol; □, acetate; ■, glycerol..