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Enhanced production of xylitol from xylose by expression of *Bacillus subtilis* arabinose:H^+ symporter and *Scheffersomyces stipitis* xylose reductase in recombinant *Saccharomyces cerevisiae*

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Highlights

- Xylitol is a sugar alcohol used as a low calorie sweetener and platform chemical.
- Xylose transport is a curial problem to increase xylitol productivity from xylose.
- *B. subtilis* arabinose:H^+ symporter (AraE) was expressed in *S. cerevisiae* variants.
- AraE and *XYL1* expression led to 18 fold higher xylitol production than the control.
- AraE took up xylose efficiently and gave *S. cerevisiae* high xylitol productivity.

Abstract

Inefficient transport of xylose into *Saccharomyces cerevisiae* is a major hurdle for production of xylitol, a natural sweetener with five carbons. To facilitate the xylose transport and hence increase xylose conversion to xylitol, the *araE* gene encoding an arabinose:H^+ symporter (AraE) from *Bacillus subtilis* and the *XYL1* gene from *Scheffersomyces stipitis* were expressed in *Saccharomyces cerevisiae* EBY.VW4000, a *hxt* null mutant. The resulting strain of EXHA exhibited 4.1 fold increases in xylose consumption rate and xylitol productivity, relative to the control strain without AraE. Also, overexpression of AraE in wild type *S. cerevisiae* D452-2 having all hexose transporters and the *XYL1* gene increased both xylose consumption and xylitol production considerably. In a glucose-limited fed-batch culture with intermittent addition of xylose,

the DXXA strain with multiple copies of *araE* and *XYL1* produced 177.8 g/L xylitol with 2.47 g/L-h productivity, which were 26.9 and 17.6 times higher than those for a batch culture of the DX strain expressing the *XYL1* gene only, respectively. It was concluded that *B. subtilis* AraE might be a potent xylose transporter and conferred much higher xylose-consuming and xylitol-producing abilities to *S. cerevisiae*.

Keywords: AraE, xylose, *Saccharomyces cerevisiae*, xylitol, *XYL1*, fed-batch fermentation

Introduction

Xylitol is a representative sugar alcohol with caries-reducing effect and widely used as a low calorie sweetener [1]. As a platform chemical, xylitol can be also converted to various starting materials for plastics and directly utilized as a plasticizer for polymer interactions [2]. For biological production of xylitol, various microbial processes have been considered by using either xylose fermenting or non-xylose fermenting microbes [3-5]. Among those microbes, *Saccharomyces cerevisiae* is a GRAS microorganism used for various foods and biochemicals manufacturing. Even though *S. cerevisiae* does not metabolize xylose because of the absence of the xylose metabolic pathways, expression of the *XYL1* gene encoding xylose reductase (XR) conferred the ability of producing xylitol from xylose with the theoretical yield of ~1 to recombinant *S. cerevisiae* (Fig. 1) [5, 6]. Various strategies have been reported to elevate xylitol productivity. Recently, co-expression of two types of xylose reductase enabled to utilize both NADPH and NADH cofactors and enhanced xylitol productivity. Additional expression of the *ZWF* and *ACS1* genes increased final xylitol concentration by 1.23 fold, relative to that without their expression [7]. Sequential consumption of glucose and xylose was overcome by expression of a cellobiose transporter and a β-glucosidase and a 40% enhancement in xylitol production was achieved [8]. Other metabolic engineering strategies including cofactor regeneration mediated by the *ZWF1, GND1, PGI1, ALD6* and *ACS1* genes and

fermentation strategies escaping from the glucose repression were well summarized in a review [5].

Among several points for consideration, xylose transport is one of key factors affecting efficient xylitol production. A kinetic analysis of xylose transport revealed that *S. cerevisiae* takes up xylose via high-affinity glucose transporters at low glucose concentration [9]. And any natural and endogenous sugar transporter specific for xylose only has not been found in wild types of *S. cerevisiae* to date, even though many xylose transporters were identified in other yeasts. Recently, evolutionary engineering of xylose-fermentable recombinant *S. cerevisiae* resulted in the identification of endogenous HXT7 (F79S) mutant with an improved xylose uptake rate [10]. The *S. cerevisiae* GAL2 (N376F) mutant was constructed to not only lose the transporting ability of hexoses but also possess the highest affinity for xylose (91.4 mM of Km) [11].

Meanwhile, xylose has a similar structure to arabinose, of which transport is mediated by arabinose: H^+ symporter [12]. The symporter named AraE has been found in many bacteria including *Bacillus subtilis* and *Corynebacterium glutamicum*, and its arabinose-transporting ability has been investigated in many reports [13-15]. Overexpression of AraE in *B. subtilis* increased cell growth and xylose consumption [16], and AraE from *C. glutamicum* and its mutant were also proven to increase xylose transport capacity in *S. cerevisiae* [17].

In this study, the *B. subtilis araE* gene was expressed in a wild type of *S. cerevisiae* D452-

2 and *hxt* null mutant of *S. cerevisiae* EBY.VW4000 harboring the *XYL1* gene from *Scheffersomyces stipitis* in order to identify its functional ability of xylose transport and increase xylitol production from xylose. Expression of GFP-fused AraE was tried to investigate the exact localization of AraE in the cytoplasmic membrane. *S. cerevisiae* EBY.VW 4000 without eighteen hexose transporters was used to exactly evaluate the xylose-transporting activity of AraE without hindrance of endogenous hexose transporters, previously known as xylose transporter in *S. cerevisiae*. A series of *XYL1* or AraE expression systems were constructed to optimize xylitol-producing *S. cerevisiae* strains, of which performances were assessed in batch cultures with xylose and maltose or glucose as energy source. Finally, fed-batch cultures with a glucose-limited strategy and intermittent xylose addition were carried to maximize xylitol production from xylose.

Materials and Methods

Strains and plasmids

S. cerevisiae D452-2 and EBY.VW4000 strains [18] were used as host strains for expression of EGFP and AraE, and production of xylitol. *Escherichia coli* TOP 10 (Invitrogen, Carlsbad, CA, USA) was used for genetic manipulation. Plasmids pDGYC2-AraE [16] and pITy_GPD_wXR [7] were the gene sources for the *B. subtilis araE* and *S. stipitis XYL1* genes, respectively. Expression of all recombinant genes were under the control of constitutive promoters: *GPDp, TEFp, HXTp,* and *PGKp*. All yeast strains and plasmids used in this study are listed in Table 1.

Genetic manipulation

To investigate the localization of AraE in the cytoplasmic membrane, the gene coding for enhanced green fluorescent protein (EGFP) was PCR-amplified from plasmid EGFP-pBAD using two DNA digomers of F-PstI-EGFP and R-SalI-EGFP. After digestion of the PCR product with *Pst*I and *Sal*I, the *egfp* gene was cloned into pRS425GPD vector treated with the same restriction enzymes, resulting in the construction of plasmid p425GPD_EGFP. The *araE* gene without the translational stop codon was PCR-amplified from plasmid pDGYC2-AraE using the primers of F-BamHI-AraE and R-PstI-AraE-no. To combine AraE with N-terminus of EGFP protein, the amplified *araE* gene and plasmid

pRS425GPD_EGFP were digested with *Bam*HI and *Pst*I, and the *araE* gene was inserted in front of the *EGFP* gene, resulting in the construction of plasmid p425GPD_AraE+EGFP. To introduce the *araE* gene in the chromosome of *S. cerevisiae* EBY.VW4000 and D452-2 strains, four *araE* expression cassettes consisting of a promoter sequence, *araE* gene and *CYC*1 terminator in a row were designed. The *araE* gene amplified with the primers of F-BamHI-AraE and R-PstI-AraE was treated with *Bam*HI and *Pst*I restriction enzymes, and combined with plasmids p425GPD, p425TEF, p425HXT and p425PGK digested with the same enzymes. Finally, the *araE* expression plasmids with different promoters (*GPDp, TEFp, HXTp* and *PGKp*) were constructed: p425GPD_AraE, p425TEF_AraE, p425HXT_AraE and p425PGK_AraE. The four *araE* expression cassettes were amplified from each plasmid with the name of the promoter by using PCR primer sets: a forward primer of F-SacI-GPD, F-SacI-HXT, F-SacI-TEF or F-SacI-PGK, and a backward primer of R-SacI-CYC. Each amplified *araE* expression cassette was treated with *Sac*I and ligated with plasmid pRS405 restricted with *Sac*I, resulting in the construction of plasmids p405GPD_AraE, p405TEF_AraE, p405HXT_AraE and p405PGK_AraE. For multiple chromosomal integration of the *araE* gene, the *HXT*p_*araE_CYC*t cassette was amplified from plasmid p425HXT_AraE with F-SacI-HXT and R-SacI-CYC primers, treated with *Sac*I restriction enzyme, and ligated with plasmid pδNEO. The resulting plasmid was named pδNEO_HXT_AraE. To express the *XYL1* gene under the control of the *GPD* promoter, the *GPD*p_*XYL1_CYC*t cassette was amplified from pITy_GPD_wXR [7] vector

with two PCR primers of F-SacI-GPD and R-SacI-CYC. The PCR product was digested with *Sac*I and combined with plasmids pRS403 and pRS406, resulting in the construction of p403GPD_XR and p406GPD_XR, respectively. The chromosomal integration in all strains were confirmed by DNA sequencing. All PCR primers used in this study are listed in Table S1.

E. coli transformation followed the chemical method using $CaCl_2$ [19]. Transformation of *S. cerevisiae* strains was carried out using the Alkali-Cation™ Yeast Transformation kit (MP Biomedicals, Santa Ana, CA). Yeast transformants were then spread on YNB medium with 20 g/L gluco1se or maltose, 20 g/L bacto-agar, and appropriate amino acids and nucleotide supplements, and incubated at 30°C for 2-3 ds. Variants of plasmid pRS403 were linearized by the *Nhe*I restriction enzyme, and pRS405 and pRS406 were digested by the *Xcm*I enzyme prior to yeast transformation. For multi-copy integration into the δ-sequence, plasmid pδNEO_HXT_AraE was cut with *Sal*I and pITy_GPD_wXR was treated with *Xho*I prior to transformation. The transformants grown in YP medium with 20 g/L glucose and 1 g/L of G418 antibiotic (Calbiochem Co., USA) were selected. Chromosomal integration was confirmed by PCR.

Culture conditions

YNB medium (0.67% yeast nitrogen without amino acid (Sigma, St. Louis, MO, USA) and 0.192% yeast dropout medium supplement (Sigma, USA)) [20] with 20 g/L glucose

or 20 g/L maltose and appropriate amino acids or nucleotide was used for transformant selection and seed culture. Pre-cultivation was carried out at 30°C in YP medium (10 g/L yeast extract and 20 g/L peptone) with 20 g/L glucose or 20 g/L maltose. Batch fermentations of recombinant yeast cells were performed in duplicate at 30°C and 250 rpm in a 500 mL-scale baffled flask (Thermo Fisher Scientific, Waltham, MA, USA) with 100 mL of YP medium containing 35-60 g/L xylose and 20 g/L maltose for EBY.VW4000 variants or 20 g/L glucose for D452-2 variants. For fed-batch fermentation, the pre-grown cells were transferred into an 1 L baffled flask with 500 mL of YP medium with 20 g/L glucose. After 20 h of incubation at 30°C and 250 rpm, the cells were harvested and suspended in sterile water. The suspended cells were inoculated to a 2.5 L bioreactor (KoBiotech, Incheon, Korea) with an 1 L working volume of YP medium containing 20 g/L glucose and 60 g/L xylose. An initial optical density of the culture was adjusted to be about 5.0. An agitation speed, aeration rate and culture temperature were set at 500 rpm, 1 vvm and 30°C, respectively. Acidity was controlled at pH 5.0 by addition of 2 N HCl and 2 N NaOH. After depletion of glucose initially added, a concentrated xylose solution (600 g/L) was supplemented intermittently into the culture broth in order to control xylose concentration between 20 and 60 g/L, and a concentrated glucose solution (600 g/L) was fed continuously at 1.8 g/L-h of feed rate..

Xylose reductase activity assay

Recombinant *S. cerevisiae* strains expressing the *XYL1* and *araE* genes were grown in 10 mL YP medium with 20 g/L glucose at 30°C and 200 rpm for 10 h. The cells were harvested by centrifugation at 13,000 rpm and room temperature for 10 min. After removal of the supernatant, appropriate amount of the Y-per solution (Pierce, Rockford, IL, USA) was mixed with the cell pellets and the cell suspension was kept at room temperature for 20 min with gentle agitation. After centrifugation at 13,000 rpm and room temperature for 10 min, the supernatants with crude enzymes were collected for enzyme activity assay. Protein concentration in the crude extract was measured by the Bradford method using bovine serum albumin (RMBIO, Missoula, MT, USA) as the standard. The reaction mixture consisted of 0.4 mM NADPH, 50 mM potassium phosphate buffer (pH 6.0), 100 mM xylose solution and the crude enzyme solution with appropriate dilution. NADPH oxidation was monitored by a spectrophotometer (Biotek, VT, USA) at 340 nm. One unit of xylose reductase activity was defined as the amount of enzyme able to oxidize 1μmol of NADPH per minute at the above condition.

Instrumental analysis

Green fluorescence on yeast cells were visualized by a Zeiss Axiovert 2000M fluorescence microscope (Carl Zeiss, Germany). Images were acquired by a charge-coupled device (AxioCam MRm, Carl Zeiss, Germany), and pseudo-color images were produced by the

AxioVision 4.5 software (Carl Zeiss, Germany). Metabolites in culture broth were analyzed by a high performance liquid chromatography (1200 series, Agilent Technologies, Santa Clara, CA, USA) equipped with a H^+ packed column (Rezex ROA organic acid, Phenomenex Inc., Torrance, CA, USA) heated at 60°C [21]. A solvent of 5 mM H_2SO_4 at a flow rate of 0.6 mL/min was used as mobile phase [19]. A refractive index (RI) detector and variable wavelength (VW) detector (1200 series, Agilent Technologies Inc, USA) were used for detection. Optical density was measured by a spectrophotometer (Ultrospec 8000, GE Healthcare Co., USA) at 600 nm. Optical density value was then multiplied with a pre-calculated conversion factor of 0.44 OD_{600nm}/DCW to obtain dry cell weight (DCW) [21].

Results

Expression and localization of B. subtilis AraE in S. cerevisiae

Before evaluation of *B. subtilis* AraE as a potential xylose transporter, its exact localization in the cytoplasmic membrane of *S. cerevisiae* was explored. Recombinant *S. cerevisiae* D452-2 expressing EGFP or EGFP-tagged AraE were constructed and visualized with a fluorescence microscopy. *S. cerevisiae* D452-2 (Fig. 2A) and its recombinant strain harboring plasmid pRS425GPD_AraE (Fig. 2B) showed no fluorescence. Green fluorescence was spread inside the yeast cells expressing EGFP only, whereas ring-shaped fluorescence was observed in the yeast cells expressing the AraE-EGFP fusion protein (Fig. 2D). According to a report in which GFP-fused membrane protein showed ring-shaped fluorescence [17], it was certain that AraE was successfully expressed and localized in the cytoplasmic membrane of recombinant *S. cerevisiae* D452-2 harboring plasmid pRS425GPD_AraE+EGFP, and might play its role exactly as a sugar transporter.

D-Xylose transporting ability of B. subtilis AraE in hxt null mutant

Since some endogenous hexose transporters in wild type *S. cerevisiae* are able to take up xylose as well as glucose, a *hxt* null mutant of *S. cerevisiae* EBY.VW4000 without eighteen hexose transporters and unable to grow on glucose only was used to exactly

analyze the xylose-transporting activity of AraE [11, 22]. As *S. cerevisiae* cannot metabolize D-xylose [23, 24], expression of xylose reductase in the EBY.VW4000 strain and measurement of xylose consumption rate and xylitol productivity could be indirect methods for analysis of xylose-transporting activity of AraE. The *araE* gene from *B. subtilis* and the *XYL1* gene coding for xylose reductase (XR) from *S. stipitis* were expressed in *S. cerevisiae* EBY.VW4000. The AraE expression cassettes were constructed with a series of constitutive promoters (*TDH3p*, *HXTp*, *TEFp*, and *PGKp*) and introduced into the chromosome in order to investigate the effect of AraE expression level on xylose transport. The yeast strains expressing the *araE* and *XYL1* genes were cultured in YP medium with 50 g/L xylose and 20 g/L maltose. After 48 h of batch cultures, xylose and maltose consumption rates, and xylitol productivity were obtained (Fig. 3). Irrespective of promoter types, AraE expression increased both the rates of xylose consumption and xylitol production by 2.5-4.1 folds, relative to those for the control without the AraE expression (EX strain). The *HXTp-araE-CYC1t* cassette in the EXHA strain gave the best performance of 5.14 g/L xylose consumption with 0.11 g/L-h of rate and 4.08 g/L xylitol production with 0.09 g/L-h of rate. Meanwhile, AraE expression did not exert the change of maltose utilization (Fig. 3A). In addition, the EXHA cells did not form colonies on YP medium with 20 g/L glucose, galactose, fructose or mannose as a sole carbon source (Figure S1). Considering the batch culture using the *hxt* null mutant of EBY.VW400 strains, it was clearly accessed that *B. subtilis* AraE protein is a sugar transporter specific

for xylose in *S. cerevisiae*. The *HXT* promoter controlling the transcription of the hexose transporter 7 gene is a strong and constitutive promoter and has been used to express various heterologous genes in *S. cerevisiae* [25, 26]. Since hexose transporters are ready to take up the corresponding sugars in the early growth phase [27], the *HXT* promoter might be the best choice for expression of heterologous sugar transporter. The AraE expression cassette with the *HXT* promoter was used for further investigation of the enhanced xylose transport phenomena.

Expression of B. subtilis AraE in a yeast strain with all hexose transporters

As described above, expression of AraE recovered the xylose-transporting activity in a specific condition using the *hxt* null mutant of EBY.VW4000 strain. To evaluate the xylose transporting-ability of AraE in general condition, a wild type of *S. cerevisiae* D452-2 with all hexose transporters was engineered to the DXA strain by the chromosomal integration of multiple copies of the *araE* gene and a single copy of the *XYL1* gene, of which transcription were controlled by the *HXT* and *GPD* promoters, respectively. As the control, the DX strain, a variant of D452-2 strain with a single copy of *XYL1* was constructed. The *XYL1* gene expression conferred 0.37-0.45 U/mg of specific XR activities to the DX and DXA strains, respectively, whereas the D452-2 strain showed a basal level of XR activity (Table 2). As shown in batch cultures in YP medium with 35 g/L xylose and 20 g/L glucose, the control DX strain showed typical profiles of

sugar consumption and xylitol production (Fig. 4A). After complete consumption of glucose, xylose was converted to xylitol, accompanied by consumption of ethanol produced from glucose. Since xylose is transported into *S. cerevisiae* cells by hexose transporters [22], xylitol could be produced from xylose in the DX strain without any xylose-specific transporters. Meanwhile, AraE expression in the DXA strain significantly increased xylose consumption rate (0.39 g/L-h) and xylitol production (18.1 g/L), relative to 0.14 g/L-h of the consumption rate and 6.6 g/L of xylitol concentration for the DX strain (Fig 4B). Titers of by-products of glycerol and acetate were kept at less than 0.04 g/L for both the strains throughout the cultures.

To increase xylose-consuming and xylitol-producing abilities, the DXA strain was additionally transformed with plasmid pδNEO_HXT_AraE and its transformants were selected against over 1 g/L G418 antibiotics. Higher concentration of G418 led to the δ-integration of more copy numbers of target gene [28]. In the same conditions of batch culture for DXA, the resulting strain with more copy numbers of the *araE* gene than the DXA strain did not show any improvement of xylose consumption and xylitol production (data not shown), suggesting that the AraE expression level in the DXA strain might be enough for xylose delivery. Based on a suggestion that additional expression of *XYL1* was essential for increasing xylitol metabolism in yeast [29], the transformation of plasmid p403GPD_XR into the DX and DXA strains resulted in the construction of the DXX and DXXA strains with two copies of the *XYL1* gene. By additional *XYL1* expression, the

DXX and the DXXA strains possessed about 1.4-fold higher specific activities than the DX and DXA strains (Table 2). To assess whether this enhanced XR activity increased xylitol production, the DXX and DXXA strains were grown in the same condition of the batch cultures for DX and DXA (Fig. 4C and 4D). As expected, the DXXA strain consumed 21.3 g/L xylose and produced 21.2 g/L xylitol with 0.44 g/L-h productivity, whereas the DXX strain produced 13.0 g/L xylitol with 0.27 g/L-h productivity. As a result, multiple expression of the *araE* and *XYL1* genes in the DXXA strain considerably enhanced xylose transport, xylose consumption and xylitol production.

Effect of AraE expression on fed-batch production of xylitol

Expression of AraE certainly conferred the enhanced ability of xylose inflow and xylitol production to the yeasts even though they possessed endogenous hexose transporters. As shown in Fig. 4, however, the catabolite repression phenomenon inhibited xylose uptake in the presence of glucose, leading to decreasing xylitol productivity [30, 31]. To escape from the glucose repression and expand the xylitol production stage, glucose-limited fed-batch cultures were performed by continuous supply of glucose and intermittent addition of concentrated xylose solution (Fig. 5). After depletion of initial glucose, the concentrated glucose solution was fed into the culture broth at a low feed rate to provide cellular energy and minimize the inhibitory effects of glucose on xylose consumption. In the fed-batch stage, glucose was not detected in the culture broth, and the glucose feeding

considerably increased the xylose-consuming ability of the recombinant *S. cerevisiae* strains and xylitol was produced linearly depending on xylose consumption (Fig. 5). The target concentration of xylose (20-60 g/L) was maintained well by one to three times addition of the concentrated xylose solution. The DXA strain metabolized xylose with 1.14 g/L-h of rate and produced 83.9 g/L xylitol finally, which were about 8% increments compared to the corresponding values for the DX strain. Especially, the same fed-batch cultures of the DXXA strain gave the best performance of xylose consumption and xylitol production (Fig. 5D). In 72 h of the fed batch culture, the DXXA strain produced 177.8 g/L xylitol with 2.47 g/L-h xylose consumption rate, while the DXX strain did 125.3 g/L xylitol with 1.74 g/-h xylose consumption rate.

Discussion

The absence of xylose-specific transporter and low competitiveness of xylose against hexoses onto sugar transporters are major hurdles to develop a xylose-based platform using *S. cerevisiae*. To solve this problem and increase xylitol productivity, in this study, *B. subtilis* arabinose:H^+ symporter of AraE was expressed in *S. cerevisiae* harboring the *XYL1* gene and able to convert xylose to xylitol. Because of the similar chemical structure of arabinose to xylose, heterogeneous arabinose transporters from *E. coli* and *C. glutamicum* were able to transport D-xylose into each host cell [32, 33]. Recently, expression of *C. glutamicum* AraE increased intracellular concentration of xylose in *S. cerevisiae* [17]. Based on our previous results showing that overexpression of *B. subtilis* AraE enhanced xylose transport in recombinant *B. subtilis* [16], we chose the *araE* gene from *B. subtilis* for our purpose. As expected, *B. subtilis* AraE was properly embedded in the cytoplasmic membrane of *S. cerevisiae* and its expression enhanced xylose transport and xylitol production in both the *hxt* null mutant and wild type of *S. cerevisiae*, of which results in batch and fed-batch cultures were summarized in Table 3. In batch cultures of recombinant S. cerevisiae with all hexose transporters, the DXS strain with AraE expression possessed about 2.8 times higher xylose-consuming and xylitol-producing performance than the DX strain without AraE introduction. Moreover, additional expression of *XYL1* in the DXXA strain showed more increment of xylose consumption

rate and xylitol production rate by 15%, relative to those for the DXA strain. These phenomena were also found in the fed-batch cultures. The glucose-limited fed-batch cultures of the DXXA strain showed 1.4-2.33 fold increases in all parameters for xylose consumption and xylitol production, compared with those for the DX, DXA and DXX strains. In addition, this fed-batch culture of the DXXA strain exhibited 17.6 times higher xylose-consuming and xylitol-producing rates than, and a 26.9 fold enhancement in final xylitol concentration relative to the batch culture of the DX strain. As expected, the yields of xylitol to consumed xylose in all cultures reached the theoretical yield of ~1. Considering all results in the batch and fed-batch cultures, it was certain that AraE expression in wild type of *S. cerevisiae* D452-2 led to a significant increment of xylose transport into the cells, and multiple expression of AraE and xylose reductase conferred a remarkable enhancement of xylitol production from xylose to recombinant *S. cerevisiae* DXXA.

Meanwhile, expression of heterogeneous xylose transporters has been attempted to enhance xylose uptake in *S. cerevisiae*. For example, xylose transporters of At5g59250 and At5g17010 from *Arabidopsis thaliana* provided 0.10 - 0.12 g/L-h of xylose consumption rate whereas the control showed 0.07 g/L-h [34]. Recently, xylose-transporting activities of *Candida intermedia* Gxf1, *S. stipitis* Sut1 and *A. thaliana* At5g59250 were evaluated by aerobic batch culture with 4 g/L xylose. *S. cerevisiae* TMB3043 expressing Gxf1 was grown to an OD_{620nm} of 3 in 260 h culture, which was

much higher than the cases of Sut1 and At5g59250 [35]. But, this enhanced xylose-transporting activity disappeared at over 15 g/L xylose. AraE from *C. glutamicum* and its mutant AraE(I178S) gave recombinant *S. cerevisiae* higher xylose-transporting capacity than *S. cerevisiae* HXT7p and Gal2p [17], but this activity was assessed in a spot assay using xylose. On the other hand, endogenous hexose transporters in *S. cerevisiae* have been engineered to be able to transport glucose and xylose simultaneously. A hexose transporter of HXT36 was able to take up both glucose and xylose with different rates and specificities as the same as other HXTs, but *S. cerevisiae* expressing HXT35-N367A mutant co-consumed 6 g/L xylose and 6 g/L glucose in 14 h culture [36]. Also, HXT7 (F79S) was able to deliver 3 g/L xylose in 50 h culture without a loss of glucose-transporting ability [10]. Gal2-N376F mutant showed xylose-specific transporting activity without D-glucose inhibition, but this characteristics was not evaluated in liquid-type culture [11]. Considering other reports described above, xylose concentration used in these studies was relatively low for their exact evaluation for mass production of biochemicals. In some cases using a *hxt* null *S. cerevisiae* only, it was unclear whether those transporters might be effective in wild type *S. cerevisiae* with all hexose transporters. It might be impossible to exactly compare xylose-transporting activity between *B. subtilis* AraE and other xylose or hexose transporters able to deliver xylose on the basis of the current reports. However, based on all results in the batch and fed-batch cultures in this study, it was firmly declared that *B. subtilis* AraE specifically transported xylose into *S.*

cerevisiae, which considerably enforced xylose metabolism to xylitol.

In conclusion, AraE was properly embedded in the cytoplasm membrane, which remarkably enhanced xylose transport in both the *hxt* null mutant and wild type of *S. cerevisiae*. Finally, multiple expression of AraE and xylose reductase in the DXXA strain triggered both xylose consumption and xylitol production by minimum 40%, relative to the cases without AraE expression. Considering these results, it was certain that *B. subtilis* AraE conferred high xylose-transporting activity to *S. cerevisiae.*

Author Agreement

Upon the submission of a manuscript entitled "Enhanced production of xylitol from xylose by expression of *Bacillus subtilis* arabinose:H^+ symporter and *Scheffersomyces stipitis* xylose reductase in recombinant *Saccharomyces cerevisiae*" written by coworkers and myself, all authors agree to submit this work to *Enzyme and Microbiology Technology*, and this work has not been published, submitted or being submitted to *Enzyme and Microbiology Technology* and other journals.

I appreciate your consideration of the manuscript for possible publication in *Enzyme and Microbiology Technology.*

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals by any of the authors.

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Figure Captions

Fig. 1. A scheme for xylitol production from xylose in recombinant *S. cerevisiae*. AraE is an arabinose:H+ symporter from *B. subtilis*, XR is xylose reductase, and PPP is a pentose phosphate pathway. HXT 1-17 and GalT represent seventeen hexose transporters and galactose transporter respectively.

Fig. 2. Microscopic pictures of representative fluorescence (left panels), differential interference contrast (middle panels), and merged (right panels) images of recombinant *S. cerevisiae* D452-2 cells transformed with following vectors: (A) pRS425GPD, (B) pRS425GPD_AraE, (C) pRS425GPD_EGFP, and (D) pRS425GPD_AraE+EGFP.

Fig. 3. Effects of AraE expression on xylose and maltose consumption (A), and xylitol production (B). Recombinant *S. cerevisiae* EBY.VW4000 strains harboring each AraE expression cassette and the *XYL1* gene were batch-wise cultivated in 100 ml of YP medium with 50 g/L xylose and 20 g/L maltose at 30 ºC and 250 rpm. All values were calculated after 48 h culture. Black bar indicates xylose consumption rate and white bar does maltose consumption rate in panel (A).

Fig. 4. Batch cultures of the DX (A), DXA (B), DXX (C) and DXXA (D) strains in 100

mL of YP medium containing 35 g/L xylose and 20 g/L glucose at 30°C and 250 rpm. The symbols are denoted as follows: ●, dry cell weight (DCW); ○, glucose; ▽, xylose; ■, ethanol; ▲, xylitol.

Fig. 5. Glucose-limited fed-batch cultures of the DX (A), DXA (B), DXX (C) and DXXA (D) strains in 1 L of YP medium with 60 g/L xylose and 20 g/L glucose at 30°C, pH 5.5, 1vvm and 500 rpm. The arrow with solid line indicates the addition of 80 mL of 600 g/L xylose. The arrow with dotted line designates the start point of continuous feeding of 600 g/L glucose at 1.8 g/L-h. The symbols are denoted as follows: ●, dry cell weight (DCW); ○, glucose; ▽, xylose; ■, ethanol; ▲, xylitol.

Table 1. The list of yeast strains and plasmids used in this study

Name	Description	Source
S. cerevisiae		
D452-2	*MAT α, leu2, his3, ura3, can1*	[9]
EBY.VW4000	*hxt17Δ hxt13Δ::loxP hxt15Δ::loxP hxt16Δ::loxP hxt14Δ::loxP hxt12Δ::loxP hxt9Δ::loxP hxt11Δ::loxP hxt10Δ::loxP hxt8Δ::loxP hxt514Δ::loxP hxt2Δ::loxP hxt367Δ::loxP gal2Δ stl1Δ::loxP agt1Δ::loxP ydl247wΔ::loxP yjr160cΔ::loxP*	[11]
EHA	EBY.VW4000 + p405HXT_AraE	In this study
EX	EBY.VW4000 + p403GPD_XR	In this study
EXHA	EX + pRS405HXT_AraE	In this study
EXPA	EX + pRS405PGK_AraE	In this study
EXGA	EX + pRS405GPD_AraE	In this study
EXTA	EX + pRS405TEF_AraE	In this study
DX	D452-2 + pRS406GPD_XR	In this study
DXA	DX + pδNEO_HXT_AraE	In this study
DXX	DX + pRS403GPD_XR	In this study
DXXA	DXX+ pδNEO_HXT_AraE	In this study
Plasmids		
pDGYC2-AraE	Amp^r, Cm^r (*B. subtilis*), *amyE* segments + P_{xylA}_*araE*	[16]
pRS425GPD	*LEU2*, 2-μ origin, GPD_p-MCS-$CYC1_t$	ATCC87359
pRS425TEF	*LEU2*, 2-μ origin, TEF_p-MCS-$CYC1_t$	ATCC87367
pRS425HXT	*LEU2*, 2-μ origin, HXT_p-MCS-$CYC1_t$	ATCC77106
pRS425PGK	*LEU2*, 2-μ origin, PGK_p-MCS-$CYC1_t$	[19]
pRS403	*HIS3*, 2-μ origin	[7]
pRS405	*LEU2*, 2-μ origin	[7]
pRS406	*URA3*, 2-μ origin	[7]
EGFP-pBAD	EGFP, 6xHis, Amp^r	Addgene Co.
p425GPD_EGFP	pRS425GPD with *EGFP*	In this study
p425GPD_AraE	pRS425GPD with *araE*	In this study
p425GPD_AraE+EGFP	pRS425GPD with *araE* +*EGFP*	In this study
p403GPD_XR	pRS403 with GPD_p + *XYL1* + $CYC1_t$	In this study
p406GPD_XR	pRS406 with GPD_p + *XYL1* + $CYC1_t$	In this study
p425TEF_AraE	pRS425TEF with *araE*	In this study
p425HXT_AraE	pRS425HXT with *araE*	In this study

P425PGK_AraE	pRS425PGK with *araE*	In this study
p405GPD_AraE	pRS405 with GPD_p + *araE* + $CYC1_t$	In this study
p405TEF_AraE	pRS405 with TEF_p + *araE* + $CYC1_t$	In this study
p405HXT_AraE	pRS405 with HXT_p + *araE* + $CYC1_t$	In this study
p405PGK_AraE	pRS405 with PGK_p + *araE* + $CYC1_t$	In this study
pδNEO	$G418^R$, δ-sequence	[9]
pδNEO_HXT_AraE	pδNEO with HXT_p + *araE* + $CYC1_t$	In this study

ATCC: American Tissue Culture Collection

Table 2. Specific activity of xylose reductase expressed in recombinant *S. cerevisiae* constructed in this study. Error bars represent standard deviations associated with four independent experiments.

Strains	XR activity (U/mg)
D452-2	0.012 ± 0.001
DX	0.374 ± 0.002
DXA	0.449 ± 0.002
DXX	0.537 ± 0.003
DXXA	0.613 ± 0.003

Table 3. Summarized results of culture parameters in batch and fed-batch production of xylitol in recombinant *S. cerevisiae*

Culture type	Strains	Dry cell mass (g/l)	Total consumed glucose (g)	Total consumed xylose (g/L)	Xylose consumption rate (g/L-h)	Xylitol productivity (g/L-h)	Xylitol concentration (g/L)	Xylitol yield (g/g)
Batch	DX	6.56 ± 0.34	19.7 ± 0.76	6.79 ± 0.59	0.14 ± 0.01	0.14 ± 0.01	6.6 ± 0.40	~ 1
	DXA	8.10 ± 0.50	19.5 ± 0.09	18.7 ± 0.71	0.39 ± 0.02	0.38 ± 0.01	18.1 ± 0.18	~ 1
	DXX	6.77 ± 0.48	19.4 ± 0.16	13.2 ± 0.12	0.28 ± 0.01	0.27 ± 0.02	13.0 ± 0.03	~ 1
	DXXA	6.83 ± 0.05	20.2 ± 0.75	21.3 ± 0.40	0.44 ± 0.01	0.44 ± 0.01	21.2 ± 0.34	~ 1
Fed-batch	DX	15.6	97.1	76.3	1.06	1.08	77.8	~ 1
	DXA	15.9	92.7	82.4	1.14	1.17	83.9	~ 1
	DXX	19.6	92.1	125.3	1.74	1.70	122.1	~ 1
	DXXA	21.1	87.6	177.8	2.47	2.47	177.8	~ 1

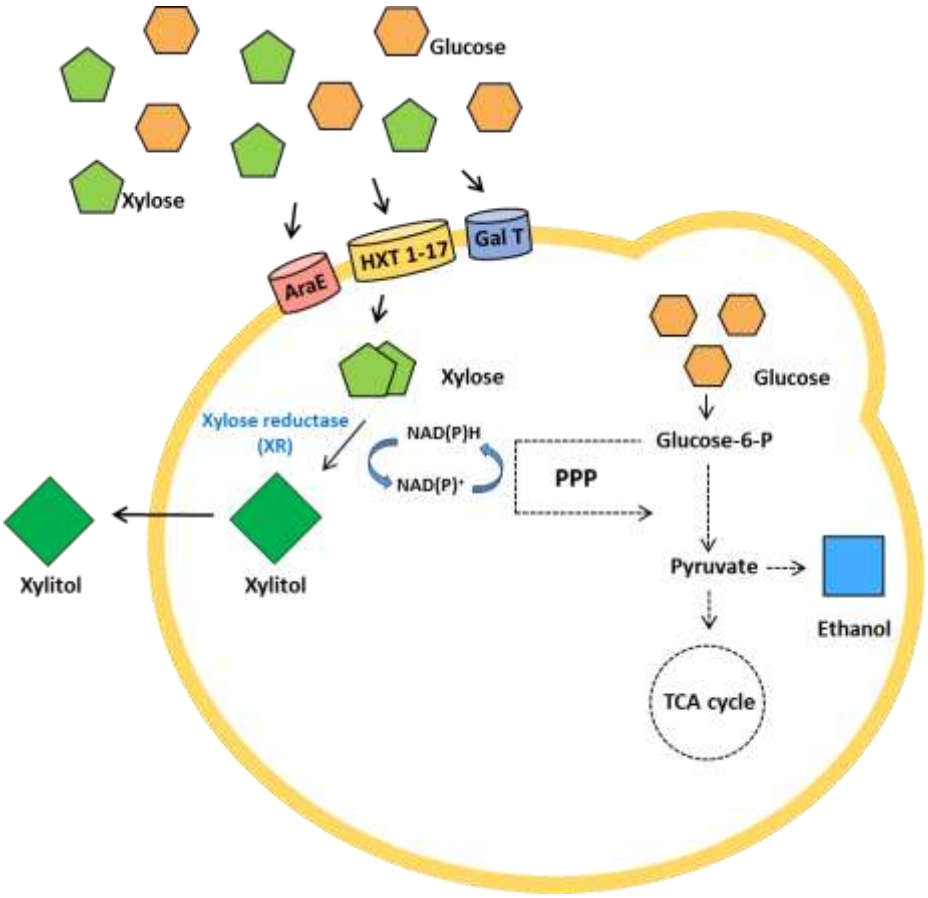

Fig. 1.

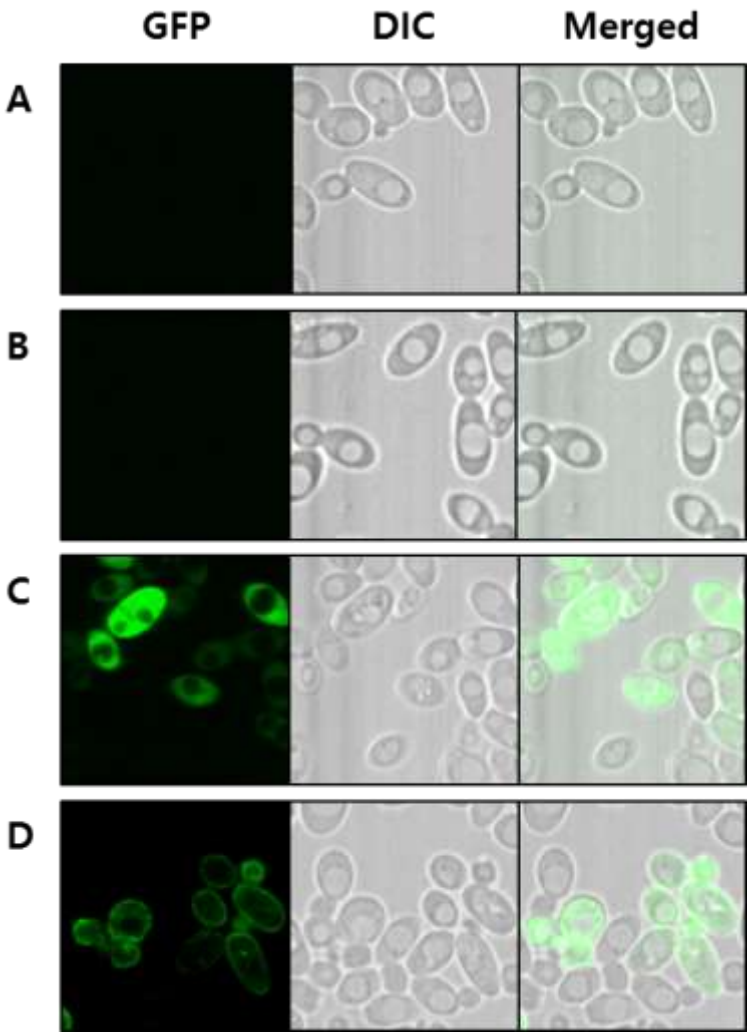

Fig. 2.

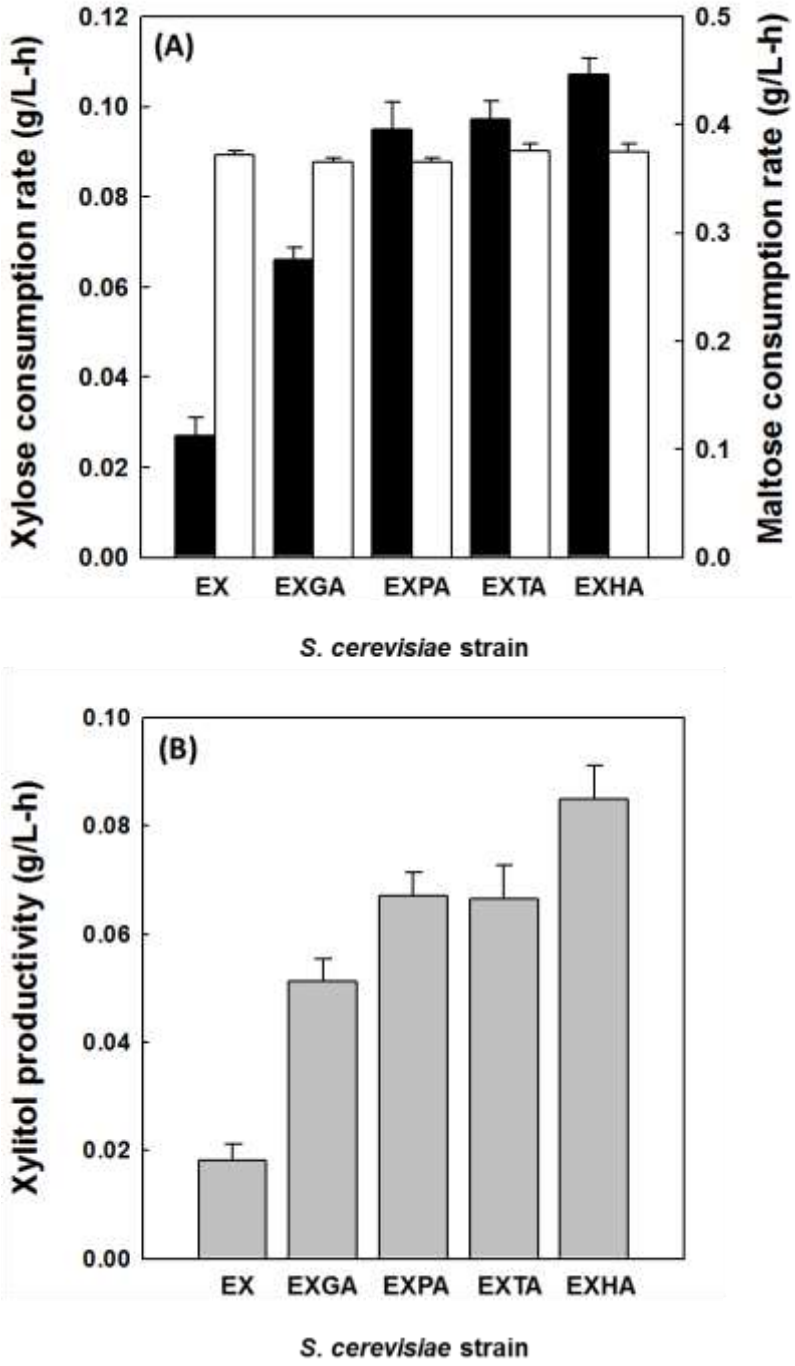

Fig. 3.

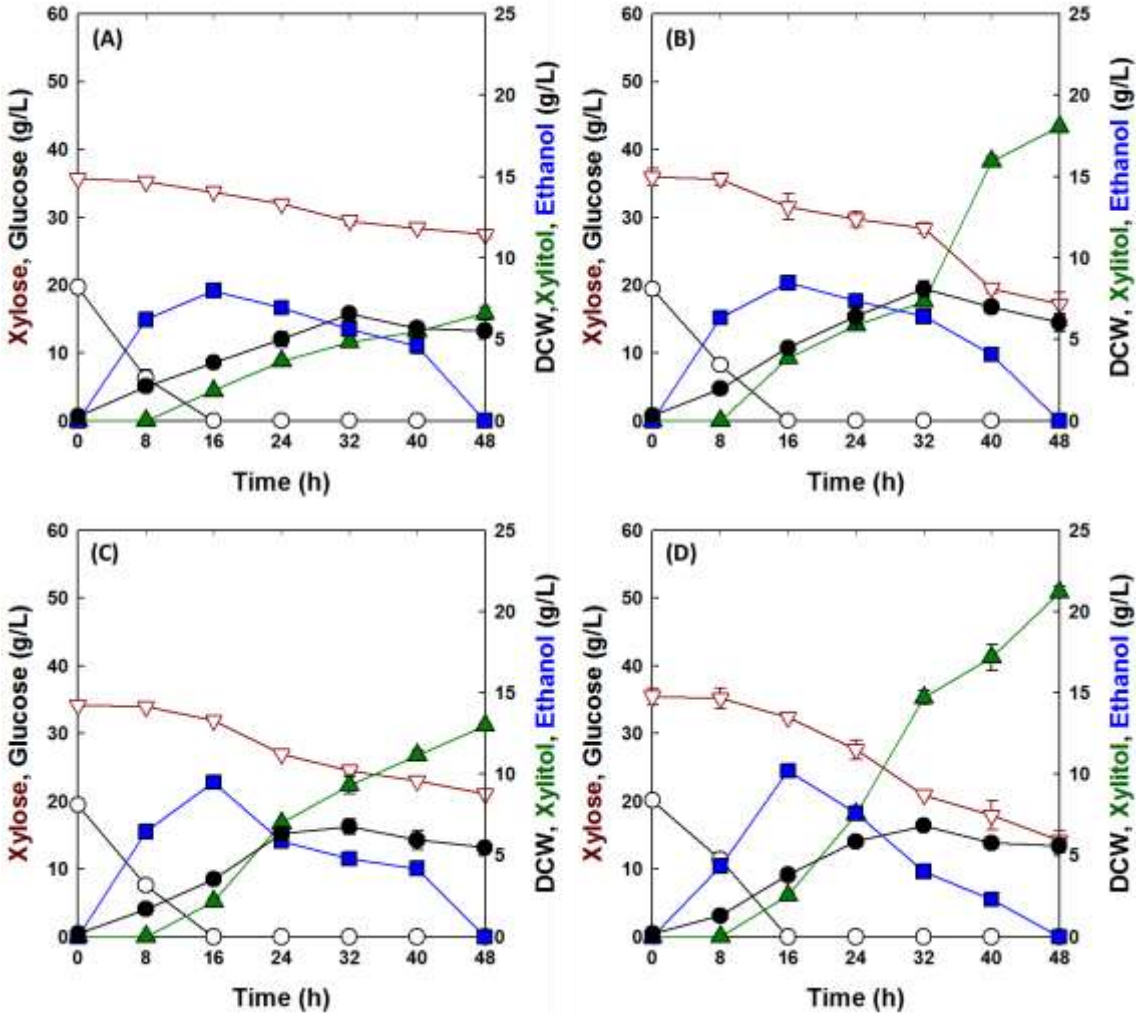

Fig. 4.

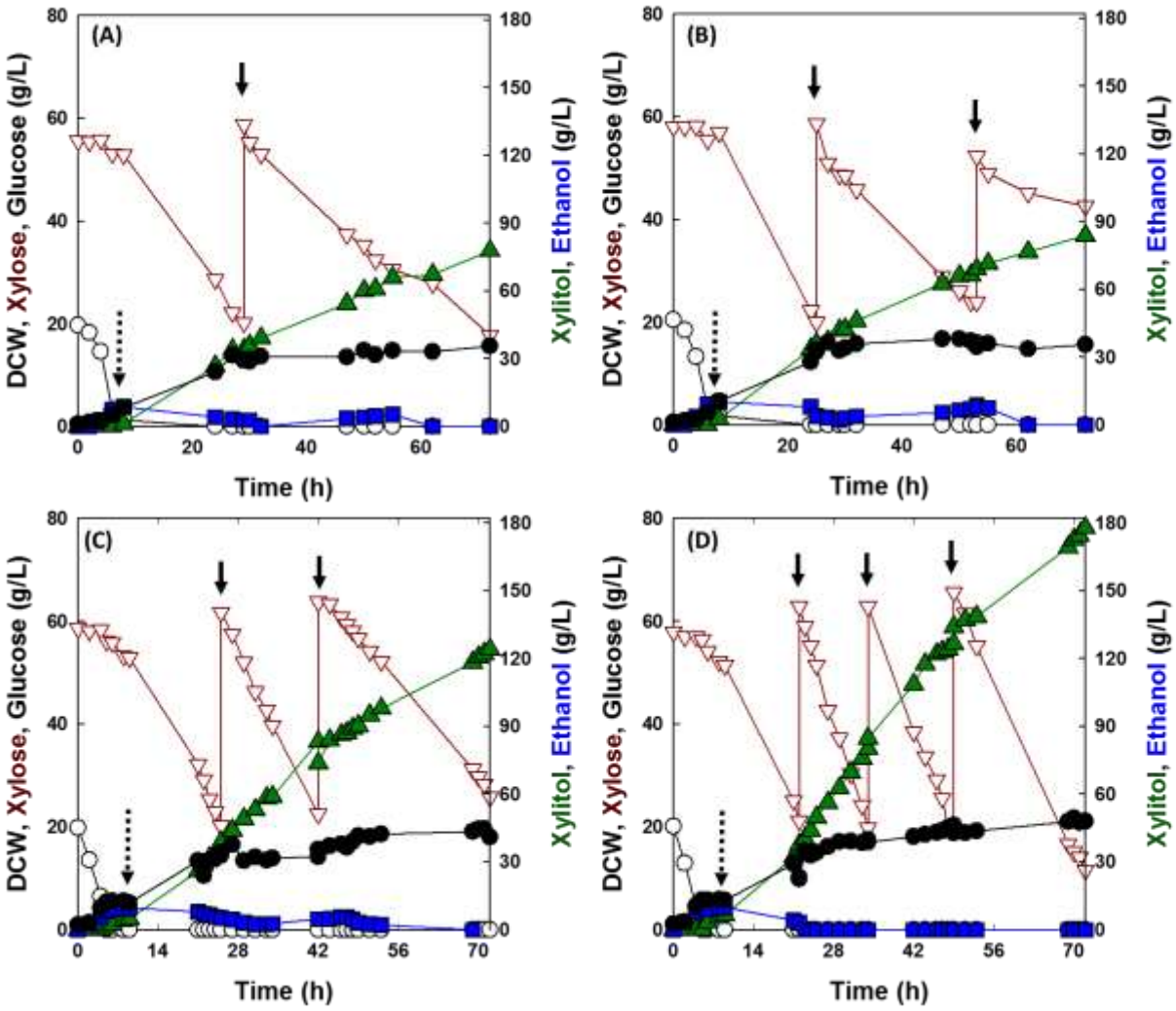

Fig. 5.