Simultaneous Saccharification and Co-fermentation with a Thermotolerant *Saccharomyces cerevisiae* to Produce Ethanol from Sugarcane Bagasse under High Temperature Conditions

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Lignocellulosic ethanol production at high temperature offers advantages such as the decrease of contamination risk and cooling cost. Recombinant xylose-fermenting *Saccharomyces cerevisiae* has been considered a promising strain for ethanol production from lignocellulose for its high inhibitor tolerance and superior capability to ferment glucose and xylose into ethanol. To improve the ethanolic fermentation by xylose at high temperature, the strain YY5A was subjected to the ethyl methanesulfonate (EMS) mutagenesis. A mutant strain T5 was selected from the EMS-treated cultures to produce ethanol. However, the xylose uptake by T5 was severely inhibited by the high ethanol concentration during the co-fermentation in defined YPDX medium at 40 °C. In this study, the simultaneous saccharification and co-fermentation (SSCF) and the separate hydrolysis and co-fermentation (SHCF) processes of sugarcane bagasse were assessed to solve this problem. The xylose utilization by T5 was remarkably improved using the SSCF process compared to the SHCF process. For the SHCF and SSCF processes, 48% and 99% of the xylose in the hydrolysate was consumed at 40 °C, respectively. The ethanol yield was enhanced by the SSCF process. The ethanol production can reach to 36.0 g/L using this process under high-temperature conditions.

Keywords: Lignocellulosic ethanol; Saccharomyces cerevisiae; Inhibitor tolerance; Mutagenesis; Sugarcane bagasse; Simultaneous saccharification and co-fermentation

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INTRODUCTION

Bioethanol is currently the dominant biofuel used in the worldwide transportation sector to replace or blend with other biofuels (Jin *et al.* 2019; Rajak *et al.* 2020). Lignocellulosic biomass, such as wood and agricultural residues, is an attractive feedstock for ethanol production due to its abundance, sustainable supply, and non–conflict with food supply. However, despite nearly a century of research to develop the cost-effective technology toward the commercial deployment of lignocellulosic ethanol, the recalcitrance of lignocellulose is still a major barrier to be economically viable *via* lignocellulosic ethanol production (Lynd *et al.* 2002; Sims *et al.* 2010).

The main constituents of lignocellulose are cellulose, hemicellulose, and lignin. The fractions of cellulose and hemicellulose can be used to produce biofuel by fermentation. The critical part of using lignocellulose to produce biofuel is obtaining enough fermentable monosaccharide from lignocellulose to serve as a carbon source during

fermentation (Robak and Balcerek 2018). The separate hydrolysis and fermentation (SHF) process is commonly employed to maximize the performance of both enzymatic hydrolysis and fermentation processes for lignocellulosic ethanol production, but this increases capital and operational costs. Enzymatic hydrolysis can be performed simultaneously with fermentation in a process referred to as simultaneous saccharification and fermentation (SSF). The SSF process is considered to be favorable for lignocellulosic ethanol production due to several advantages, including removal of end-products of enzymatic hydrolysis that inhibit cellulases, higher ethanol productivity, and higher yield than that of the SHF process (Erdei *et al.* 2012; Jin *et al.* 2012). The main problem in the SSF process is the different optimum temperatures for enzymatic hydrolysis and fermentation. The optimal temperature for cellulases (50 °C) is remarkably higher than the fermenting temperature suitable for *Saccharomyces cerevisiae* (30 °C), the most used organism for industrial ethanol production. The SSF process has been currently exerted at lower temperatures to coordinate with the growth temperature of fermenting strains, which slows enzymatic hydrolysis efficiency and results in reduced fermentation rates and yields (Eklund *et al.* 1990; Sassner *et al.* 2006). To improve the SSF process for efficient conversion of lignocellulose into ethanol, using thermotolerant organisms to compromise the optimal temperature for yeast fermentation and enzymatic hydrolysis has been considered a promising solution (Banat *et al.* 1992; Fonseca *et al.* 2008; Yanase *et al.* 2010). In addition, high-temperature fermentation offers several advantages, such as reduction of contamination risk and cooling cost, especially in tropical countries (Chamnipa *et al.* 2018).

Besides glucose, xylose is the most abundant sugar in lignocellulosic biomass. Thus, an ideal organism for lignocellulosic ethanol production with both superior glucose and xylose co-fermentation capacity and high-temperature tolerance is required. Some studies have identified genetically engineered thermotolerant yeasts *Kluyveromyces marxianus* and *Hansenula polymorpha* capable of converting xylose to ethanol at elevated temperatures (42 to 45 °C), whereas the low ethanol productivity and slow xylose utilization are still the major problems that need to be overcome (Dmytruk *et al.* 2008; Zhang *et al.* 2015). To date, *S. cerevisiae* is still considered a favorable microorganism for industrial ethanol production because of its high ethanol productivity and high inhibitor tolerance (Cunha *et al.* 2019; Favaro *et al.* 2019). Numerous efforts have engineered *S. cerevisiae* to ferment xylose by introducing homologous or heterologous xylose metabolic pathways, resulting in promising xylose fermentation properties (Kuyper *et al.* 2005; Hahn-Hägerdal *et al.* 2007; Watanabe *et al.* 2007). The *S. cerevisiae* strains with increased thermotolerance have been obtained by genetic engineering or evolutionary engineering strategies. For example, the evolved *S. cerevisiae* strain ISO12 developed by long-term adaptation strategy can grow and ferment the non-detoxified spruce hydrolysate at 39 °C (Wallace-Salinas and Gorwa-Grauslund 2013). In another study, the engineered *S. cerevisiae* strain Z-06 could enhance cell stress tolerance and glycolysis flux at high temperature by overexpression of the TSL1 gene (Ge *et al.* 2013). In addition, other researchers have identified other genes, such as *TPS1*, *RSP5*, *HSP100*, *SSK2*, *PPG1*, and *PAM1*, that are important for the thermotolerance of *S. cerevisiae* (An *et al.* 2011; Kim *et al.* 2011; Shahsavarani *et al.* 2012). There have been a considerable number of studies for improving fermentation of glucose at elevated temperature by *S. cerevisiae* (Benjaphokee *et al.* 2012; Lu *et al.* 2012). However, the number of studies for alcoholic fermentation of xylose at elevated temperatures is still limited (Ismail *et al.* 2013; Jin *et al.* 2013).

The inhibition of xylose consumption by recombinant xylose-fermenting *S. cerevisiae* in glucose and xylose co-fermentation at elevated temperature was observed in a previous study (Jin *et al.* 2013). However, there is a need for more detailed information about the inhibitory factors affecting xylose fermentation by recombinant *S. cerevisiae* at elevated temperatures. The effects of glucose and ethanol on xylose fermentation by xylose-fermenting *S. cerevisiae* at elevated temperature have not been reported. In this study, the effects of glucose and ethanol on xylose utilization in a synthetic medium by a recombinant xylose-utilizing *S. cerevisiae* strain YY5A were tested. In addition, improving the thermotolerance of YY5A by ethyl methanesulfonate (EMS) mutagenesis was demonstrated in this work. In contrast, to assess a better process for xylose conversion in non-detoxified lignocellulosic hydrolysate from sugarcane bagasse at high-temperature, SSCF and SHCF processes were tested and discussed in this study.

EXPERIMENTAL

Strain and Medium

The genetically engineered strain YY5A used in this study has been described elsewhere (Ma *et al.* 2012). The strain was maintained on a yeast extract peptone dextrose (YPD) agar plate (10 g/L yeast extract, 20 g/L peptone, 20 g/L D-glucose, and 20 g/L agar) at 30 °C. The mutagenized cells by EMS (Sigma-Aldrich, St. Louis, MO, USA) treatment were selected on the yeast extract peptone xylose (YPX) agar plate (10 g/L yeast extract, 20 g/L peptone, 20 g/L D-xylose, and 20 g/L agar) at 40 °C.

Fermentation in a Synthetic Medium

The seed cultures were aerobically grown in 50 mL of YPD in 250-mL Erlenmeyer flasks at 30 °C for 16 h. For xylose fermentation, experiments were carried out at different temperatures, at which 10 mL of the seed culture was inoculated into 40 mL of YPX medium in a 250-mL Erlenmeyer flask. The initial fermentation broth contained 10 g/L yeast extract, 20 g/L peptone, and 40 to 50 g/L D-xylose. An initial dry cell weight (DCW) of approximately 0.8 to 1.2 g/L was used. The fermentation processes were performed in triplicate at 30, 37, or 40 °C with agitation at 150 rpm. For glucose and xylose co-fermentation experiments at elevated temperatures in a 250-mL Erlenmeyer flask, 10 mL of the seed culture was inoculated into 40 mL of yeast extract peptone dextrose (YPDX) medium in the presence of different initial concentrations of glucose and xylose. The initial fermentation broth contained 10 g/L yeast extract, 20 g/L peptone, 18 to 74 g/L glucose, and 40 to 50 g/L xylose. An initial DCW of approximately 1.2 g/L was used, and all fermentation processes were performed in triplicate at 37 or 40 °C with agitation at 150 rpm. For xylose fermentation with varying concentrations of initial ethanol at different temperatures in a 250-mL Erlenmeyer flask, 10 mL of the seed culture was inoculated into 40 mL of YPX medium in the presence of varying initial concentrations of ethanol. The initial fermentation broth contained 10 g/L yeast extract, 20 g/L peptone, 47 g/L xylose, and 2.5, 12, 23, or 33 g/L ethanol. An initial DCW of approximately 1.2 g/L was used, and all fermentation processes were performed in triplicate at 30, 37, and 40 °C with agitation at 150 rpm.

Strain Mutagenesis by EMS Treatment

Strain mutagenesis was carried out with EMS. The seed cultures were aerobically grown in 10 mL of YPD in 50-mL Erlenmeyer flasks at 30 °C for 16 h. Cells in a 500 µL fresh culture were collected by centrifugation, washed twice with sterile water, and resuspended in 1 mL EMS solution (4% (v/v)). The cells were incubated with EMS at 30 °C for 50 min to obtain a survival rate of 10%. The mutagenesis was then stopped by adding 500 µL freshly made sodium thiosulfate (% (w/v)). The mutagenized cells were then collected by centrifugation, washed twice with 500 µL freshly made 5% (w/v) sodium thiosulfate, and resuspended in sterile water. Following mutagenesis, the cells were immediately plated on a YPX agar plate and incubated at 40 °C. Well-grown colonies with different xylose fermentation capacities were selected for further studies.

Pretreated Sugarcane Bagasse

The pretreated sugarcane bagasse was provided by Dr. Wen-Hua Chen of Institute of Nuclear Energy Research (INER), Taoyuan, Taiwan. Sugarcane bagasse with a moisture content of 61.3% was chopped into smaller pieces (≤ 2 cm) with a shredder and used as raw material. The acid-catalyzed steam explosion experiments were performed in a pilot-scale continuous pretreatment system at the Institute of Nuclear Energy Research, Taiwan (Chen *et al.* 2013). The reaction condition of the acid-catalyzed steam explosion process was 200 °C for a residence time of 1 min. Subsequently, the pretreated sugarcane bagasse slurry was collected and separated into solid residues and liquid portions by a separator for further study.

Separate Hydrolysis and Co-fermentation

In the SHCF process, the enzymatic hydrolysis process was performed in a 5-L fermenter with a working volume of 3 L by fed-batch mode. The two-fold diluted liquid fractions were transferred to a 5-L fermenter, and the pH was adjusted to 6.0 by adding 10 M NaOH. Subsequently, the CTec3 cellulase enzyme (Novozymes, Bagsværd, Denmark) was added at 0 h. The enzyme activity was set at 15 FPU/g water-insoluble solids (WIS), and the enzymatic hydrolysis reaction was performed at 50 °C for 120 h. The solid residues were added at time periods of 0, 2, and 4 h to a final WIS of 13%. The pH was maintained at around 5.5 to 5.8 using 10 M NaOH with agitation at 250 rpm. After enzymatic hydrolysis, the hydrolysate slurry was adjusted to 6 pH, and the fermentation was performed in a 5-L fermenter with a working volume of 2 L. Then, 400 mL of the seed culture was centrifuged and inoculated into 2 L of the sugarcane bagasse hydrolysate and initiated with an inoculum of 1.2 g/L DCW at 40 °C with agitation at 250 rpm.

Simultaneous Saccharification and Co-fermentation

The SSCF process was performed in a 5-L fermenter with a working volume of 2 L by fed-batch mode. The liquid fractions of pretreated sugarcane bagasse were transferred to a 5-L fermenter, and the pH was adjusted to 6.0 by adding 10 M NaOH. The yeast cells and CTec3 were added to the liquid fractions at 0 h, with an inoculum of 1.2 g/L DCW. The enzyme activity was set at 15 FPU/g WIS. The enzymatic hydrolysis and co-fermentation reaction was performed at 40 °C for 120 h. The solid residues were added at time periods of 0, 2, and 4 h to a final WIS of 13%. The pH was maintained around 5.5 to 5.8 using 10 M NaOH with agitation at 250 rpm.

Analysis

Concentrations of ethanol, glucose, xylose, xylitol, glycerol, and acetic acid were determined by high performance liquid chromatography (HPLC) (Jasco, Tokyo, Japan) equipped with a refractive index detector. An HPX-87H ion-exclusion column (Bio-Rad Laboratories, Hercules, CA, USA) was used for separation. The HPLC apparatus was operated using 5 mM H_2SO_4 as the mobile phase with a flow rate of 0.6 mL/min. Cell growth was monitored by measuring the absorbance at 600 nm using a spectrophotometer U-3000 (HITACHI, Chiyoda, Tokyo, Japan). The DCW was determined in triplicate using an infrared moisture analyzer FD-720 (Kett Electric Laboratory, Otaku, Tokyo, Japan). Next, 10 mL of cell cultures were rinsed with sterile water, and the DCW was determined by the FD-720. The identified cellulose, hemicellulose, and lignin component contents of the pretreated sugarcane bagasse were determined according to laboratory analytical procedures from the National Renewable Energy Laboratory (Sluiter *et al.* 2008), Table 1. The carbohydrate content was determined by measuring the hemicellulose (xylan and arabinan) and cellulose (glucan) compositions of the derived sugars.

Table 1. Composition of the Pretreated Sugarcane Bagasse[a]

Name	Content in Liquid Fraction (g/L)	Name	Content in Solid Fraction (%)[b]
Glucose	17.8 ± 0.9	Glucan	47.0 ± 0.4
Xylose	57.6 ± 0.1	Xylan	2.9 ± 0.6
HMF	0.3 ± 0.1	Araban	0.3 ± 0.1
Furfural	2.4 ± 0.1	Lignin	26.2 ± 1.2
Acetic acid	5.0 ± 0.1	-	-

[a]: The pretreatment using acid-catalyzed steam explosion process at 200 °C for 1 min with 1% dilute sulfuric acid solution
[b]: Values are the averages the standard deviations of three independent experiments based on dry weight

RESULTS AND DISCUSSION

Effect of Temperature on Xylose Fermentation by YY5A

A microbial strain that can efficiently ferment glucose and xylose into ethanol and tolerate both high-temperature and multiple inhibitors is a prerequisite for efficiently converting lignocellulosic biomass into ethanol. Previous studies have successfully developed the recombinant xylose-utilizing *S. cerevisiae* strain YY5A (Hahn *et al.* 2007). However, the thermotolerance of YY5A in xylose fermentation remains unclear. To assess the effect of temperature on xylose fermentation performance of YY5A, ethanolic xylose fermentation in the YPX medium (41.5 g/L xylose as a carbon source) was conducted at 30, 37, and 40 °C. The fermentation by YY5A was initiated with an inoculum of approximately 0.8 g/L DCW. In the xylose fermentation, cell concentrations of YY5A were 6.8, 5.5, and 2.3 g/L DCW at 30, 37, and 40 °C after 24 h fermentation, respectively (Fig. 1a). The results indicate an inhibition effect on the cell growth of YY5A at 40 °C. After 24 h of fermentation, YY5A produced 13.6 g/L ethanol at 30 °C, with a yield of 0.37 $g/g_{consumed\ xylose}$; 15.0 g/L ethanol at 37 °C, with a yield of 0.38 $g/g_{consumed\ xylose}$; and 7.6 g/L ethanol at 40 °C, with a yield of 0.35 $g/g_{consumed\ xylose}$ (Fig. 1c).

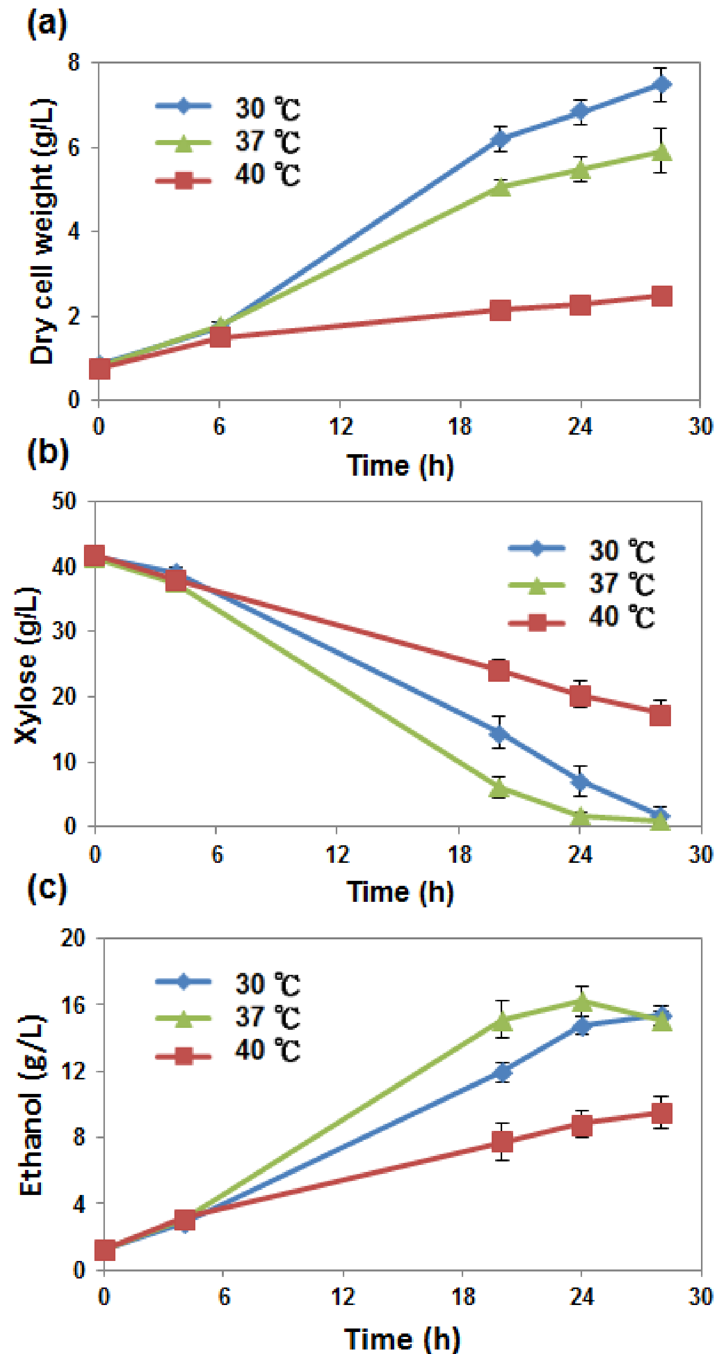

Fig. 1. The variation of (a) cell growth; (b) xylose consumption; and (c) ethanol production during xylose fermentation by the recombinant yeast strain YY5A at 30, 37, and 40 °C

The results showed a higher ethanol yield by YY5A at 30 °C and 37 °C than at 40 °C. Moreover, the specific consumption rates of xylose were different from volumetric xylose consumption rates in the initial 24 h of fermentation. The total consumptions of xylose in the initial 24 h of fermentation were 36.7, 39.8, and 21.6 g/L at 30, 37, and 40 °C, respectively. The total increments of dry cell weight in the initial 24 h of fermentation were 6.1, 4.7, and 1.5 g/L at 30, 37, and 40 °C, respectively. According to above results, the specific xylose consumption rates at 30, 37 and 40 °C were 0.25, 0.35, and 0.60

g/g·DCW/h, and the volumetric xylose consumption rates were 1.53, 1.66, and 0.90 g/L/h, respectively (Fig. 1a and 1b). The specific xylose consumption rate represents how much xylose can be consumed by 1 g YY5A/h and volumetric xylose consumption rate represents the consumption of xylose in the medium per hour. The specific growth rate and the volumetric xylose consumption rate for the xylose fermentation at 40 °C was lower than that at 30 and 37 °C, whereas the higher specific xylose consumption rate was at 40 °C rather than that at 30 and 37 °C. The results suggested that the elevated temperature inhibits the cell growth of YY5A but stimulates the xylose assimilation efficiency by fermenting cells. Consistent with this work, it has been observed that the activities of xylose reductase from *Neurospora crassa* and xylitol dehydrogenase and from *Scheffersomyces stipitis* increased with the elevated reaction temperature during an *in vitro* enzyme activity assay (Zang *et al.* 2015). Therefore, the increase of the specific xylose consumption rates by YY5A at elevated temperatures could be explained by the elevated activity of xylose metabolizing enzymes.

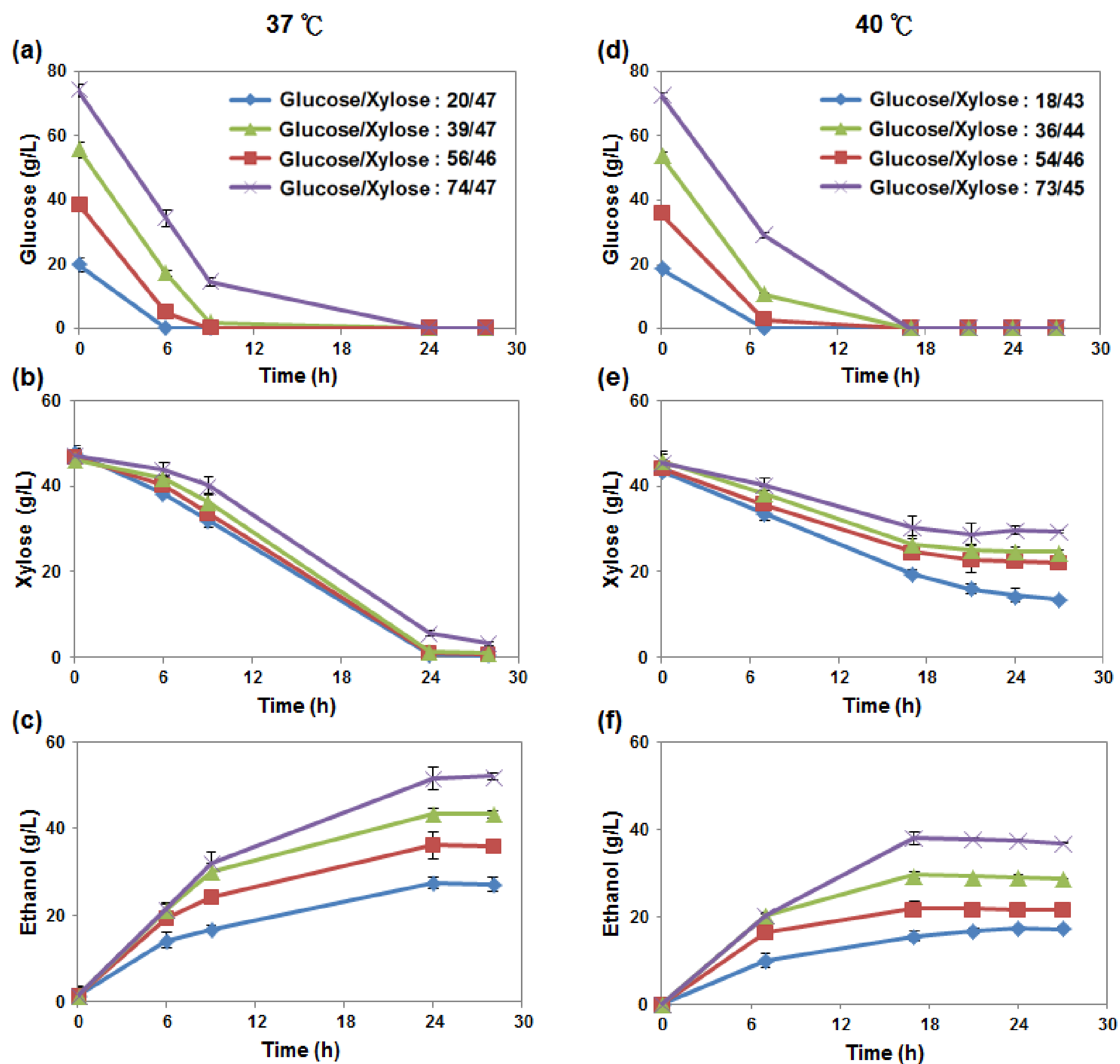

Fig. 2. Variation of (a and d) glucose consumption; (b and e) xylose consumption; and (c and f) ethanol production during co-fermentation by the recombinant yeast strain YY5A at 37 °C or 40 °C

Effect of Initial Glucose Concentration on Co-fermentation of a Glucose and Xylose Mixture by YY5A at Different Temperatures

Previous research has shown that the xylose utilization by recombinant xylose-fermenting *S. cerevisiae* at a high temperature of 40 °C is inhibited in glucose and xylose co-fermentation (Jin *et al.* 2013). It is still unclear how well the recombinant strain YY5A could co-ferment glucose and xylose at elevated temperatures. To assess this, ethanolic co-fermentation by YY5A in YPDX medium with different initial concentrations of glucose were conducted at 37 °C and 40 °C (Fig. 2). The co-fermentation by YY5A was initiated with an inoculum of approximately 1.2 g/L DCW. After 24 h of co-fermentation at 37 °C, the consumptions of xylose were 46.9 g/L, 45.9 g/L, 44.6 g/L, and 41.5 g/L (Fig. 2b). The concentrations of ethanol were 51.5 g/L, 43.3 g/L, 36.1 g/L, and 27.4 g/L (Fig. 2c) at different initial concentrations of glucose (20, 39, 56, and 74 g/L, respectively) (Fig. 2a). After 24 h of co-fermentation at 40 °C, the consumptions of xylose were 28.9 g/L, 21.8 g/L, 21.1 g/L, and 15.7 g/L (Fig. 2e), and the concentrations of ethanol were 37.4 g/L, 29.1 g/L, 21.7 g/L, and 17.3 g/L (Fig. 2f) at different initial concentrations of glucose (18, 36, 54, and 73 g/L, respectively) (Fig. 2d). In addition, the volumetric xylose consumption rate for YY5A in the YPDX medium increased at both 37 °C (all conditions) and 40 °C. This was more than the case of YPX fermentation (Fig. 1), in which xylose was the only carbon source. Similar results were observed in a previous study, in which the xylose consumption rate for the recombinant *S. cerevisiae* increased during the co-fermentation of glucose and xylose, which could be explained by the increased biomass concentration *via* glucose assimilation (Ma *et al.* 2012).

Effect of Initial Ethanol Concentration on Xylose Fermentation by YY5A at Different Temperatures

Ethanol is an inhibitory factor for yeast growth. It can affect the RNA and protein synthesis, denature cellular proteins, and disrupt the membrane integrity (Birch and Walker 2000; Ding *et al.* 2009). The xylose conversion by recombinant *S. cerevisiae* has been found to be inhibited by ethanol, which interferes with the cell membrane to inhibit the xylose transport (Meinander and Hahn-Hägerdal 1997). In contrast, the yeast fermentation at high temperature increases the fluidity in membranes (Suutari *et al.* 1990). Thus, it could be speculated that the higher ethanol concentration produced from the higher initial glucose concentration in glucose and xylose co-fermentation could aggravate the inhibition for xylose metabolism of YY5A at high temperature.

To understand the effect of ethanol on xylose fermentation at different temperatures, YY5A was used in YPX medium (47 g/L xylose as a carbon source) to ferment xylose in the presence of the same initial concentrations of ethanol at 30, 37, and 40 °C. During 24 h of fermentation, it was observed that the decreases of biomass growth rates and xylose consumption rates for YY5A as the initial ethanol concentrations increased under all conditions of temperature (Fig. 3a, 3d, and 3g), particularly at the elevated temperatures of 37 °C and 40 °C. A comparison of the profiles revealed the effect of ethanol on xylose fermentation by YY5A at different temperatures (Fig. 3b, 3e, and 3h). There were no remarkable differences in ethanol yield for YY5A at 30 °C under all conditions of different initial ethanol concentrations (Fig. 3c, 3f, and 3i), whereas decreases were observed in an initial ethanol concentration above 23 g/L at 37 °C, and that above 12 g/L at 40 °C (Fig. 3). The inhibitory effect of ethanol on xylose utilization for YY5A strictly corresponded to the varying temperature. Lower ethanol concentrations in synthetic medium were preferred for xylose fermentation by YY5A at elevated temperatures.

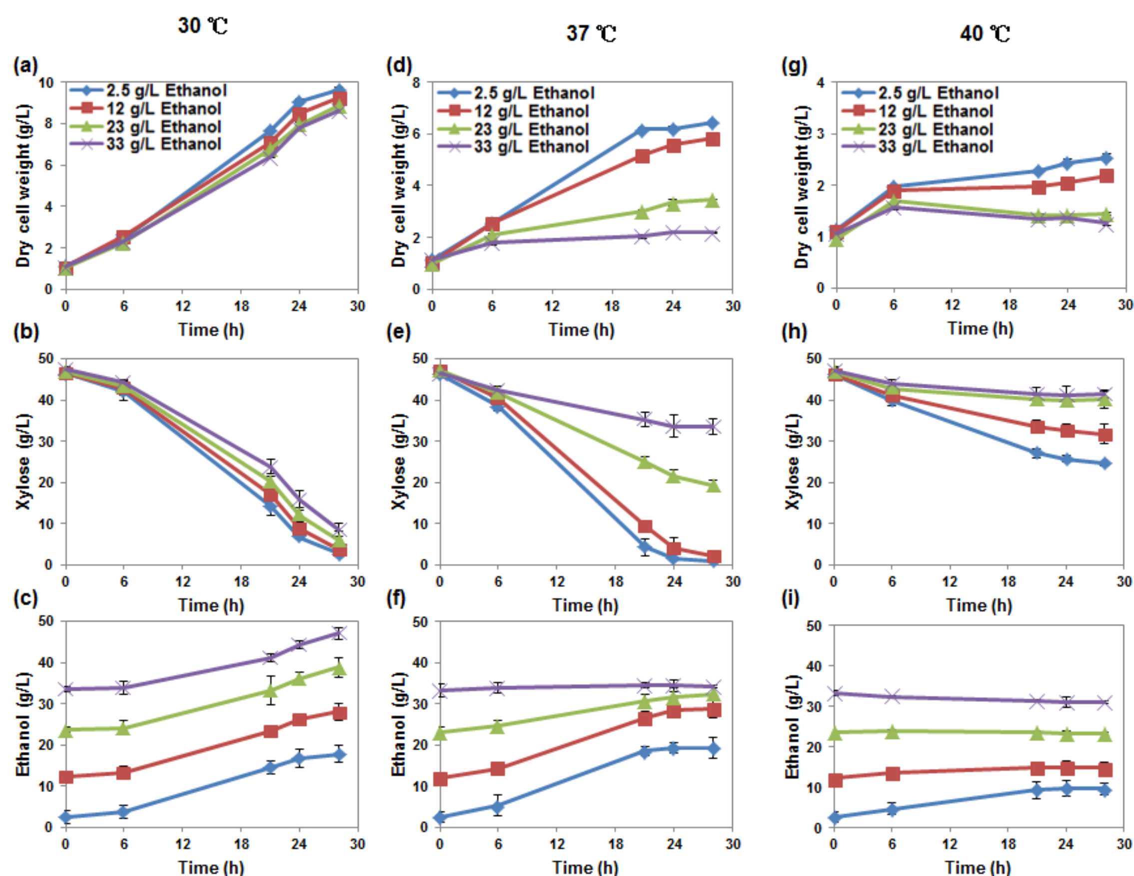

Fig. 3. Comparison of xylose fermentation by the recombinant yeast strain YY5A in the presence of varying initial ethanol concentrations at 30, 37, and 40 °C. (a) Cell growth at 30 °C; (b) xylose consumption at 30 °C; (c) ethanol production at 30 °C; (d) Cell growth at 37 °C; (e) xylose consumption at 37 °C; (f) ethanol production at 37 °C; (g) Cell growth at 40 °C; (h) xylose consumption at 40 °C; and (i) ethanol production at 40 °C

Improving Xylose Fermentation of YY5A at High Temperature by EMS Mutagenesis

To improve the xylose fermentation ability of YY5A strain at high temperature, it was subjected to mutagenesis by EMS. Sixteen well-grown colonies on YPX agar plates at 40 °C were selected from the EMS-treated cultures. After the screening of 16 mutants through a comparison of ethanol yield and xylose consumption rate in YPX medium at 40 °C, the best mutant strain with a superior xylose fermentation capacity was selected and designated as strain T5. To further compare the fermentation capacity between T5 and YY5A at high temperature, ethanolic fermentation was evaluated in YPX medium (1% yeast extract, 2% peptone, and 4.5% xylose) and YPDX medium (1% yeast extract, 2% peptone, 6% glucose, and 4.5% xylose) at 40 °C. The xylose consumption of YY5A and T5 was 27.8 g/L (Fig. 4a) and 34.3 g/L (Fig. 4c) at 24 h, respectively. During the glucose and xylose co-fermentation, the xylose consumption of YY5A and T5 was 21 g/L (Fig. 4b) and 19 g/L (Fig. 4d) at 24 h, respectively. After 16 h of co-fermentation, the xylose consumption rates of both strains were slowed after glucose was completely consumed, this phenomenon was consistent with the above results in co-fermentation (Fig. 2). In contrast, the slightly higher ethanol concentration was obtained by T5 (13.5 g/L) compared

to YY5A (10.4 g/L) at 40 h (Fig. 4a and 4c). The results indicate that the xylose fermentation capacity of YY5A at high temperature was improved by EMS mutagenesis, especially in the fermentation of YPX medium. However, there still was inhibition of high ethanol concentration on xylose utilization by the thermotolerant T5 strain during the co-fermentation at high temperature.

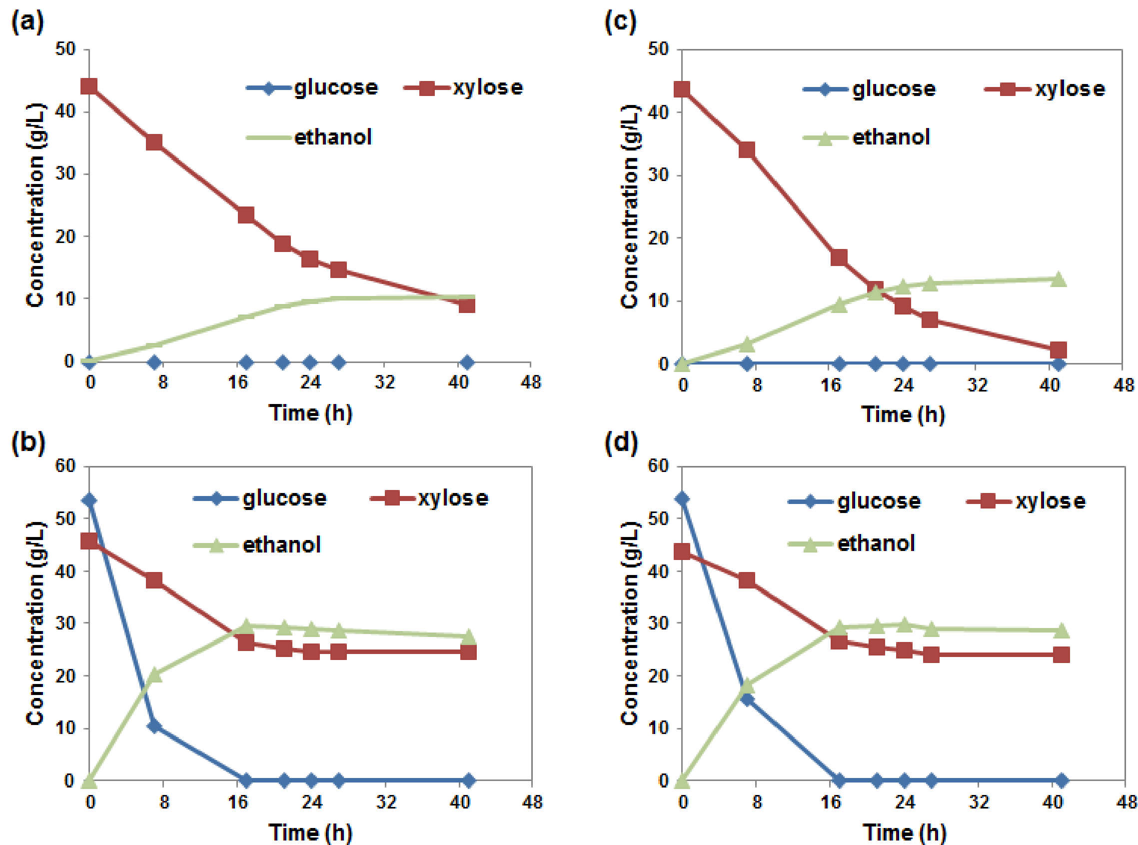

Fig. 4. Comparison of ethanol fermentation in YPX and YPDX medium by the strain YY5A and T5 at 40 °C. (a) xylose fermentation by YY5A in YPX medium; (b) glucose/xylose co-fermentation by YY5A in YPDX medium; (c) xylose fermentation by T5 in YPX medium; and (d) glucose/xylose co-fermentation by T5 in YPDX medium

SHCF and SSCF Processes for Lignocellulosic Ethanol Production at High-Temperature

The SHCF process is commonly employed to maximize the performance of both enzymatic hydrolysis and fermentation processes for lignocellulosic ethanol production. The high concentration of sugar mixture can be used to produce ethanol by co-fermentation. However, the high concentration of ethanol produced from glucose in the sugar mixture could be an inhibitory factor for further consuming xylose by recombinant co-fermenting *S. cerevisiae* at high temperature. Therefore, the SHCF process might not be suitable for lignocellulosic ethanol production at high temperature. However, the SSCF process is more favorable for efficient xylose fermentation at high temperature due to the lower initial concentrations of glucose and ethanol to inhibit xylose assimilation by recombinant *S. cerevisiae*.

To assess the fermentation capacity of the thermotolerant mutant strain T5 in lignocellulosic hydrolysates by SHCF and SSCF processes, the fermentation in sugarcane bagasse hydrolysates at 40 °C was evaluated. The composition of the pretreated sugarcane bagasse is summarized in Table 1. During 24 h of SHCF, glucose was consumed completely within 17 h. The xylose consumption was 15.3 g/L (48% of the initial xylose) and the ethanol production was 31.9 g/L (Fig. 5a). The xylose consumption rate was slowed greatly after 17 h of co-fermentation, which was consistent with the results of co-fermentation in defined medium at 40 °C (Figs. 2 and 4). The results correspond to the abovementioned hypothesis, which indicate that the high concentration of ethanol produced from glucose during co-fermentation is an important inhibitory factor for consuming xylose by co-fermenting *S. cerevisiae* at high temperature. During 24 h of SSCF, the xylose consumption was 19.7 g/L (78% of the initial xylose). After 120 h of SSCF, the xylose consumption was 24.9 g/L (99% of the initial xylose), and the ethanol production was 36.0 g/L (Fig. 5b). The results indicate that the xylose utilization in lignocellulosic hydrolysates by T5 was improved dramatically when using the SSCF process compared to the SHCF process at 40 °C. In addition, the higher ethanol yield was obtained by the SSCF process. Enzymatic prehydrolysis process at high temperature (50 °C) is often adopted prior to the SSF or SSCF process (hybrid process) for the liquefaction of pretreated lignocellulosic solid residues. The decreased viscosity in slurry allowed for easier pumping, stirring, and effectively increased the enzymatic hydrolysis efficiency (Öhgren *et al.* 2007; Cannella and Jørgensen 2014). However, releasing excessed glucose during the prehydrolysis might inhibit the xylose conversion for recombinant co-fermenting *S. cerevisiae* strain in the following SSCF process at high temperature. Therefore, the SSCF process without the prehydrolysis is a preferred manner to reduce the co-stress of ethanol and temperature for xylose conversion in lignocellulosic ethanol production by xylose-fermenting *S. cerevisiae* at high temperature.

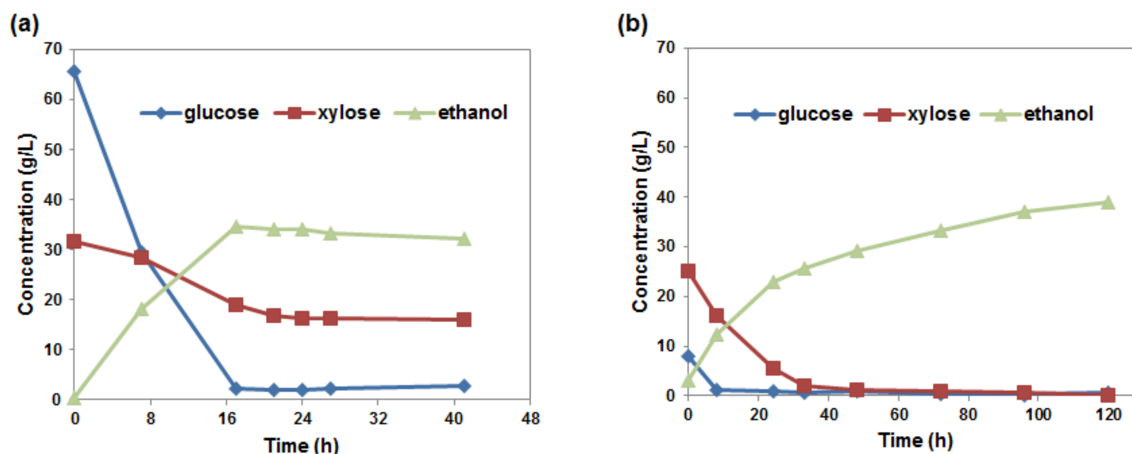

Fig. 5. (a) SHCF; and (b) SSCF of pretreated sugarcane bagasse hydrolysates by the mutant strain T5 at 40 °C

CONCLUSIONS

1. The xylose fermentation by YY5A is impaired greatly at a high temperature of 40 °C whether in the defined medium of YPX or YPDX.

2. The inhibition of ethanol on xylose utilization by recombinant xylose-fermenting *Saccharomyces cerevisiae* YY5A strictly corresponds to the conditions of temperature.
3. Although the xylose assimilation capacity of YY5A at high temperature could be improved markedly by EMS mutagenesis, the inhibition of ethanol on xylose utilization remains during the co-fermentation of glucose and xylose.
4. The simultaneous saccharification and co-fermentation (SSCF) process with a thermotolerant xylose-fermenting *S. cerevisiae* might be a crucial strategy for ethanol production from lignocellulose at high temperature.

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