*fermentation*

MDPI

*Article*

# Recombinant Diploid *Saccharomyces cerevisiae* Strain Development for Rapid Glucose and Xylose Co-Fermentation

**Tingting Liu, Shuangcheng Huang and Anli Geng ***

School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, Singapore 599489, Singapore; lttf7@sina.com (T.L.); higle945@yahoo.com (S.H.)

* Correspondence: gan2@np.edu.sg; Tel.: +65-6460-8617

Received: 25 June 2018; Accepted: 25 July 2018; Published: 30 July 2018


**Abstract:** Cost-effective production of cellulosic ethanol requires robust microorganisms for rapid co-fermentation of glucose and xylose. This study aims to develop a recombinant diploid xylose-fermenting *Saccharomyces cerevisiae* strain for efficient conversion of lignocellulosic biomass sugars to ethanol. Episomal plasmids harboring codon-optimized *Piromyces* sp. E2 xylose isomerase (*PirXylA*) and *Orpinomyces* sp. ukk1 xylose (*OrpXylA*) genes were constructed and transformed into *S. cerevisiae*. The strain harboring plasmids with tandem *PirXylA* was favorable for xylose utilization when xylose was used as the sole carbon source, while the strain harboring plasmids with tandem *OrpXylA* was beneficial for glucose and xylose cofermentation. *PirXylA* and *OrpXylA* genes were also individually integrated into the genome of yeast strains in multiple copies. Such integration was beneficial for xylose alcoholic fermentation. The respiration-deficient strain carrying episomal or integrated *OrpXylA* genes exhibited the best performance for glucose and xylose co-fermentation. This was partly attributed to the high expression levels and activities of xylose isomerase. Mating a respiration-efficient strain carrying the integrated *PirXylA* gene with a respiration-deficient strain harboring integrated *OrpXylA* generated a diploid recombinant xylose-fermenting yeast strain STXQ with enhanced cell growth and xylose fermentation. Co-fermentation of 162 g $L^{-1}$ glucose and 95 g $L^{-1}$ xylose generated 120.6 g $L^{-1}$ ethanol in 23 h, with sugar conversion higher than 99%, ethanol yield of 0.47 g $g^{-1}$, and ethanol productivity of 5.26 g $L^{-1} \cdot h^{-1}$.

**Keywords:** *Saccharomyces cerevisiae*; diploid; xylose isomerase; xylose fermentation; glucose and xylose co-fermentation; biomass hydrolysate; cellulosic ethanol

## 1. Introduction

Ethanol can be produced from renewable resources such as crops or agricultural waste. It is therefore a sustainable and clean fuel. Further growth in bioethanol production largely depends on the effective conversion of lignocellulosic feedstock such as agricultural and forestry wastes to bioethanol because they are the most abundant polymers of fermentable sugars [1–3].

*Saccharomyces cerevisiae* is the most effective microorganism for fermenting sugars to ethanol due to its rapid sugar consumption rate, high sugar and ethanol tolerance, and resistance to biomass-derived inhibitors [4,5]. Much research has been done to genetically engineer *S. cerevisiae* strains for xylose fermentation [6–10].

Two xylose-assimilating pathways were heterologously engineered in *S. cerevisiae* for xylose-fermenting yeast construction. One focused on the D-xylose isomerase (XI) pathway [11,12], the other focused on the D-xylose reductase (XR) and xylitol dehydrogenase (XDH) pathway [10,13–15]. In the XI pathway, xylose is first isomerized into xylulose by XI and xylulose was then phosphorylated

into xylulose 5-phosphate by xylulokinase. Xylulose is subsequently metabolized to ethanol through glycolysis in the pentose phosphate pathway [11]. As no xylitol is produced in this pathway, much research focused on the XI pathway construction in xylose-fermenting yeast [12].

However, for all the engineered strains developed, rapid glucose and xylose co-fermentation is still challenging, in particular when sugar concentration is high [4,8–10]. Because fermentation time on mixed-substrate hydrolysates is still not cost-effective, strategies in evolutionary engineering were used to improve fermentation kinetics [16–18] and much research focused on the search for new or engineered sugar transporters [19–21]. More recently, robust diploid *S. cerevisiae* strains were developed for rapid xylose-fermentation [22–25].

This study aims to develop a recombinant *S. cerevisiae* strain for rapid glucose and xylose co-fermentation through metabolic engineering, evolutionary engineering and strain mating. Firstly, four episomal plasmids containing the two-copy codon-optimized *Piromyces* sp. E2 XI gene (*PirXylA*, GenBank accession number AJ249909.1), one-copy codon-optimized *Orpinomyces* sp. ukk1 XI gene (*OrpXylA*, GenBank accession number EU411046), one-copy *PirXylA* and *OrpXylA* in tandem, and two-copy *OrpXylA*, were individually constructed. They were subsequently transformed to an evolved respiration-deficient yeast strain. Four engineered strains with episomal XI genes were generated and optimal XI functional expression was identified. Afterwards, *PirXylA* and *OrpXylA* were separately integrated into the genome of two evolved *S. cerevisiae* strains (one respiration-efficient and the other respiration-deficient) in multiple copies according the methods described previously [26,27]. Four engineered yeasts with integrated XI genes were generated and screened for xylose fermentation or glucose/xylose co-fermentation. In the end, a diploid recombinant xylose-fermenting *S. cerevisiae* was constructed by mating a respiration-efficient haploid strain with a respiration-deficient haploid strain. To the best of our knowledge, this is the first report on diploid xylose-fermenting yeast strain construction by such strain mating. The resulted diploid yeast strain displayed superior glucose and xylose co-fermentation performance, which far exceeded that by engineered *S. cerevisiae* reported to-date [28,29].

## 2. Materials and Methods

### *2.1. Plasmid Construction*

All plasmids used in this work are listed in Table 1. All primers used in this study are listed in Table S1.

*E. coli* strain DH5α (Life Technologies, Rockville, MD, USA) were used as the transformation host for plasmid construction. *E. coli* were grown in LB medium containing 100 μg/mL ampicillin at 30 °C or 37 °C for plasmid maintenance [25]. The *PGK1* promoter was amplified from genomic DNA of *S. cerevisiae* strain ATCC 24860 and was ligated to pYES2 (Thermo-Fischer Scientific, Singapore) to replace the *GAL1* promoter, resulting in pPY1. *PirXylA* and *OrpXylA* were synthesized by Integrated DNA Technologies Singapore). Cassettes *PGK1*p-*OrpXylA-CYC1*t, *PGK1*p-*PirXylA-CYC1*t-*PGK1*p-*OrpXylA-CYC1*t, *PGK1*p-*OrpXylA-CYC1*t-*PGK1*p-*OrpXylA-CYC1*t and *PGK1*p-*PirXylA-CYC1*t-*PGK1*p-*PirXylA-CYC1*t were cloned into pPY1 individually, resulting in plasmids pPYXo, pPYXpXo, pPYXoXo and pPYXpXp (Figure 1A–D).

NTS2-2 partial fragment (pNTS) was obtained by overlap extension polymerase chain reaction (OE-PCR) of *S. cerevisiae* ATCC 24860 genomic DNA over 274 bp upstream and 245 bp downstream homologous regions of NTS2-2. The XXUN plasmid (Figure 1E) is an integrating yeast plasmid based on pPYXpXp whereby the 2 μ origin was replaced with pNTS. The *loxP-KanMX4-loxP* cassette was amplified from the plasmid pUG6 [30]. Cassettes *PGK1*p-*OrpXylA-CYC1*t and *loxP-KanMX4-loxP*-pNTS were obtained by OE-PCR and then subsequently constructed into plasmid pUC19 [12], resulting in plasmid XoNK (Figure 1F). Both plasmids were digested with *Swa*I for XI genome integration using NTS2-2 homologous recombinant arms.

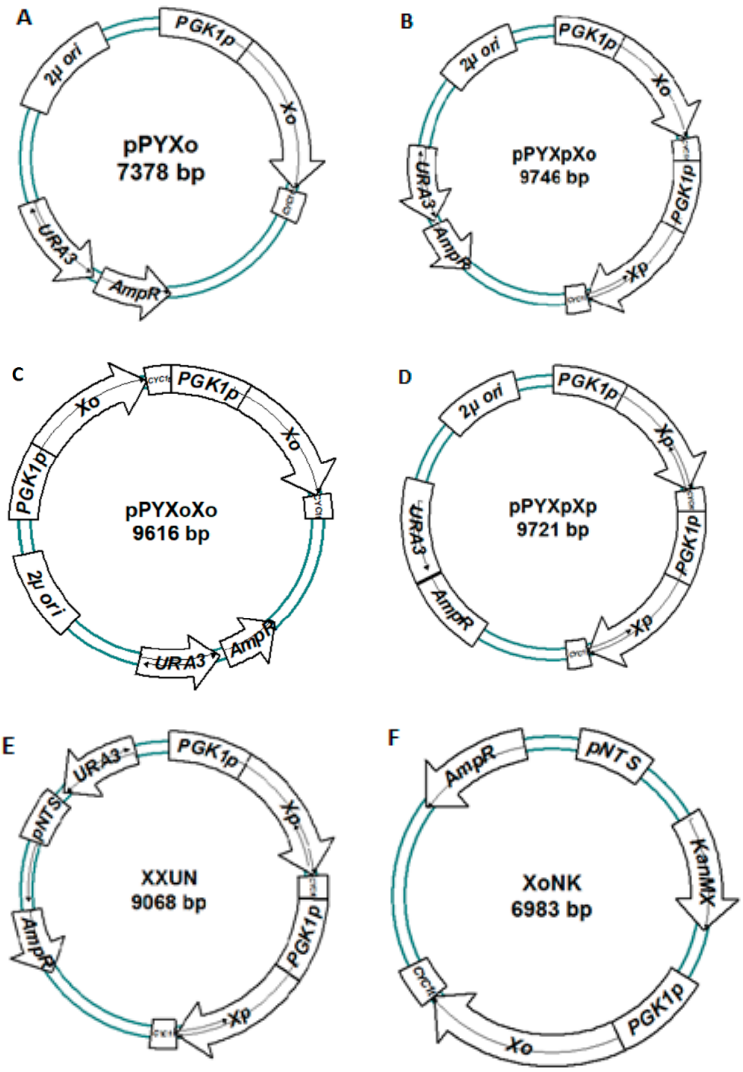


**Figure 1.** Map of plasmids. (**A**) pPYXo; (**B**) pPYXpXo; (**C**) pPYXoXo; (**D**) pPYXpXp; (**E**) XXUN; and (**F**) XoNK.

### *2.2. Strain Construction and Adaptive Evolution*

All strains used in this work are listed in Table 1. *S. cerevisiae* haploid strains JUK36α and JUK39a were isolated from the diploid strain *S. cerevisiae* ATCC 24860. They were both overexpressed with the non-oxidative pentose phosphate pathway (PPP) genes and xylulokinase gene, *XKS1*. *URA3* and *GRE3* genes were disrupted in both haploid strains. In addition, the *CYC3* gene, encoding cytochrome c heme lyase, was knocked out in strain JUK39a to eliminate respiration [12].

Plasmid pPYXpXp was transformed into strain JUK39a using the LiAc/SS carrier DNA/PEG method [31], resulting in recombinant *S. cerevisiae* 39aXpXp. The respiration-deficient strain 39aXpXp was evolved by continuous transfer and cultivation in a synthetic medium (SM) containing 6.7 g $L^{-1}$ yeast nitrogen base without amino acids (YNB) (Difco Laboratories Inc., Detroit, MI, USA) and 20 g $L^{-1}$ xylose (SMX) under oxygen-limited conditions according to the protocols described in our earlier report [12]. After 75-day continuous transfer, cell doubling time was reduced from 15.9 h to 6.4 h. Samples were taken on day 75 and streaked on SMX plates containing 20 g $L^{-1}$ xylose and 20 g $L^{-1}$ agar. Fifteen large single colonies were selected. They were then incubated in 50 mL SMX medium in 100 mL Erlenmeyer flasks capped with rubber stoppers, shaken at 200 rpm and 30 °C. Weight loss of the cultures from the 15 colonies was individually measured on Day 4. The best ethanol-producing strain

was indicated by the highest weight loss [18]. This strain was denoted 39aXpXp2415, and plasmid pPYXpXp in this strain was removed by streaking the culture on 5-FOA plates [12]. This generated the background strain $39a_2$ (Table 1). On the other hand, strain JUK51a_2 (Table 1) was evolved anaerobically in a chemostat with an increase in the dilution rate from 0.01 to 0.14 $h^{-1}$ on xylose using the method described in our earlier report [12]. The fastest growing strain was selected, and the plasmid pJFX11 was removed according to the above-described method on 5-FOA plates. The background strain $36\alpha_2$ was later obtained (Table 1).

**Table 1.** Strains and plasmids used in this study.

| Strains/Plasmid | Genotype/Phenotype | References |
|---|---|---|
| *Saccharomyces cerevisiae* strains | | |
| *Saccharomyces cerevisiae* ATCC 24860 | Obtained from American Type Culture Collection (ATCC) | |
| JUK36α | *S. cerevisiae* ATCC 24860 segregant; *MAT*; *ura3::loxP*; *TKL1::RKIt-RKI1-ADH1p-RPE1t-RPE1-TPI1p-loxP-XKS1t-XKS1-PGK1p-PDC1p-TAL1-TAL1t-FBA1p*; *gre3::loxP* | [12] |
| JUK39a | *S. cerevisiae* ATCC 24860 segregant; *MATa*; *ura3::loxP*; *TKL1::RKIt-RKI1-ADH1p-RPE1t-RPE1-TPI1p-loxP-XKS1t-XKS1-PGK1p-PDC1p-TAL1-TAL1t-FBA1p*; *gre3::loxP*; *cyc3::loxP* | [12] |
| JUK51a_2 | JUK36α derivative; {pJFX11}/(*BvuXylA*, *XK*, *PPP*, *gre3*Δ) | [12] |
| 39aXpXp | JUK39a derivative; {pPYXpXp}/(two-copy *PirXylA*, *XK*, *PPP*, *gre3*Δ, *cyc3*Δ) | This work |
| 39aXpXp2415 | 39aXpXp derivative; {pPYXpXp}/(two-copy *PirXylA*, *XK*, *PPP*, *gre3*Δ, *cyc3*Δ, AE) | This work |
| $36\alpha_2$ | Isolate from chemostat anaerobic and adaptive evolution at a dilution rate of 0.15 $h^{-1}$ on xylose of JUK51a_2 and loss of plasmid pJFX11 | This work |
| $39a_2$ | Isolate from 39aXpXp2415 and loss of plasmid pPYXpXp | This work |
| $39a_2$XpXp | $39a_2$ derivative; {pPYXpXp}/(two-copy *PirXylA*, *XK*, *PPP*, *gre3*Δ, *cyc3*Δ) | This work |
| $39a_2$XpXo | $39a_2$ derivative; {pPYXpXo}/(*OrpXylA*, *PirXylA*, *XK*, *PPP*, *gre3*Δ, *cyc3*Δ) | This work |
| $39a_2$Xo | $39a_2$ derivative; {pPYXo}/(*OrpXylA*, *XK*, *PPP*, *gre3*Δ, *cyc3*Δ) | This work |
| $39a_2$XoXo | $39a_2$ derivative; {pPYXoXo}/}/(two-copy *OrpXylA*, *XK*, *PPP*, *gre3*Δ, *cyc3*Δ) | This work |
| $36\alpha_2$XpXpUN | $36\alpha_2$ derivative;*NTS2-2*::two-copy *PirXylA*, *ura3*, *XK*, *PPP*, *gre3*Δ | This work |
| $36\alpha_2$XoNK | $36\alpha_2$ derivative;*NTS2-2*::*OrpXylA-KanMX4*, *XK*, *PPP*, *gre3*Δ, *ura3*Δ | This work |
| $39a_2$XpXpUN | $39a_2$ derivative; *NTS2-2*::two-copy *PirXylA*, *ura3*, *XK*, *PPP*, *gre3*Δ, *cyc3*Δ | This work |
| $39a_2$XoNK | $39a_2$ derivative; *NTS2-2*::*OrpXylA-KanMX4*, *XK*, *PPP*, *gre3*Δ, *cyc3*Δ, *ura3*Δ | This work |
| STXQ | Isolate from mating of $36\alpha_2$XpXpUN with $39a_2$XoNK | This work |
| **Plasmids** | | |
| pUG6 | *E. coli* plasmid with segment *loxP–KanMX4–loxP* | [30] |
| pJFX11 | YEp, *TEF1*p-*BvuXylA-CYC1t* | [12] |
| pPY1 | pPYES2; *GAL1*p replaced by *PGK1*p | This work |
| pPYXo | pPY1; *PGK1*p-*OrpXylA-CYC1*t | This work |
| pPYXpXp | pPY1; 2 copies of *PGK1*p-*PirXylA-CYC1*t in tandem | This work |
| pPYXpXo | pPY1; *PGK1*p-*PirXylA-CYC1*t-*PGK1*p-*OrpXylA-CYC1t* | This work |
| pPYXoXo | pPY1; 2 copies of *PGK1*p-*OrpXylA-CYC1t* in tandem | This work |
| XXUN | pPYXpXp-based yeast integration plasmid; 2 μ and *ura3* were replaced with *ura3* and *NTS2-2* partial fragment | This work |
| XoNK | pUC19-based yeast integration plasmid; *loxP-KanMX4-loxP-pNTS-PGK1*p-*OrpXylA-CYC1*t | This work |

Plasmids pPYXo, pPYXpXo, pPYXoXo and pPYXpXp were individually transformed into $39a_2$, resulting in strains $39a_2$Xo, $39a_2$XpXo, $39a_2$XoXo and $39a_2$XpXp, respectively. Plasmids XoNK and XXUN were digested with *Swa*I and linearized. The linearized fragments were integrated into the genome of $39a_2$ and $36\alpha_2$ at the NTS2-2 site, individually, resulting in recombinant strains $39a_2$XoNK, $39a_2$XpXpUN, $36\alpha_2$XoNK and $36\alpha_2$XpXpUN. For each plasmid transformation, a pool of transformants were generated. The best transformant was isolated based on its cell growth and ethanol production in xylose medium as described in the isolation of 39aXpXp2415. Recombinant strains, $36\alpha_2$XpXpUN and $39a_2$XoNK, were later mated to obtain strain STXQ through screening on SMX agar plates containing 20 g $L^{-1}$ xylose at pH 6 followed by fermentation in SMX medium containing up to 250 g $L^{-1}$ xylose.

### 2.3. Enzyme Activity Assay

Cells were grown to the exponential phase in SMX medium containing 20 g $L^{-1}$ xylose. After centrifugation, cells were washed twice with chilled distilled water, and then lysed in chilled extraction buffer (100 mM Tris-HCl, 10 mM $MgSO_4$, pH 7.5) by vortex mixing using a Vortex Mixer (Mixer UZUSIO, Tokyo, Japan) with 0.5 mg of 0.5 mm glass beads (Sigma-Aldirich, Singapore). Protease inhibitor cocktail set V (Merck Millipore, Singapore) was added to inhibit serine and cysteine generated in the lysis process. Cell debris was removed by centrifugation (Microcentrifuge D3024, DR. LAB Technology Hong Kong, Hong Kong, China) at 4000× *g* for 10 min at 4 °C, and the crude extract was stored for enzyme activity assay. The protein concentration of the cell extract was determined by the Bradford Assay using a Coomassie Protein Assay Kit (Thermo Scientific, Singapore), and bovine serum albumin (BSA) was used as the standard. Extraction of raw proteins from the yeast strains was performed in duplicate.

The in-vitro XI activity was determined by measuring NADH absorbance using sorbitol dehydrogenase (SDH) (Sigma Aldrich, Singapore). The assay mixture (1 mL) contained extraction buffer, 0.15 mM NADH, 1 U SDH, and 50 µL crude extract. It was equilibrated at 30 °C for 2 min. The reaction was started by the addition of D-xylose to a final concentration of 500 mM. The change of NADH concentration within 3 min was detected using a UV-visible spectrophotometer (Shimadzu, Tokyo, Japan) at wavelength 340 nm, and the specific activity of XI in the recombinant strains was determined [32]. A molar extinction coefficient of 6.25 $(mM\ cm)^{-1}$ at 340 nm for NADH was used to calculate specific activity. Specific activity was expressed as units per mg protein. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of coenzyme/min, under the specified reaction conditions [12].

### 2.4. Glucose and Xylose Fermentation by the Recombinant Strains

The preculture of the evolved engineered strains was prepared by growing them in 40 mL SMX medium containing 20 g $L^{-1}$ xylose in 100 mL Erlenmeyer flaks at 200 rpm and 30 °C for 24 h. The oxygen-limited conditions in the flasks was maintained by capping the flasks with rubber stoppers pierced with a needle to allow the release of $CO_2$. Cells in the exponential phase were harvested by centrifugation (Microcentrifuge D3024, DR. LAB Technology Hong Kong, Hong Kong, China) at 14,000× *g* for 1 min. Cell pellets were washed twice and were then inoculated into SM medium supplemented with 20 g $L^{-1}$ xylose with (SMGX) or without 20 g $L^{-1}$ glucose (SMX). The initial optical cell density at 600 nm (OD600) of the culture was about 2 unless otherwise stated. Fermentation was conducted in 100 mL Erlenmeyer shaking flasks under oxygen-limited conditions with a working volume of 40 mL at 200 rpm and 30 °C. The pH value was adjusted at 5.0–6.0 using 3 M NaOH during fermentation. All fermentation experiments were performed in duplicate. Samples were taken periodically to measure OD600, sugar and metabolite concentration.

### 2.5. Analytical Methods

Cell densities (OD600) were determined using a UV-visible spectrophotometer (Shimadzu, Tokyo, Japan). Fifty-mL cell cultures with varying OD600 (1–5) were filtered with 0.22 µm glass fiber filter membrane (Merck Millipore, Singapore) using Aspirator A-3S (Fisher Scientific, Tokyo, Japan). Cells were washed twice with distilled water, dried at 105 °C in an oven for 24 h, and then weighed. One OD600 unit corresponded to 0.241 g $L^{-1}$ dry cell weight (DCW). Concentrations of glucose, xylose, xylitol, acetate, glycerol and ethanol produced in fermentation were determined by Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector RID-10A using an Aminex HPX-87H ion exchange column (Bio-Rad Laboratories, Woodinville, WA, USA). The column was eluted at 60 °C with 5 mM of sulfuric acid as the mobile phase at a flow rate of 0.6 mL $min^{-1}$.

### *2.6. Quantitative Reserve Transcription Polymerase Chain Reaction (RT-PCR)*

Recombinant yeast strains $39a_2Xo$, $39a_2XpXo$, $39a_2XoXo$ and $39a_2XpXp$ were individually cultivated in 40 mL SMX medium containing 20 g $L^{-1}$ xylose under oxygen-limited conditions at 200 rpm and 30 °C. The expression of XI gene transcripts was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Primers for RT-PCR are listed in Table S1. Samples were taken at 24 h, and cells were harvested by centrifuging 2-mL culture at 14,000× *g* and 4 °C for 1 min. Cell pellets were washed twice using double distilled water and total RNA was isolated by using the E.Z.N.A™ Yeast RNA Kit (Omega Bio-tek, Norcross, GA, USA). First-strand cDNA was obtained by using the SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Such cDNA was then used as the template for qRT-PCR using iCycler iQ™ Real-time PCR Detection System (Bio-Rad Laboratories, Woodinville, WA, USA) and FastStart Universal SYBR Green Master (Roche Applied Science, Penzberg, Germany). The cycle threshold values were calculated with the Optical System Software Version 3.1 (Bio-Rad Laboratories, Woodinville, WA, USA), and the detection threshold over the cycle range was set at 2 to 10. Each PCR was carried out in duplicate. All kits were used under conditions recommended by the manufacturers. The $2^{-\Delta\Delta Ct}$ method [33] was used to analyze the relative changes in gene expression using the housekeeping *ACT1* gene as the reference.

### *2.7. Biomass Hydrolysate Fermentation Using Strain S. cerevisiae STXQ*

The diploid recombinant *S. cerevisiae* strain STXQ (Table 1) was inoculated into YP medium (10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ peptone, pH 5.0) containing 132 g $L^{-1}$ xylose or mixture of 162 g $L^{-1}$ glucose and 95 g $L^{-1}$ xylose at an initial OD600 of about 13. Fermentation was conducted in 40 mL YP medium in 100 mL shaking flasks under oxygen limited conditions at 200 rpm and 30 °C.

Oil palm empty fruit bunch (OPEFB) hydrolysate was obtained using crude cellulase from *Trichoderma reesei* Rut-C30 according to the protocols described in our earlier report [34]. OPEFB hydrolysate was sterilized using 0. 22 μm filter membrane (Merck Millipore, Singapore) and was supplemented with 7 g $L^{-1}$ yeast extract 2 g $L^{-1}$ peptone, 2 g $L^{-1}$ $(NH_4)_2SO_4$, 2.05 g $L^{-1}$ $KH_2PO_4$, and 0.25 g $L^{-1}$ $Na_2HPO_4$. The diploid recombinant *S. cerevisiae* strain STXQ was inoculated into the above OPEFB hydrolysate medium with an initial OD600 about 5. Fermentation was carried out in 40 mL fermentation medium in 100 mL shaking flasks under oxygen-limited conditions at 200 rpm and 30 °C with an initial pH of 4.48. Samples were taken periodically for OD600, sugar and metabolite analysis. Experiments were conducted in duplicate.

## 3. Results

### *3.1. Expression of XIs with Various Combinations*

XI activities were assayed for $39a_2$ recombinant strains with episomal XI genes (Table 2). XI activity of $39a_2XoXo$ was three times higher than that of $39a_2XpXp$ and 2.5 times higher than that of $39a_2Xo$. Strain $39a_2XoNK$ presented the highest XI activity among all the yeast strains with integrated XI genes, followed by $39a_2XpXpUN$. Quantitative RT-PCR showed that the transcription level of XI gene in the engineered strains significantly increased compared to that in the parent strain $39a_2$ (Table 3). In $36\alpha_2XpXpUN$ and $39a_2XoNK$, respective 1.04-fold and 1.41-fold increases in XI gene transcription levels were observed compared to those in $39a_2XpXp$ and $39a_2XoXo$. Inconsistent XI activity and transcription level were observed.

**Table 2.** Specific activity of xylose isomerase in the recombinant strains.

| Strains | Specific Activity (U $mg^{-1}$ Protein) |
|---|---|
| $39a_2$XpXp | 0.10 ± 0.003 |
| $39a_2$XpXo | 0.11 ± 0.019 |
| $39a_2$Xo | 0.12 ± 0.041 |
| $39a_2$XoXo | 0.30 ± 0.079 |
| $36\alpha_2$XpXpUN | 0.11 ± 0.007 |
| $36\alpha_2$ XoNK | 0.04 ± 0.005 |
| $39a_2$XpXpUN | 0.26 ± 0.004 |
| $39a_2$XoNK | 0.72 ± 0.006 |

The results represent the mean ± standard deviation of duplicate independent experiments.

**Table 3.** Fold-change in xylose isomerase (XI) mRNA expression.

| Strain | Fold-Change [a] | |
|---|---|---|
| | ***PirXylA*** | ***OrpXylA*** |
| $39a_2$XpXp | 50.21 (47.81–52.74) | nil |
| $39a_2$XpXo | 29.86 (28.43–31.36) | 11.71 (10.62–12.92) |
| $39a_2$Xo | nil | 59.71 (51.98–68.59) |
| $39a_2$XoXo | nil | 59.71 (55.72–64) |
| $36\alpha_2$XpXpUN | 51.98 (49.50–54.60) | nil |
| $39a_2$XoNK | nil | 84.45 (59.71–119.43) |

[a] Fold-change of XI mRNA level in the recombinant yeast strains compared to the parent strain $39a_2$. Results were based on duplicate independent real-time RT-PCR reactions.

### *3.2. Glucose and Xylose Fermentation by the Engineered $39a_2$ Strains Harboring Episomal XI Genes*

Fermentation performance of $39a_2$ recombinant strains with episomal XI genes was tested in SMX medium containing 20 g $L^{-1}$ xylose under oxygen-limited conditions. Apparently, strain $39a_2$XpXp displayed the fastest xylose utilization rate, followed by $39a_2$XoXo (Figure 2). Strain $39a_2$XpXo utilized xylose more slowly than $39a_2$XpXp, though it demonstrated almost the same XI activity (Table 2). On the other hand, strain $39a_2$XoXo showed a faster xylose utilization rate than strain $39a_2$Xo; however, the former displayed much higher activity than the latter. On the contrary, xylose utilization results accorded quite well with the results of qRT-PCR analysis showing that strain $39a_2$XpXp had higher XI gene transcription levels than strain $39a_2$XpXo (Table 3) and a faster xylose utilization rate (Figure 2). However, strains $39a_2$XoXo and $39a_2$Xo displayed identical XI gene transcription levels (Table 3), almost the same xylose consumption rate (Figure 2) and xylose conversion (Table 4). Interestingly, strain $39a_2$XpXp also exhibited the maximal ethanol yield of 0.472 g $g^{-1}$ (Table 4). Such results suggest that expression of two-copy *PirXylA* is favorable for xylose alcoholic fermentation when xylose is used as the sole carbon source.

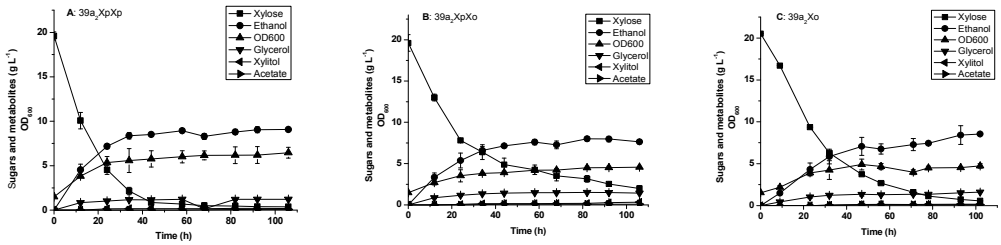


**Figure 2.** *Cont.*

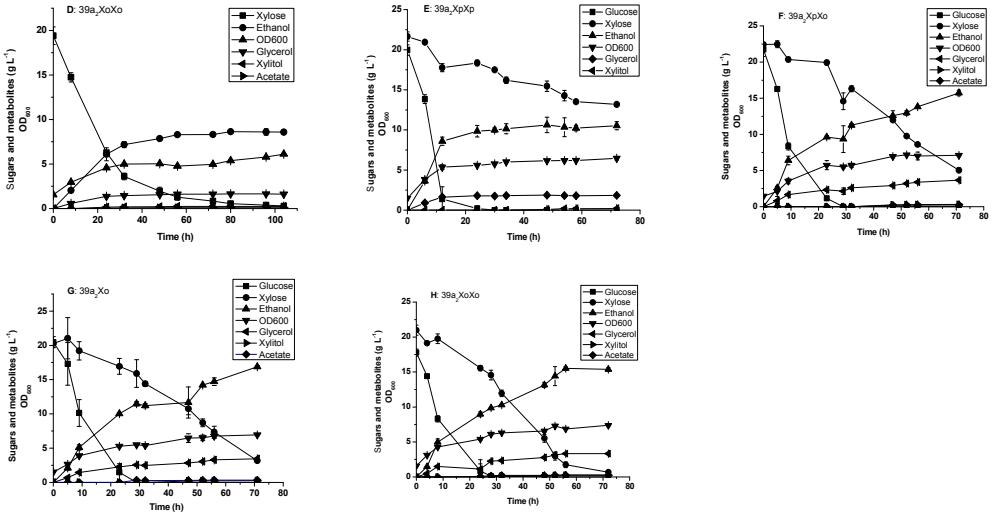


**Figure 2.** Sugar fermentation under oxygen-limited conditions by the engineered *S. cerevisiae* strains in SM medium containing 20 g $L^{-1}$ xylose (SMX) or 20 g $L^{-1}$ glucose and 20 g $L^{-1}$ xylose (SMGX). (**A**) 39$a_2$XpXp SMX; (**B**) 39$a_2$XpXo SMX; (**C**) 39$a_2$Xo SMX; (**D**) 39$a_2$XoXo SMX; (**E**) 39$a_2$XpXp SMGX; (**F**) 39$a_2$XpXo SMGX; (**G**) 39$a_2$Xo SMGX; (**H**) 39$a_2$XoXo SMGX. The results represent the mean ± standard deviation of duplicate independent experiments ($p < 0.01$).

**Table 4.** Glucose and xylose fermentation by recombinant xylose-fermenting yeast strains.

| Strains | Initial glucose (g $L^{-1}$) | Initial Xylose (g $L^{-1}$) | Xylose Conversion (%) | Ethanol Yield (g $g^{-1}$) | Ethanol Productivity (g $h^{-1}$ $L^{-1}$) | Specific Growth Rate ($h^{-1}$) |
|---|---|---|---|---|---|---|
| 39$a_2$xpXp [a] | - | 20 | 98% ± 0.004 | 0.472 ± 0.013 | 0.098 ± 0.004 | 0.014 ± 0.001 |
| 39$a_2$XpXo [a] | - | 20 | 89.8% ± 0.011 | 0.434 ± 0.017 | 0.098 ± 0.004 | 0.011 ± 5.89 × $10^{-6}$ |
| 39$a_2$Xo [a] | - | 20 | 97.3% ± 0.0138 | 0.428 ± 0.026 | 0.084 ± 0.001 | 0.011 ± 0.0003 |
| 39$a_2$XoXo [a] | - | 20 | 98.5% ± 0.0057 | 0.449 ± 0.038 | 0.108 ± 0.001 | 0.013 ± 2.29 × $10^{-5}$ |
| 36$\alpha_2$XpXpUN [a] | - | 20 | 86.4% ± 0.052 | 0.318 ± 0.01 | 0.069 ± 0.007 | 0.014 ± 0.0003 |
| 36$\alpha_2$XoNK [a] | - | 20 | 6.42% ± 0.057 | ND | ND | 0.003 ± 0.0006 |
| 39$a_2$XpXpUN[a] | - | 20 | 67.0% ± 0.089 | 0.398 ± 0.048 | 0.067 ± 0.001 | 0.013 ± 0.0002 |
| 39$a_2$XoNK [a] | - | 20 | 99.0% ± 0.002 | 0.368 ± 0.029 | 0.107 ± 0.007 | 0.0098 ± 0.0003 |
| 39$a_2$XpXp [a] | 20 | 20 | 22.0% ± 0.003 | 0.444 ± 0.029 | 0.221 ± 0.020 | 0.020 ± 0.0007 |
| 39$a_2$XpXo [a] | 20 | 20 | 77.5% ± 0.017 | 0.392 ± 0.001 | 0.081 ± 0.006 | 0.022 ± 0.0001 |
| 39$a_2$Xo [a] | 20 | 20 | 84.3% ± 0.0007 | 0.428 ± 0.01 | 0.238 ± 0.003 | 0.021 ± 0.0004 |
| 39$a_2$XoXo [a] | 20 | 20 | 96.7% ± 0.011 | 0.449 ± 0.022 | 0.213 ± 0.004 | 0.021 ± 0.001 |
| 36$\alpha_2$XpXpUN [a] | 20 | 20 | 73.8% ± 0.011 | 0.348 ± 0.049 | 0.225 ± 0.007 | 0.029 ± 0.0006 |
| 36$\alpha_2$XoNK [a] | 20 | 20 | 2.62% ± 0.000 | 0.390 ± 0.043 | 0.222 ± 0.003 | 0.016 ± 0.0013 |
| 39$a_2$XpXpUN [a] | 20 | 20 | 25.6% ± 0.097 | 0.405 ± 0.049 | 0.213 ± 0.002 | 0.013 ± 0.0002 |
| 39$a_2$XoNK [a] | 20 | 20 | 97.3% ± 0.006 | 0.387 ± 0.002 | 0.243 ± 0.003 | 0.020 ± 0.0005 |
| 36$\alpha_2$XpXpUN [b] | - | 40 | 75.9% ± 0.041 | 0.384 ± 0.033 | 0.108 ± 0.011 | 0.013 ± 0.0004 |
| 39$a_2$XoNK [b] | - | 40 | 81.6% ± 0.004 | 0.421 ± 0.004 | 0.131 ± 0.000 | 0.010 ± 0.0003 |
| STXQ [b] | - | 40 | 93.3% ± 0.019 | 0.393 ± 0.024 | 0.146 ± 0.009 | 0.017 ± 0.0002 |
| STXQ [b] | - | 132 | 100% | 0.498 ± 0.006 | 1.13 ± 0.01 | 0.014 ± 0.0004 |
| STXQ [c] | 162 | 95 | 99.27% ± 0.002 | 0.475 ± 0.01 | 5.24 ± 0.02 | 0.024 ± 0.0001 |

[a] Fermentation in SM medium in 72 h; [b] Fermentation in YP medium in 102 h; [c] Fermentation in YP medium. The results represent the mean ± standard deviation of duplicate independent experiments.

Co-fermentation of 20 g $L^{-1}$ glucose and 20 g $L^{-1}$ xylose was carried out in SM medium under oxygen-limited conditions. It can be seen that for all strains, glucose was almost completely consumed at 24 h (Figure 2). However, xylose utilization varied greatly for the four engineered 39$a_2$ strains. Noticeably, in the presence of 20 g $L^{-1}$ glucose, strain 39$a_2$XpXp utilized xylose quite slowly and about 22% xylose was consumed at 72 h (Table 4). On the other hand, xylose utilization was improved to 77.5% by strain 39$a_2$XpXo. Moreover, xylose consumption was improved to 84.3% by strain 39$a_2$Xo and it was further improved to 97% by 39$a_2$XoXo (Table 4). It is worth noting that strain 39$a_2$XoXo presented the highest ethanol yield (0.449 g $g^{-1}$). The above results suggest that the expression of two-copy of *OrpXylA* is beneficial for glucose and xylose co-fermentation.

### *3.3. Glucose and xylose Fermentation by the Engineered Yeast Strains Harboring Integrated XI Genes*

Fermentation performance of $39a_2$ recombinant strains with integrated XI genes was tested in SM medium containing 20 g $L^{-1}$ xylose (SMX) or 20 g $L^{-1}$ glucose and 20 g $L^{-1}$ xylose (SMGX) under oxygen-limited conditions (Figure 3). In SMX fermentation, the specific growth rate of $36\alpha_2$XpXpUN on xylose was 0.014 $h^{-1}$, much higher than that of $36\alpha_2$XoNK (0.003 $h^{-1}$), whereas xylose conversion by strain $39a_2$XoNK was 99.0%, much higher than that by $39a_2$XpXpUN (67.0%) (Table 4). In SMGX fermentation, the specific growth rate of $36\alpha_2$XpXpUN on xylose was 0.029 $h^{-1}$, much higher than that of $36\alpha_2$XoNK (0.016 $h^{-1}$), whereas xylose conversion by strain $39a_2$XoNK was 97.30%, much higher than that by $39a_2$XpXpUN (73.8%) (Table 4). In both SMX and SMGX fermentation, strain $36\alpha_2$XpXpUN presented the best cell growth, whereas strain $39a_2$XoNK displayed the best xylose conversion. Furthermore, strain $39a_2$XoNK exhibited the highest ethanol productivity in both SMX and SMGX fermentation. Despite the possible XI gene copy number variation in these strains, the above results demonstrated that the elimination of respiration was favorable for xylose fermentation, which corroborated quite well with previous reports [12,14]. Strains $36\alpha_2$XpXpUN and $39a_2$XoNK were therefore selected for mating to generate the diploid recombinant strain STXQ to attain good cell growth and xylose fermentation.

### *3.4. Glucose and xylose Fermentation by Diploid Recombinant Strain STXQ*

Fermentation performance of STXQ was tested in YP medium containing 40 g $L^{-1}$ xylose under oxygen-limited conditions. As expected, strain STXQ inherited the advantages of both parent strains, $36\alpha_2$XpXpUN and $39a_2$XoNK. It presented 93.3% xylose conversion and a specific growth rate of 0.017 $h^{-1}$; both were higher than those of its parent strains (Table 4). In addition, ethanol productivity was greatly improved (Figure 3, Table 4). A very minimum amount of glycerol was produced by STXQ, although glycerol production was notable for the respiration-deficient parent strain $39a_2$XoNK (Figure 3). Moreover, production of xylitol and acetate was almost undetectable.

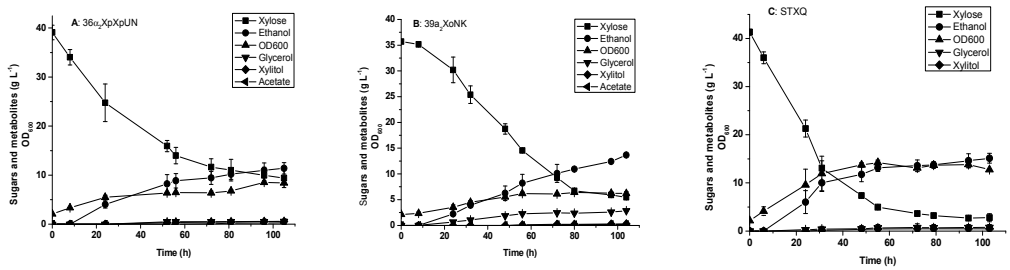


**Figure 3.** Xylose fermentation in YPX medium containing 40 g $L^{-1}$ xylose under oxygen-limited conditions. (**A**) $36\alpha_2$XpXpUN; (**B**) $39a_2$XoNK; (**C**) STXQ. The results represent the mean ± standard deviation of duplicate independent experiments ($p < 0.01$).

Fermentation performance of strain STXQ was further invested in YP medium containing high-concentration sugar. With an initial OD600 of about 13, strain STXQ consumed 100% xylose in fermenting 132 g $L^{-1}$ xylose and produced 65.8 g $L^{-1}$ ethanol at 46 h. The corresponding ethanol yield was 0.498 g $g^{-1}$ (Figure 4A, Table 4). In fermentation of 162 g $L^{-1}$ glucose and 95 g $L^{-1}$ xylose with about the same initial OD600, more than 99% glucose and xylose were co-utilized within 23 h (Figure 4B, Table 4). Ethanol titer reached 120.6 g $L^{-1}$ corresponding to an ethanol volumetric productivity of 5.26 g $L^{-1}$ $h^{-1}$. These are so far the highest values compared to those reported in the literature. The above results indicate that the strain development strategy elaborated in this study is efficient in generating a robust *S. cerevisiae* strain with improved xylose fermentation capabilities.

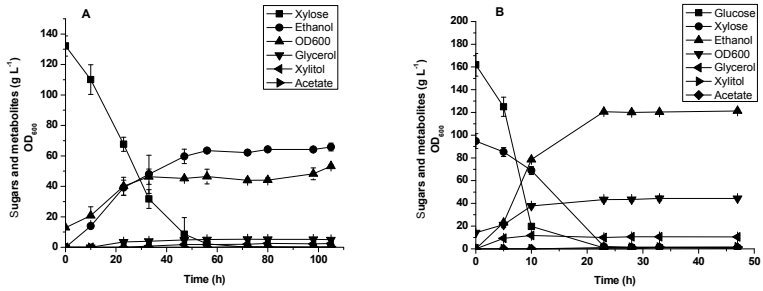


**Figure 4.** High-titer sugar fermentation under oxygen-limited conditions by strain STXQ in YP media. (**A**) Xylose fermentation in YPX medium containing 132 g $L^{-1}$ xylose; (**B**) Glucose and xylose co-fermentation in YPGX medium containing 162 g $L^{-1}$ glucose and 95 g $L^{-1}$ xylose. The results represent the mean ± standard deviation of duplicate independent experiments ($p < 0.01$).

### *3.5. Oil Palm Empty Fruit Bunch Hydrolysate Fermentation by Diploid Recombinant Strain STXQ*

Fermentation performance of strain STXQ was further tested in OPEFB hydrolysate containing 41.81 g $L^{-1}$ glucose, 30.00 g $L^{-1}$ xylose, 7 g $L^{-1}$ yeast extract, 2 g $L^{-1}$ peptone, 2 g $L^{-1}$ $(NH4)_2SO_4$, 2.05 g $L^{-1}$ $KH_2PO_4$, and 0.25 g $L^{-1}$ $Na_2HPO_4$ under oxygen-limited conditions with an initial OD600 of about 10. Strain STXQ consumed 95.3% glucose and 88.9% xylose (Figure 5). The pH value decreased from 4.48 to 4.00 within 72 h. Strain STXQ consumed 94.0% total sugar from the OPEFB hydrolysate without detoxification. The specific cell growth rate ($\mu_{max}$) reached 0.013 $h^{-1}$ and the ethanol yield was 0.420 g $g^{-1}$. Glucose was quickly consumed, and glucose-xylose co-fermentation was clearly observed within 24 h. Subsequently, ethanol concentration kept increasing with almost the sole consumption of xylose until it reached the final titer of 28.4 g $L^{-1}$ at 72 h.

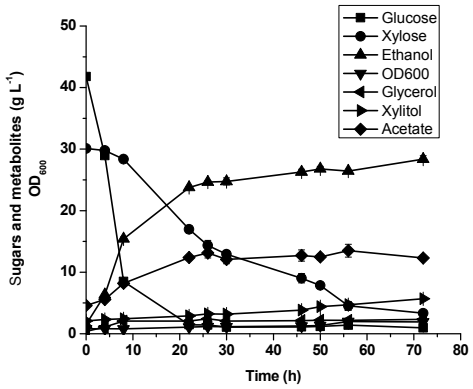


**Figure 5.** Sugar fermentation under oxygen-limited conditions by strain STXQ in oil palm empty fruit bunch hydrolysate supplemented with 7 g $L^{-1}$ yeast extract, 2 g $L^{-1}$ peptone, 2 g $L^{-1}$ $(NH4)_2SO_4$, 2.05 g $L^{-1}$ $KH_2PO_4$, and 0.25 g $L^{-1}$ $Na_2HPO_4$. The results represent the mean ± standard deviation of duplicate independent experiments ($p < 0.01$).

## 4. Discussion

Episomal plasmids containing *OrpXylA*, *PirXylA* and *OrpXylA* in tandem, two-copy *PirXylA* and two-copy *OrpXylA* expression cassettes (Figure 1) were transformed into the background strain $39a_2$ individually. Among all the $39a_2$ strains harboring episomal XI genes, strain $39a_2$XpXp presented the fastest rate of xylose utilization when xylose was used as the sole carbon source. However, it did not exhibit the highest XI activity and transcription level (Tables 2 and 3). Inconsistent XI activity and transcription level in recombinant xylose-fermenting *S. cerevisiae* strain was reported and it could be due to rearrangement of pentose phosphate pathway (PPP) genes, decreased glycolysis activity, repressed respiration activity, and enhanced gluconeogenesis [35]. Besides XI activity, the

enhanced xylose utilization and fermentation could be associated with the elevated expression of sugar transporter genes, non-oxidative pentose phosphate pathway (PPP) genes such as *TAL1*, *TKL1*, *RKI1*, and *RPE1* and xylulokinase gene, *XKS1* [19,20,25,36].

For glucose and xylose co-fermentation, strain $39a_2$XoXo presented the best glucose and xylose co-utilization (Figure 2) and exhibited the highest xylose conversion (96.7%) (Table 4). *S. cerevisiae* does not contain specific xylose transporters. Xylose was therefore transported by glucose transporters. Xylose affinity for the glucose transporters was very low at a high glucose concentration; however, it could increase to a similar level of glucose affinity at low glucose concentration [19,20]. As a result, diauxic lag is still a practical problem associated with mixed sugar utilization by xylose-fermenting yeasts. However, the diauxic growth was not significant for strains expressing *OrpXylA*, $39a_2$Xo and $39a_2$XoXo (Figure 2). The above results and analysis suggest that expression of *OrpXylA* is beneficial for glucose and xylose co-fermentation. This is consistent with a previous report [37].

Chromosomal integration of the XI gene into the *S. cerevisiae* genome has received a significant amount of attention in recent years [25,26]. It allows the recombinant strain to retain its physiological characteristics in nonselective medium. In the present study, multiple copies of XI genes were integrated at the 18S rDNA sites based on random homologous recombination. Such genome integration led to stable recombinant yeast strains (Figure S1) and boosted XI activity to 0.72 U $mg^{-1}$ protein in $39a_2$XoNK (Table 2). Such improvement might be associated with the multiple copies of XI integrated in the yeast genome [25]. This further led to high XI transcription levels (Table 3), high xylose conversion and ethanol production (Table 4). Such results accorded quite well with earlier reports [25,26,36]. Notably, among these strains, $39a_2$ strains with integrated XI genes presented higher XI activity and transcription level than $36\alpha_2$ strains (Tables 2 and 3). This further confirmed that the elimination of respiration was favorable for xylose fermentation [12,14].

*S. cerevisiae* strains are regarded as industrial working horses for ethanol production owing to their high ethanol titer and sugar tolerance. Mating is one of the traditional yeast breeding methods to develop improved *S. cerevisiae* strains without genetic modifications [38]. Through strain mating, robust diploid *S. cerevisiae* strains were developed for enhanced xylose-fermentation and inhibitor resistance [23,24]. In the present work, a recombinant diploid *S. cerevisiae* strain STXQ was obtained by mating respiration-dependent strain $36\alpha_2$XpXpUN with the respiration-deficient strain $39a_2$XoNK. Such strain mating enabled strain STXQ to present improved cell growth, xylose utilization and ethanol production (Figure 4, Table 4).

For glucose-xylose co-fermentation by engineered laboratory *S. cerevisiae* strains, higher ethanol concentration of ~60 g $L^{-1}$ was reported by Ho et al. [39] (Table 5). An ethanol titer of 47.5 g $L^{-1}$ was obtained by recombinant *S. cerevisiae* RWB218 expressing *PirXylA* [40]. About 53 g $L^{-1}$ ethanol titer was reported by Diao and his colleagues using the diploid recombinant *S. cerevisiae* strain CIBTS0735 [22]. Demeke et al. obtained an inhibitor-resistant recombinant *S. cerevisiae* through metabolic engineering and adaptive evolution [18]. It utilized glucose and xylose rapidly with ethanol titer up to 46 g $L^{-1}$ and ethanol productivity of 2.58 g $L^{-1}{\cdot}h^{-1}$. This is the highest volumetric ethanol productivity reported to-date in the literature. More recently, about 58 g $L^{-1}$ ethanol titer was obtained by a diploid recombinant yeast strain LF1 developed from a wild-type *S. cerevisiae* strain in YP medium [25]. Strain STXQ yielded 65.8 g $L^{-1}$ ethanol with an ethanol yield of 0.50 g $g^{-1}$ at 56 h in YPX medium containing 132 g $L^{-1}$ xylose (Figure 4A, Table 4). Further glucose-xylose co-fermentation produced 120.6 g $L^{-1}$ ethanol with an ethanol yield of 0.48 g $g^{-1}$ at 23 h in YP medium containing 162 g $L^{-1}$ glucose and 95 g $L^{-1}$ xylose. The corresponding volumetric ethanol productivity reached 5.24 g $L^{-1}{\cdot}h^{-1}$. (Figure 4B, Table 4). Both ethanol titer and volumetric productivity far exceeded the results in previous reports.

**Table 5.** Comparison of fermentation performance of engineered xylose-fermenting *S. cerevisiae*.

| Strain | Description | Inoculum Biomass (g DCW $L^{-1}$) | Initial Glucose (g $L^{-1}$) | Initial Xylose (g $L^{-1}$) | Final Ethanol (g $L^{-1}$) | Ethanol Yield (g $g^{-1}$) | Volumetric Ethanol Productivity (g $L^{-1} \cdot h^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|---|
| CIBTS0735 | *PirXylA*; *XKS1*; PPP; ci*GXF1*; adaptive evolution | Rich medium, initial inoculum size at 0.63 g DCW $L^{-1}$ | | 40 | 17.47 | 0.44 | 1.09 | [18] |
| | | Rich medium, initial inoculum size at 0.63 g DCW $L^{-1}$ | 80 | 40 | 53 | 0.45 | 2.22 | |
| RWB218 | *PirXylA*; *XKS1*; PPP; *gre3*Δ; adaptive evolution) | defined synthetic medium, initial inoculum size at 1.1 g DCW $L^{-1}$ | 100 | 25 | 47.5 | 0.38 | 1.98 | [40] |
| 1400 (pLNH32) | *XR*; *XDH*; *XK*; adaptive evolution | Rich medium; OD 40–45 | 50 | | 24 | 0.48 | 0.52 | [39] |
| | | Rich medium; OD 40–45 | 80 | 40 | 60 | 0.45 | 1.3 | |
| H31-A3-AL$^{CS}$ | *PirXylA*; *XKS1*; PPP; *gre3*Δ; adaptive evolution | Defined medium;initial inoculum size at 0.05 g DCW $L^{-1}$ | | 40 | 16.4 | 0.41 | 0.55 | [28] |
| GS1.11-26 | *cpXylA*; *XKS1*; PPP; *HXT7*; *AraT*; *AraA*; *AraB*; *AraD*; *TAL2*; *TKL2* mutagenesis; genome shuffling; adaptive evolution | Rich medium; initial inoculum size at 1.3 g DCW $L^{-1}$ | 36 | 37 | 33.6 | 0.46 | 2.58 | [18] |
| LF1 | *Ru-XylA*, *XKS1*, PPP, *gre3::MGT05196*$^{N360F}$, adaptive evolution | Rich medium, initial inoculum size at 1.00 g DCW $L^{-1}$ | 80 | 42 | 58.0 | 0.47 | 3.60 | [25] |
| STXQ | *OrpXylA*, *PirXylA*, *XKS1*, PPP, *gre3*Δ; adaptive evolution | Rich medium, initial inoculum size at 3.13 g DCW $L^{-1}$ | | 132 | 65.8 | 0.50 | 1.13 | This study |
| | | Rich medium, initial inoculum size at 3.43 g DCW $L^{-1}$ | 162 | 95 | 120.6 | 0.48 | 5.24 | This study |

The OPEFB hydrolysate fermentation result demonstrated that strain STXQ could co-ferment glucose and xylose without detoxification, with 95.3% glucose conversion and 88.9% xylose conversion at 72 h (Figure 5). Ethanol titer reached 28.4 g $L^{-1}$ at 72 h with an ethanol yield of 0.420 g $g^{-1}$. Strain STXQ has potential in the conversion of lignocellulosic biomass hydrolysate to ethanol. These results suggest that the strain development protocols outlined in this study are effective in obtaining robust xylose-fermenting yeast strains for industrial applications.

## 5. Conclusions

A recombinant haploid strain containing plasmids harboring two-copy *PirXylA* presented the best xylose utilization among the engineered yeast strains harboring episomal XI genes when xylose was used as the sole carbon source. On the other hand, the strain containing plasmids with two-copy *OrpXylA* exhibited the best glucose and xylose co-fermentation. Respiration-deficient $39a_2$ strains harboring the *OrpXylA* gene were favorable for xylose fermentation and glucose-xylose co-fermentation in the engineered yeast strains. Chromosome integration of XI genes in *S. cerevisiae* resulted in high XI activity, high XI transcription levels, and improved xylose fermentation. Mating the respiration-efficient strain $36\alpha_2$XpXpUN with the respiration-deficient strain $39a_2$XoNK resulted in a diploid recombinant *S. cerevisiae* strain STXQ with enhanced cell growth and xylose fermentation. Strain STXQ demonstrated superior glucose and xylose co-fermentation performance. It produced 120.6 g $L^{-1}$ ethanol with a volumetric productivity of 5.24 g $L^{-1}$ $h^{-1}$, the highest among those reported to-date. Such superior performance by strain STXQ is largely associated with its development process, in particular with strain adaptive evolution, XI gene chromosome integration and strain mating.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2311-5637/4/3/59/s1.

**Author Contributions:** Conceptualization, A.G. and T.L; Methodology, T.L.; Investigation, T.L.; Data Curation, T.L. and S.H.; Writing—Original Draft Preparation, T.L.; Writing—Review & Editing, A.G.; Supervision, A.G.; Project Administration, A.G.; Funding Acquisition, A.G.

**Funding:** This research was funded by the Science and Engineering Research Council of the Agency for Science Technology and Research (A*STAR) Singapore, grant number 092-139-0035.

**Acknowledgments:** The authors would like to thank Ngee Ann Polytechnic for providing the internship opportunities to Tingting Liu and Shuangcheng Huang during the period of this project.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Somerville, C.; Youngs, H.; Taylor, C.; Davis, S.C.; Long, S.P. Feedstocks for lignocellulosic biofuels. *Science* **2010**, *329*, 790–792. [CrossRef] [PubMed]
2. Miao, Z.; Grift, T.E.; Hansen, A.C.; Ting, K.C. An overview of lignocellulosic biomass feedstock harvest, processing and supply for biofuel production. *Biofuels* **2013**, *4*, 5–8. [CrossRef]
3. Fatma, S.; Hameed, A.; Noman, M.; Ahmed, T.; Shahid, M.; Tariq, M.; Sohail, I.; Tabassum, R. Lignocellulosic biomass: A sustainable bioenergy source for the future. *Protein Pept. Lett.* **2018**, *25*, 148–163. [CrossRef] [PubMed]
4. Moyses, D.N.; Reis, V.C.; de Almeida, J.R.; de Moraes, L.M.; Torres, F.A. Xylose fermentation by *Saccharomyces cerevisiae*: Challenges and prospects. *Int. J. Mol. Sci.* **2016**, *17*, 207. [CrossRef] [PubMed]
5. Li, Y.C.; Gou, Z.X.; Zhang, Y.; Xia, Z.Y.; Tang, Y.Q.; Kida, K. Inhibitor tolerance of a recombinant flocculating industrial *Saccharomyces cerevisiae* strain during glucose and xylose co-fermentation. *Braz. J. Microbiol.* **2017**, *48*, 798–800. [CrossRef] [PubMed]
6. Hahn-Hägerdal, B.; Karhumaa, K.; Jeppsson, M.; Gorwa-Grauslund, M.F. Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. *Adv. Biochem. Eng. Biotechnol.* **2007**, *108*, 147–177. [CrossRef] [PubMed]
7. Madhavan, A.; Srivastava, A.; Kondo, A.; Bisaria, V.S. Bioconversion of lignocellulose-derived sugars to ethanol by engineered *Saccharomyces cerevisiae*. *Crit. Rev. Biotechnol.* **2012**, *32*, 22–48. [CrossRef] [PubMed]
8. Zhang, G.C.; Liu, J.J.; Kong, I.I.; Kwak, S.; Jin, Y.S. Combining C6 and C5 sugar metabolism for enhancing microbial bioconversion. *Curr. Opin. Chem. Biol.* **2015**, *29*, 49–57. [CrossRef] [PubMed]

9. Kwak, S.; Jin, Y.S. Production of fuels and chemicals from xylose by engineered *Saccharomyces cerevisiae*: A review and perspective. *Microb. Cell Factories* **2017**, *16*, 82. [CrossRef] [PubMed]
10. Hou, J.; Qiu, C.; Shen, Y.; Li, H.; Bao, X. Engineering of *Saccharomyces cerevisiae* for the efficient co-utilization of glucose and xylose. *FEMS Yeast Res.* **2017**, *17*. [CrossRef] [PubMed]
11. Kuyper, M.; Hartog, M.M.; Toirkens, M.J.; Almering, M.J.; Winkler, A.A.; van Dijken, J.P.; Pronk, J.T. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res.* **2005**, *5*, 399–409. [CrossRef] [PubMed]
12. Peng, B.; Huang, S.; Liu, T.; Geng, A. Bacterial xylose isomerases from the mammal gut Bacteroidetes cluster function in *Saccharomyces cerevisiae* for effective xylose fermentation. *Microb. Cell Factories* **2015**, *14*, 70. [CrossRef] [PubMed]
13. Johansson, B.; Hahn-Hägerdal, B. The non-oxidative pentose phosphate pathway controls the fermentation rate of xylulose but not of xylose in *Saccharomyces cerevisiae* TMB3001. *FEMS Yeast Res.* **2002**, *2*, 277–282. [PubMed]
14. Peng, B.; Shen, Y.; Li, X.; Chen, X.; Hou, J.; Bao, X. Improvement of xylose fermentation in respiratory-deficient xylose-fermenting *Saccharomyces cerevisiae*. *Metab. Eng.* **2012**, *14*, 9–18. [CrossRef] [PubMed]
15. Lee, S.H.; Kodaki, T.; Park, Y.C.; Seo, J.H. Effects of NADH-preferring xylose reductase expression on ethanol production from xylose in xylose-metabolizing recombinant *Saccharomyces cerevisiae*. *J. Biotechnol.* **2012**, *158*, 184–191. [CrossRef] [PubMed]
16. Sonderegger, M.; Sauer, U. Evolutionary engineering of Saccharomyces cerevisiae for anaerobic growth on xylose. *Appl. Environ. Microbiol.* **2003**, *69*, 1990–1998. [CrossRef] [PubMed]
17. Scalcinati, G.; Otero, J.M.; Vleet, J.R.; Jeffries, T.W.; Olsson, L.; Nielsen, J. Evolutionary engineering of *Saccharomyces cerevisiae* for efficient aerobic xylose consumption. *FEMS Yeast Res.* **2012**, *12*, 582–597. [CrossRef] [PubMed]
18. Demeke, M.M.; Dietz, H.; Li, Y.; Foulquie-Moreno, M.R.; Mutturi, S.; Deprez, S.; Den Abt, T.; Bonini, B.M.; Liden, G.; Dumortier, F.; et al. Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. *Biotechnol. Biofuels* **2013**, *6*, 89. [CrossRef] [PubMed]
19. Sedlak, M.; Ho, N.W. Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant *Saccharomyces* yeast. *Yeast* **2004**, *21*, 671–684. [CrossRef] [PubMed]
20. Runquist, D.; Hahn-Hagerdal, B.; Radstrom, P. Comparison of heterologous xylose transporters in recombinant *Saccharomyces cerevisiae*. *Biotechnol. Biofuels* **2010**, *17*, 3–5. [CrossRef] [PubMed]
21. Goncalves, D.L.; Matsushika, A.; de Sales, B.B.; Goshima, T.; Bon, E.P.; Stambuk, B.U. Xylose and xylose/glucose co-fermentation by recombinant *Saccharomyces cerevisiae* strains expressing individual hexose transporters. *Enzyme Microb. Technol.* **2014**, *63*, 13–20. [CrossRef] [PubMed]
22. Diao, L.; Liu, Y.; Qian, F.; Yang, J.; Jiang, Y.; Yang, S. Construction of fast xylose-fermenting yeast based on industrial ethanol-producing diploid *Saccharomyces cerevisiae* by rational design and adaptive evolution. *BMC Biotechnol.* **2013**, *13*, 110. [CrossRef] [PubMed]
23. Demeke, M.M.; Dumortier, F.; Li, Y.; Broeckx, T.; Foulquié-Moreno, M.R.; Thevelein, J.M. Combining inhibitor tolerance and D-xylose fermentation in industrial *Saccharomyces cerevisiae* for efficient lignocellulose-based bioethanol production. *Biotechnol. Biofuels* **2013**, *6*, 120. [CrossRef] [PubMed]
24. Kim, S.R.; Lee, K.S.; Kong, I.I.; Lesmana, A.; Lee, W.H.; Seo, J.H.; Kweon, D.H.; Jin, Y.S. Construction of an efficient xylose-fermenting diploid *Saccharomyces cerevisiae* strain through mating of two engineered haploid strains capable of xylose assimilation. *J. Biotechnol.* **2013**, *164*, 105–111. [CrossRef] [PubMed]
25. Li, H.; Shen, Y.; Wu, M.; Hou, J.; Jiao, C.; Li, Z.; Liu, X.; Bao, X. Engineering a wild-type diploid *Saccharomyces cerevisiae* strain for second-generation bioethanol production. *Bioresour. Bioprocess.* **2016**, *3*, 51. [CrossRef] [PubMed]
26. Tanino, T.; Hotta, A.; Ito, T.; Ishii, J.; Yamada, R.; Hasunuma, T.; Ogino, C.; Ohmura, N.; Ohshima, T.; Kondo, A. Construction of a xylose-metabolizing yeast by genome integration of xylose isomerase gene and investigation of the effect of xylitol on fermentation. *Appl. Microbiol. Biotechnol.* **2010**, *88*, 1215–1221. [CrossRef] [PubMed]
27. Bamba, T.; Hasunuma, T.; Kondo, A. Disruption of PHO13 improves ethanol production via the xylose isomerase pathway. *AMB Express* **2016**, *6*, 4. [CrossRef] [PubMed]

28. Zhou, H.; Cheng, J.S.; Wang, B.L.; Fink, G.R.; Stephanopoulos, G. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. *Metab. Eng.* **2012**, *14*, 611–622. [CrossRef] [PubMed]
29. Ko, J.K.; Um, Y.; Woo, H.M.; Kim, K.H.; Lee, S.M. Ethanol production from lignocellulosic hydrolysates using engineered *Saccharomyces cerevisiae* harboring xylose isomerase-based pathway. *Bioresour. Technol.* **2016**, *209*, 290–296. [CrossRef] [PubMed]
30. Güldener, U.; Heck, S.; Fiedler, T.; Beinhauer, J.; Hegemann, J.H. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* **1996**, *24*, 2519–2524. [CrossRef] [PubMed]
31. Gietz, R.D.; Schiestl, R.H. Large-scale high-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* **2007**, *2*, 38–41. [CrossRef] [PubMed]
32. Kersters-Hilderson, H.; Callens, M.; Opstal, O.V.; Vangrysperre, W.; Bruyne, C.K.D. Kinetic characterization of d-xylose isomerases by enzymatic assays using d-sorbitol dehydrogenase. *Enzyme Microb. Technol.* **1987**, *9*, 145–148. [CrossRef]
33. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2–ΔΔCT method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
34. Wang, Z.; Ong, H.X.; Geng, A. Cellulase production and oil palm empty fruit bunch saccharification by a new isolate of *Trichoderma koningii* D-64. *Proc. Biochem.* **2012**, *47*, 1564–1571. [CrossRef]
35. Qi, X.; Zha, J.; Liu, G.G.; Zhang, W.; Li, B.Z.; Yuan, Y.J. Heterologous xylose isomerase pathway and evolutionary engineering improve xylose utilization in *Saccharomyces cerevisiae*. *Front. Microbiol.* **2015**, *6*, 1165. [CrossRef] [PubMed]
36. Vilela Lde, F.; de Araujo, V.P.; Paredes Rde, S.; Bon, E.P.; Torres, F.A.; Neves, B.C.; Eleutherio, E.C. Enhanced xylose fermentation and ethanol production by engineered *Saccharomyces cerevisiae* strain. *AMB Express* **2015**, *5*, 16. [CrossRef] [PubMed]
37. Madhavan, A.; Tamalampudi, S.; Srivastava, A.; Fukuda, H.; Bisaria, V.S.; Kondo, A. Alcoholic fermentation of xylose and mixed sugars using recombinant *Saccharomyces cerevisiae* engineered for xylose utilization. *Appl. Microbiol. Biotechnol.* **2009**, *82*, 1037–1047. [CrossRef] [PubMed]
38. Perez-Traves, L.; Lopes, C.A.; Barrio, E.; Querol, A. Evaluation of different genetic procedures for the generation of artificial hybrids in *Saccharomyces* genus for winemaking. *Int. J. Food Microbiol.* **2012**, *156*, 102–111. [CrossRef] [PubMed]
39. Ho, N.W.; Chen, Z.; Brainard, A.P. Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. *Appl. Environ. Microbiol.* **1998**, *64*, 1852–1859. [PubMed]
40. Kuyper, M.; Harhangi, H.R.; Stave, A.K.; Winkler, A.A.; Jetten, M.S.; de Laat, W.T.; den Ridder, J.J.; Op den Camp, H.J.; van Dijken, J.P.; et al. High-level functional expression of a fungal xylose isomerase: The key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Res.* **2003**, *4*, 69–78. [CrossRef]