



Enhanced ethanol production from industrial lignocellulose hydrolysates by a hydrolysate-cofermenting *Saccharomyces cerevisiae* strain

Shuangcheng Huang¹ · Tingting Liu¹ · Bingyin Peng¹ · Anli Geng¹

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Abstract

Industrial production of lignocellulosic ethanol requires a microorganism utilizing both hexose and pentose, and tolerating inhibitors. In this study, a hydrolysate-cofermenting *Saccharomyces cerevisiae* strain was obtained through one step in vivo DNA assembly of pentose-metabolizing pathway genes, followed by consecutive adaptive evolution in pentose media containing acetic acid, and direct screening in biomass hydrolysate media. The strain was able to coferment glucose and xylose in synthetic media with the respective maximal specific rates of glucose and xylose consumption, and ethanol production of 3.47, 0.38 and 1.62 g/g DW/h, with an ethanol titre of 41.07 g/L and yield of 0.42 g/g. Industrial wheat straw hydrolysate fermentation resulted in maximal specific rates of glucose and xylose consumption, and ethanol production of 2.61, 0.54 and 1.38 g/g DW/h, respectively, with an ethanol titre of 54.11 g/L and yield of 0.44 g/g. These are among the best for wheat straw hydrolysate fermentation through separate hydrolysis and cofermentation.

Keywords *Saccharomyces cerevisiae* · Lignocellulose hydrolysate · Cellulosic ethanol · In vivo DNA assembly · Hexose and pentose co-fermentation · Inhibitory chemicals

Introduction

Lignocellulosic biomass, such as oil palm empty fruit bunch (OPEFB) and wheat straw (WS), does not compete with humans for food and is a sustainable source of sugars for expanding biofuel and chemical production [1, 2]. Lignocellulosic biomass hydrolysates, especially those produced

from agricultural wastes, herbaceous and hardwood biomass, are rich in pentoses, such as D-xylose and L-arabinose [2, 3]. To maximize the yield of lignocellulosic ethanol, all carbohydrate fractions including hexoses (i.e. glucose and galactose, etc.) and pentoses (D-xylose and L-arabinose) should be fully utilized by the microorganisms [3, 4]. The budding yeast *Saccharomyces cerevisiae* commonly used for industrial ethanol production cannot metabolize xylose and arabinose naturally. Pentose-fermenting yeasts have been developed by intensive genetic engineering and prolonged evolutionary engineering [4, 5]. However, for industrial application in lignocellulosic hydrolysate fermentation, tolerance to inhibitory chemicals in the hydrolysates is important [6].

For xylose utilization, either xylose isomerase (XI) pathway or xylose reductase/xylitol dehydrogenase (XR/XDH) pathway was introduced into yeast strains [7, 8]. Through the XI pathway, xylose is directly converted to xylulose and it does not require any cofactor and this leads to high theoretical ethanol yield [9, 10]. On the other hand, the XR/XDH pathway offers higher metabolic fluxes than the XI pathway; however, it accumulates xylitol due to the uncoupled cofactor consumption of NADPH-preferred XR and

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✉ Anli Geng
gan2@np.edu.sg

Shuangcheng Huang
higle945@yahoo.com

Tingting Liu
litt7@sina.com

Bingyin Peng
bingyin.peng@yahoo.com

¹ School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, 535 Clementi Road, Singapore 599489, Singapore

NAD⁺-preferred XDH [11]. Numerous genetic manipulations have been applied to *S. cerevisiae* expressing XR and XDH to solve the co-factor imbalance problems and improve ethanol yield [10]. For L-arabinose utilization, three enzymes from bacterial arabinose operon (*araBAD*) were overexpressed, which include genes encoding L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), and L-ribulose-5-phosphate 4-epimerase (*araD*) [12, 13]. Efficient alcoholic fermentation of these two sugars requires combinatorial engineering of yeast strains to enhance the non-oxidative pentose phosphate pathway and prolonged evolutionary cultivation to accumulate spontaneous beneficial mutation [4, 5].

Commercial production of lignocellulosic ethanol is still challenging due to the inhibitory compounds in lignocellulose hydrolysates. Acetic acid is a primary inhibitor in the hydrolysates of various lignocellulosic materials [14–16], which negatively influences cell growth and sugar fermentation, whereas 5-hydroxymethyl-2-furaldehyde and furfural are below the inhibitory concentration [17]. It was shown that ~7 g/L acetic acid delayed xylose utilization in the evolved xylose-fermenting yeast strains [17, 18]. Although introducing heterologous acetaldehyde dehydrogenase can generate a yeast strain that converts acetate to ethanol, it is still necessary to make the yeast strain tolerant to high concentration of acetic acid for initial cell propagation [19].

Traditional method in pentose-fermenting yeast construction involves multiple steps of pentose-metabolizing pathway genetic engineering in the host strain followed by prolonged evolutionary engineering [4, 5, 17, 20]. It takes years to develop a successful pentose-fermenting yeast strain for industrial lignocellulose hydrolysate fermentation. Recently, xylose-metabolizing pathway genes were refactored in *S. cerevisiae* through combinatorial CRISPR-Cas9-mediated rational metabolic engineering and evolutionary engineering [3]. This allows rapid xylose-fermenting yeast construction with glucose and xylose cofermentation capabilities. Besides CRISPR-Cas9, DNA assembler is an effective method to assemble an entire biochemical pathway in a single step in *S. cerevisiae* via in vivo homologous recombination [21].

In this study, a hydrolysate-cofermenting yeast strain was rapidly constructed through one-step in vivo DNA assembly, followed by consecutive evolutionary engineering in pentose media containing acetic acid, and lignocellulose hydrolysate medium screening. A series of gene expression cassettes containing varied xylose isomerase (XI) genes and arabinose-metabolizing pathway genes were in vivo assembled and integrated into the genome of a uracil auxotrophic *S. cerevisiae* strain JUK36 α [8] using an integrative and replicative plasmid [22]. This allowed the rapid recombination of xylose- and arabinose-metabolizing pathway genes in the host strain with optimal pentose-metabolizing pathway combination. The obtained engineered yeast strain, 36aS1, was

subsequently enhanced for pentose utilization and hydrolysate-cofermentation through consecutive adaptive cultivation alternatively in complex media containing xylose and arabinose in the presence of 6 g/L acetic acid until no further improvement in cell growth was observed. This endowed the strain library, 36aS1.10, with optimal and stable genome integration of pentose-metabolizing pathway genes.

Two lignocellulose hydrolysate samples were collected from industries. A potential hydrolysate-cofermenting yeast strain was isolated on lignocellulose hydrolysate agar plates and liquid media. This generated an engineered yeast strain, 36aS1.10.4, which displayed superior glucose and xylose cofermentation capabilities in both synthetic and industrial lignocellulose hydrolysate media. High ethanol titre, productivity and yield were obtained from both lignocellulose hydrolysate samples. This strain is promising in efficient fermentation of industrial lignocellulose hydrolysates and can be applied as a workhorse in lignocellulosic ethanol industries.

Materials and methods

Materials

The *S. cerevisiae* strains used in this work are listed in Table 1. Diploid yeast strain *S. cerevisiae* ATCC 24860 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). *S. cerevisiae* haploid strain JUK36 α was isolated from strain *S. cerevisiae* ATCC 24860 [8]. It was overexpressed with the non-oxidative pentose phosphate pathway (PPP) genes and xylulokinase gene, *XKS1*. The auxotrophic marker gene *ura3* and the aldose reductase gene *gre3* were disrupted in JUK36 α . Strain *Escherichia coli* DH5 α was obtained from Life Technologies (Rockville, MD, USA) and grown in Luria Bertani (LB) broth containing ampicillin (50 mg/L) at 37 °C for plasmid construction and maintenance.

All chemicals were of analytical grade and were obtained from Sigma-Aldrich (Singapore) unless otherwise stated. The restriction enzymes and Phusion DNA polymerase were purchased from the New England Biolabs (Ipswich, MA, USA).

Oil palm empty fruit bunch (OPEFB) hydrolysate (hydrolysate I) was prepared by Teck Guan Holdings Sdn Bhd (Tawau, Malaysia) through hydrothermal pretreatment followed by enzymatic hydrolysis using the enzyme mixture of cellulast 1.5 L and Novozym 188 (Novozymes A/S, Bagsværd, Denmark) according to the protocols described in earlier reports [23, 24]. Wheat straw hydrolysate (hydrolysate II) was prepared by Inbicon A/S (Fredericia, Denmark) in a similar manner also according to the previous report [23].

Table 1 Strains and plasmids

Plasmids/strains	Description	Sources
<i>S. cerevisiae</i> strains		
ATCC 24860	MAT α /MAT α	ATCC
JUK36 α	<i>S. cerevisiae</i> ATCC 24860 segregant; MAT α ; <i>ura3</i> (-203,+882):: <i>loxP</i> ; <i>TKL1</i> (-286,-1):: <i>RKI1-RKI1-ADH1p-RPE1t-RPE1-TPI1p-loxP-XKS1t-XKS1-PGK1p-PDC1p-TAL1-TAL1t-FBA1p</i> ; <i>gre3</i> (+127,+712):: <i>loxP</i>	[8]
36aS1	JUK36 α derivative; pNTS2AK:: <i>PGK1p-BvuXylA-CYC1t-TEF1p-araB-araD-araA-CYC1t-TEF1p-KanMX-CYC1t</i>	This study
36aS1.10	36aS1 derivative; the 10th generation strain library derived from 36aS1	This study
36aS1.10.4	36aS1.10 derivative; the best biomass-hydrolysate-fermenting strain isolated from strain library 36aS1.10	This study
Plasmids		
pJFE11	pYES2; <i>TEF1p/CYC1t</i> , <i>ura3</i>	[8]
pJFE11-ABD	pJFE11; <i>TEF1p-araD-PGK1t-PGK1p-araB-ADH1t-TDH3p-araA-CYC1t</i> , <i>ura3</i>	This study
pJFE11-BDA	pJFE11; <i>TEF1p-araB-araD-araA-CYC1t</i> , <i>ura3</i>	This study
pPY1	pYES2, <i>PGK1p/CYC1t</i> , <i>ura3</i>	[8]
pPY1-Bvu	pPY1; codon-optimized <i>Bacteroides vulgatus xylA</i> , <i>ura3</i>	[8]
pPY1-XIq	pPY1; codon-optimized <i>Orpinomyces</i> sp. ukk1 <i>xylA</i> , <i>ura3</i>	[8]
pPY1-TAA	pPY1; codon-optimized <i>Tannerella</i> sp. 6_1_58FAA_CT1 <i>xylA</i> , <i>ura3</i>	[8]
pUG6	<i>loxP-KanMX-loxP</i>	[25]
pUC19	Ori, Amp	[26]
pNTS2AK	pUC19: ARS315, rDNA1/rDNA2, <i>TEF1p-KanMX-CYC1t</i>	This study

Table 2 Composition of lignocellulose hydrolysate fermentation media

Components	Hydrolysates fermentation media (g/L)		
	YPH1	YPH1.1	YPH2
Glucose	26.92	83.17	82.66
Xylose	5.1	43.56	43.96
Arabinose	ND	ND	ND
Acetate	6.07	6.07	7.42
Furfural	0.131	0.131	0.088
HMF	0.414	0.414	ND
Yeast extract	10	10	10
Peptone	20	20	20

YPH1 and YPH2, respectively, contained hydrolysate I (OPEFB hydrolysate) and hydrolysate II (wheat straw hydrolysate); YPH1.1 was the sugar-enriched YPH1 with initial sugar concentration equivalent to that in YPH2

ND not detected

The composition of lignocellulose hydrolysate samples and their fermentation media is listed in Table 2.

Microorganism cultivation media and lignocellulose hydrolysate fermentation media

Synthetic medium contained 1.7 g/L yeast nitrogen base w/o amino acids (YNB), 5 g/L ammonium sulphate, and specified concentration of glucose (YNBG), xylose (YNBX) or

arabinose (YNBA), or sugar mixture. YP medium contained 10 g/L yeast extract, 20 g/L peptone, supplemented with glucose (YPD), xylose (YPX) or arabinose (YPA) at specified concentration with an initial pH 5.5. All engineered yeast strains were stored at -80°C in stock medium (YPX or YPD) containing 0.9% sodium chloride and 20% (w/v) glycerol.

Lignocellulose hydrolysate samples were supplemented with 10 g/L yeast extract, 20 g/L peptone, and sugar if necessary to generate hydrolysate fermentation media with an initial pH of 5.5. The composition of lignocellulose hydrolysate fermentation media is listed in Table 2.

Twenty grams per liter Bacto agar was added to each medium and was autoclaved at 121°C for 15 min. Subsequently, the autoclaved mixture was spread into petri dishes to generate its corresponding agar plates.

In vivo DNA assembly of pentose-metabolizing pathway genes

The plasmids used in this work are listed in Table 1. The primers used in this study are listed in Table S1. The codon-optimized XI genes, *BvuXylA* (*Bacteroides vulgatus* *XylA*, GenBank accession number KY362292), *XIqXylA* (*Orpinomyces* sp. ukk1 *XylA*, GenBank accession number KY348519), *TAAxylA* (*Tannerella* sp. 6_1_58FAA_CT1 *XylA*, GenBank accession number KY362293) were synthesized by Bio Basic Asia Pacific Pte Ltd (Singapore) [8]. The arabinose pathway genes (*araA*, *araB* and *araD*) were

amplified from the genome of *Lactobacillus plantarum* through polymerase chain reaction (PCR) using Phusion DNA polymerase (New England Biolabs, Ipswich, MA, USA). The yeast expression plasmids pJFE11 and pPY1 were constructed from pYES2 previously in our laboratory [8]. All the XI genes were individually cloned into the *XhoI/XbaI* sites of plasmid pPY1 between *PGK1* promoter and *CYC1* terminator to generate the XI gene expression cassettes (Fig. 1b; Table 1). Promoters and terminators were PCR amplified from the genomic DNA of *S. cerevisiae* ATCC 24,860. The *araB-araD-araA* fragment was amplified from *L. plantarum* genome directly and was cloned into the *BamHI/XhoI* sites of plasmid pJFE11 between *TEF1* promoter and *CYC1* terminator to generate plasmid pJFE11-BDA. The arabinose-metabolizing gene cassettes *araD-PGK1t-PGK1p-araB-ADH1t-TPH3p-araA* were constructed via overlap extension PCR (OE-PCR) with *BamHI/XhoI* linearized plasmid pJFE11 to generate plasmid pJFE11-ABD (Table 1). A high effective autonomous replicative sequence in *S. cerevisiae* genome, ARS315 [22], and the homologous arms of rDNA gene in *S. cerevisiae*, *rDNA1* and

rDNA2, were amplified from the genomic DNA of *S. cerevisiae* ATCC 24,860. The yeast selection marker *KanMX* was amplified from pUG6 [25]. ARS315, *rDNA1*, *rDNA2* and *KanMX* were cloned into the plasmid pUC19 [26] to generate the integrative and replicative plasmid pNTS2AK (Fig. 1a; Table 1). Pentose-metabolizing gene cassettes with their respective flanked arms were individually amplified from their respective plasmids. Each xylose isomerase gene expression cassette was mixed with the *BamHI/XbaI* linearized plasmid pNTS2AK and was then cotransformed with each arabinose-metabolizing gene cassette into JUK36 α using LiAc/SS carrier DNA/PEG method [27]. This generated six sets of xylose and arabinose metabolic pathway gene combination. The seventh was created by transforming the mixture of all the five pentose-metabolizing pathway gene cassettes in equal amount and then cotransforming them with one part of the linearized plasmid pNTS2AK. The overlapping regions were 45–50 bp between the gene cassettes and the linearized backbone of plasmid pNTS2AK (Table S1) [21].

Strain screening for optimal cell growth in pentose media

After gene cassette transformation, recombinant yeast strain libraries with six possible combinations were generated (Fig. 1b). The transformed yeast cells were plated on the YPD agar plates containing 300 μ g/L G418. After 3 days, seven colonies from each set of transformants with the largest colony size were selected and transferred to YP agar plates containing xylose (YPX) or arabinose (YPA). Afterwards, functional strains were screened on synthetic medium agar plates supplemented with 67 mg/L uracil and 20 g/L xylose (YNBX) or 20 g/L arabinose (YNBA). They were further evaluated in 12-mL tubes containing 5 mL synthetic medium supplemented with 50 g/L xylose (YNBX). Cell growth was measured at 72 h and the strain with the highest cell density at 600 nm (OD_{600}) was selected for further strain adaptive evolution (Fig. 2).

Adaptive evolution and screening of hydrolysate-cofermenting yeast strain

Strain 36aS1 was selected due to its best growth in xylose media (Figs. 1c, 2). Series transfer with 48 h-intervals was performed alternatively in YPX (50 g/L xylose) and YPA (20 g/L arabinose) media containing 6 g/L acetic acid with an initial OD_{600} of 0.2 for each transfer. Every transferred culture was considered as one generation and all generation strains were harvested at 48 h and then individually stored at $-80\text{ }^{\circ}\text{C}$ until use. They were later on grown in 24-well plate containing YNBX (50 g/L xylose) and YNBA (20 g/L arabinose) media with an initial OD_{600} of 0.2. Cell

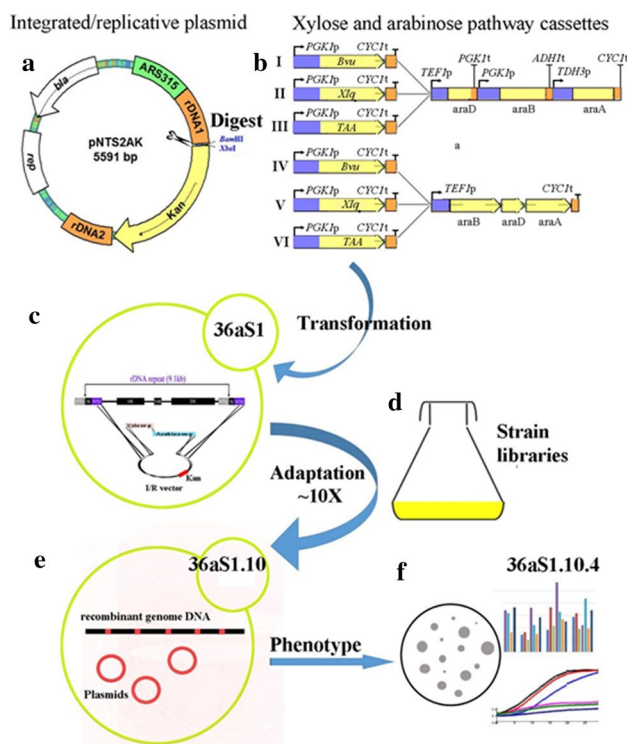


Fig. 1 Flowchart for rapid yeast strain development. **a** Linearized integrative and replicative plasmid pNTS2AK; **b** xylose and arabinose pathway gene cassettes; **c** best pentose-utilizing strain 36aS1; **d** adaptive evolution of strain 36aS1 alternatively in xylose and arabinose liquid media containing acetic acid; **e** adapted strain library 36aS1.10 with optimal pentose-metabolizing gene integration; **f** strain 36aS1.10.4 screening on OPEFB hydrolysates agar plates and in OPEFB hydrolysate liquid media

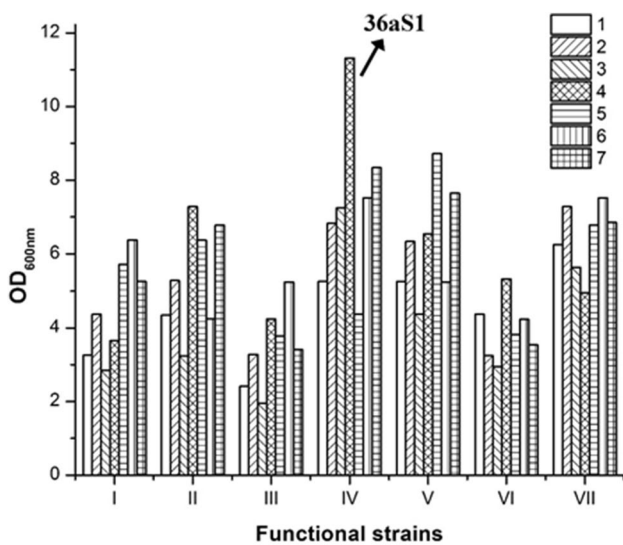


Fig. 2 Final OD₆₀₀ of pentose-utilizing strains in YNB media containing 50 g/L xylose (YNBX) at 72 h. I–VII, transformants set number; 1–7, colony number from each set of transformants

growth, OD₆₀₀, was continuously monitored in a microplate reader (Infinite 200 PRO, Tecan Group, AG, Männedorf, Switzerland) and the specific cell growth rate at 24 h was calculated (Fig. 3). Strain library obtained after 10 rounds of transfer, 36aS1.10, was further transferred in YPD medium (20 g/L dextrose) to test its genetic stability (Figs. 1d, e, 3). In the meantime, it was spread on YP agar plates containing industrial hydrolysate I (YPH1, Table 2). Fermentation performance of colonies with larger size was evaluated in YP

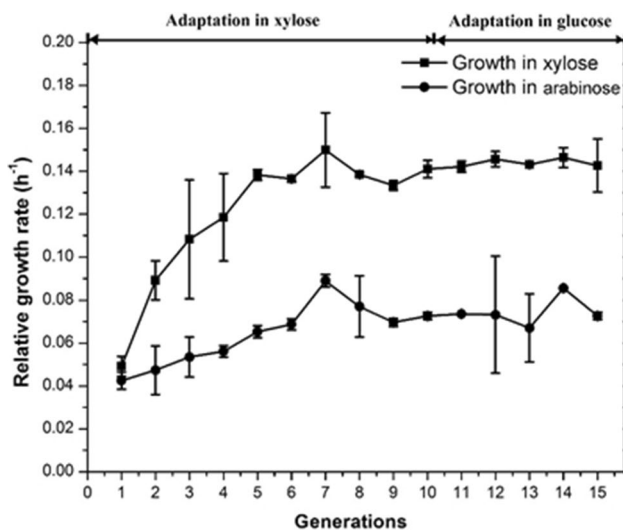


Fig. 3 Cell-specific growth rate in YNB media containing 50 g/L xylose (YNBX) or 20 g/L arabinose (YNBA) at 24 h by all generation strains. The results represent the mean ± standard deviation of duplicate independent experiments

liquid medium containing hydrolysate I (YPH1) (Fig. 1f). The best performing strain, 36aS1.10.4, was isolated according to its final OD₆₀₀ value and ethanol titre in YPH1 (Fig. 4). The assembled pentose-metabolizing pathways in strain 36aS1.10.4 were confirmed by PCR analysis.

Fermentation in both synthetic and lignocellulose hydrolysate fermentation media

Seed culture of 36aS1.10.4 was prepared by reviving the stock culture from –80 °C in 12 mL tube containing 3 mL YPX (50 g/L xylose) at 30 °C and 200 rpm for 24 h. About 10 mL of the revived culture was transferred into 500 mL flasks containing 100 mL YPX medium (50 g/L xylose) and then incubated at 30 °C and 200 rpm for 24 h. This seed culture was subsequently harvested, washed with sterile double distilled water, and later on used to inoculate fermentation

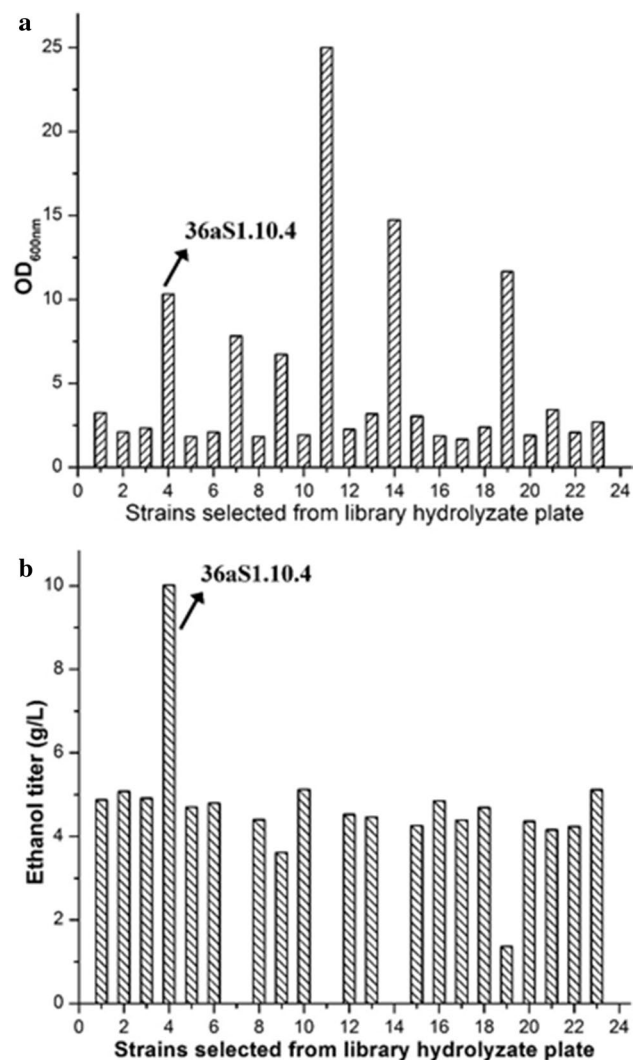


Fig. 4 Strain 36aS1.10.4 screening in an OPEFB hydrolysate liquid medium. **a** Final OD₆₀₀ at 72 h; **b** final ethanol titre at 72 h

medium at an initial OD₆₀₀ of 4.0 (approximately 1 g DW/L of yeast cells) unless otherwise stated. Fermentation was performed in 125 mL flasks containing 25 mL synthetic medium (YNB media) and hydrolysate fermentation media (Table 2) at 30 °C and 100 rpm under oxygen-limited conditions with the initial pH at 5.5. The oxygen-limited conditions in the flasks were maintained by capping the flasks with rubber stoppers pierced with a needle to allow the release of CO₂. All fermentation experiments were conducted in duplicate and average results were reported.

Cell dry weight measurement and metabolite analysis

OD₆₀₀ was monitored using a UV–visible spectrophotometer (Shimadzu, Tokyo, Japan). For determination of cell dry weight, cell cultures (50 mL) with varied OD₆₀₀ (1–5) were filtered through 0.22 µm filter paper 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 weighed. One OD₆₀₀ unit corresponded to 0.241 g/L cell dry weight. Concentrations of glucose, xylose, xylitol, acetate, glycerol, ethanol, furfural, and hydroxymethylfurfural (HMF) in lignocellulose hydrolysates or produced during 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 with 5 mM of sulphuric acid at a flow rate of 0.6 mL/min and 60 °C.

Results and discussion

Pentose-utilizing yeast strain construction

In our previous study, a background strain JUK36α with enhanced xylulokinase and non-oxidative pentose phosphate pathway was constructed by overexpressing xylulokinase (*XKS1*), and the nonoxidative pentose phosphate pathway genes, transaldolase (*TAL1*), transketolase (*TKL1*), ribose-5-phosphate isomerase (*RKII*), and ribulose 5-phosphate 3-epimerase (*RPE1*), and disrupting the aldose reductase gene *gre3* [8]. In this study, gene cassettes containing codon-optimized XI genes (*BvuXylA*, *XIqXylA* and *TAAXylA*) and L-arabinose-metabolizing pathway genes (*araA*, *araB* and *araD*) were individually or co-transformed into strain JUK36α. This was conducted by in vivo assembly of gene cassettes containing above pentose-metabolizing genes in *S. cerevisiae* with a replicative and integrative plasmid (Fig. 1a) [22]. In the end, fermentation performance of 49 transformants was evaluated in synthetic medium with

50 g/L xylose (YNBX). A colony (36aS1) with superior cell growth was selected (Figs. 1b, c, 2). It harboured *B. vulgatus XylA* (*BvuXylA*) and *TEF1p-araB-araD-araA-CYC1t* operon (Table 1). In addition, strain 36aS1 also exhibited growth on YNBA agar plates with 20 g/L arabinose (data not shown).

Metabolic pathway engineering requires expression of gene cassettes containing all pathway genes with their own promoters and terminators. In vivo DNA assembly is efficient in random assembling multiple genes to generate a targeted metabolic pathway in yeast rapidly [21, 28]. It has an advantage in that the pathway can be directly created in vivo through a single transformation, whereas other methods require multiple steps of plasmid construction and transformation [29, 30]. In this study, a series of xylose isomerase and arabinose-metabolizing gene cassettes were in vivo assembled in *S. cerevisiae* JUK36α and the best functional strain 36aS1 was isolated with optimal combination of xylose and arabinose-metabolizing pathway genes (Fig. 2). It harboured codon-optimized *B. vulgatus XylA* (*BvuXylA*) and *TEF1p-araB-araD-araA-CYC1t* operon (Table 1). This has not been reported previously [4, 12, 13].

Adaptive evolution and screening of hydrolysate-cofermenting yeast strain

To improve pentose utilization and hydrolysate fermentation capability, strain 36aS1 was alternatively cultivated in YPX (50 g/L xylose) and YPA (20 g/L arabinose) media in the presence of 6 g/L acetic acid (Fig. 1d). Cell growth was monitored in both YNBX and YNBA medium after each round of adaptive cultivation. As can be seen, cell growth on xylose was significantly enhanced until seventh round of cultivation (Fig. 3). Although cell growth on arabinose was also improved, it was not as significant as that on xylose. After round seven, no further improvement in cell growth was observed on both sugars, suggesting that the incorporation of the pentose-metabolizing genes in the yeast genome has reached its optimum. The 10th -generation culture showed a maximum specific growth rate of ~0.14 1/h in YNBX medium and ~0.07 1/h in YNBA medium. The stability of the 10th-generation culture was further assessed by consecutive transfer and cultivation in YPD medium (20 g/L glucose). Cell growth ability on xylose and arabinose did not deteriorate (Fig. 3). As a result, a stable pentose-metabolizing yeast strain library 36aS1.10 was obtained. The above strain evolution process was rapid in assembling the pentose-metabolizing-pathway gene cassettes with stable and optimal genome integration.

To isolate strains for efficient lignocellulose hydrolysate fermentation, strain library 36aS1.10 was spread on OPEFB hydrolysate YP agar plates (YPH1, Table 2). In total, 23 clones with larger colony size were selected for cell growth and fermentation performance evaluation in liquid OPEFB

hydrolysate fermentation medium YPH1 (Fig. 4). While majority of these clones displayed weak growth after 3-day cultivation, six clones showed moderately good growth (Fig. 4a). However, only one clone (clone 4) accumulated ethanol in higher titre than the rest, indicating glucose and xylose cofermentation (Fig. 4b). This clone was denoted as 36aSa.10.4 and was selected for further characterization (Fig. 1e, f).

Fermentation performance of strain 36aS1.10.4 in glucose and xylose media

Fermentation performance of strain 36aSa.10.4 was assessed in the YNB media with xylose as the sole carbon source, and with the mixture of glucose and xylose (Fig. 5; Table 3). Although strain 36aS1.10.4 harboured the arabinose-metabolizing pathway genes (Fig. 1; Table 1) and was able to utilize arabinose (Fig. 3), here only glucose and xylose fermentation results were presented as both industrial hydrolysate samples contained no arabinose (Table 2). In xylose medium (Fig. 5a), xylose was rapidly converted to ethanol in about 40 h with an ethanol yield of 0.40 g/g xylose (Table 3). The maximal specific xylose consumption rate and maximal ethanol production rate were 1.47 g/g DW/h and 0.60 g/g DW/h, respectively. In glucose and xylose mixture medium (Fig. 5b), glucose was almost consumed and xylose was slightly utilized at 24 h; a considerable xylose consumption was observed afterwards. Xylose concentration was reduced to 2.25 g/L at 48 h with about 98% total sugar conversion. Under this condition, strain 36aS1.10.4 had the maximal specific rates of glucose and xylose consumption of 3.47 g/g DW/h and 0.38 g/g DW/h, respectively. In this case, the maximal ethanol production rate was 1.62 g/DW/h and the ethanol yield was 0.42 g/g sugars (Table 3).

It can be observed that when xylose was used as the sole carbon source its consumption rate (1.47 g/g DW/h) was higher than majority of the reported results obtained for xylose fermentation (Table 3) except that reported by Zhou and his colleagues [31]. However, in the presence of glucose, xylose consumption rate was greatly reduced to 0.38 g/g DW/h (Table 3). Such result was lower than that reported by Demeke and his colleagues, 1.1 g/g DW/h [17] and that by Liu and her coworkers, 0.53 g/g DW/h [32] for glucose and xylose cofermentation. However, it was higher than or equal to those reported in majority of other research where xylose-specific consumption rates were in the range of 0.05–0.38 g/g DW/h (Table 3). Such difference was largely attributed to the different adaptive evolution procedures and duration for each strain development. In addition, it was also associated with the fermentation media composition. Generally, fermentation conducted in complex media, such as YP media, presented higher xylose consumption rates [17, 32]. It is worth to note that when glucose and xylose mixture

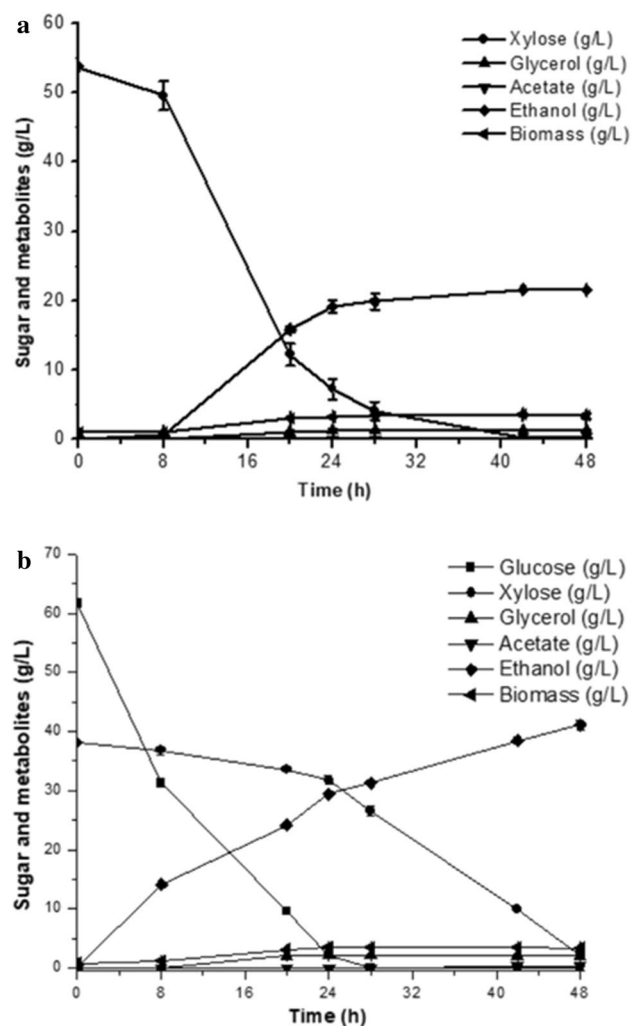


Fig. 5 Fermentation performance of strain 36aS1.10.4 in synthetic medium. **a** 50 g/L D-xylose; **b** 62 g/L D-glucose and 38 g/L D-xylose. The results represent the mean \pm standard deviation of duplicate independent experiments

was used as the carbon source, the maximal ethanol-specific production rate (1.62 g/g DW/h) was much higher than that obtained using xylose as the sole carbon source (0.60 g/g DW/h) (Table 3). While the latter is higher than majority of the reported data for xylose fermentation, the former was the highest among those in most of the earlier reports for glucose and xylose cofermentation even though synthetic media was used (Table 3). Such promising results were obtained with only 18-day strain adaptive evolution, demonstrating the high efficiency of strain development approach adopted in this study.

Furthermore, it is worthwhile to compare the ethanol yield obtained from recombinant yeast strains harbouring XI or XR/XDH pathways. Strain 36aS1.10.4 obtained an ethanol yield of 0.40 g/g for xylose fermentation and 0.42 g/g for glucose and xylose cofermentation (Table 3). Such results

Table 3 Comparison of batch fermentation performance of recombinant xylose-fermenting *S. cerevisiae* strains in synthetic or complex media

Strain name and xylose metabolizing pathway	Fermentation media	Initial sugar concentration (g/L)	Maximal sugar consumption rate (g/g DW/h)		Final ethanol volumetric productivity (g/L/h)	Maximal ethanol specific production rate (g/g DW/h)	Final ethanol titre (g/L)	References
			Glucose	Xylose				
36aS1.10.4, XI	YNB	–	–	1.47 ± 0.02	0.71 ± 0.04	0.60 ± 0.02	21.54 ± 0.08	This study
	YNB	62	3.47 ± 0.04	0.38 ± 0.01	0.86 ± 0.02	1.62 ± 0.01	41.07 ± 0.76	
BSW4XA3, XI	YPM	20	1.89	0.055	NR	NR	11.95	[4]
DXS, XR/XDH	YPM	70	NR	NR	1.85	NR	44.5	[10]
KEI-17, XR/XDH	MM	20	3.35 ± 0.05	0.10 ± 0.00	NR	0.35 ± 0.00	NR	[15]
LF1, XI	YPM	–	–	1.089	NR	0.472	NR	[16]
	YPM	80	–	0.482	NR	0.819	NR	
GSI.11-26, XI	YPM	36	2.71 ± 0.04	1.10 ± 0.00	NR	1.38 ± 0.01	NR	[17]
HI31-A3-AL ^{CS} , XI	YNB	–	–	1.866	NR	0.765	NR	[31]
STXQ, XI	YPM	–	–	0.54 ± 0.07	1.13 ± 0.01	0.34 ± 0.02	63.51 ± 0.50	[32]
	YPM	162	2.97 ± 0.20	0.53 ± 0.02	5.24 ± 0.02	1.57 ± 0.02	120.6 ± 0.41	
IBB10B05, XR/XDH	DMM	–	–	0.056 ± 0.003	NR	0.28 ± 0.04	NR	[33]
NAPX37, XR/XDH	YPM	–	–	NR	NR	NR	13.5	[34]
	YPM	75	NR	NR	NR	NR	39.9	
KE6-12A, XR/XDH	YPM	–	–	0.66 ± 0.04	NR	NR	NR	[35]
	YPM	40	1.23 ± 0.03	0.27 ± 0.02	NR	NR	NR	
IMS0629, XI	SM	10	0.28 ± 0.01	0.38 ± 0.00	NR	0.103 ± 0.02	NR	[36]

DMM: defined mineral medium: 5 g/L (NH₄)₂SO₄, 0.5 g/L MgSO₄·7H₂O, 0.42 mg/L Tween-80, 10 mg/L ergosterol, 250 µL/L anti-foam 204 (Sigma-Aldrich, Vienna, Austria), trace elements, vitamins, 14.4 g/L K₂HPO₄ buffer (pH 6.5), and sugar [33]. MM: minimal medium, and sugar [15]. SM: 2.3 g/L urea, 6.6 g/L K₂SO₄, 3 g/L KH₂PO₄, 1 mL/L trace elements solution and 1 mL/L vitamin solution, and sugar [36]. YNB: 1.7 g/L yeast nitrogen base w/o amino acids (YNB), 5 g/L ammonium sulphate, and sugar. YPM: 10 g/L yeast extract, 20 g/L peptone, and sugar. The results in this study represent the mean ± standard deviation of duplicate independent experiments

G glucose, X xylose, NR not reported

were comparable with those obtained for strains with XI pathway (Table 3). In general, strains harbouring XI pathway exhibited higher ethanol yield than those harbouring XR/XDH pathway (Table 3). This is due to the cofactor imbalance problems in XR/XDH strains [10, 11]. However, with the engineering of XR, ethanol yield could be considerably improved [10]. Moreover, the presence of glucose [16, 34, 35] or the high initial cell density [32] enhanced the ethanol yield (Table 3).

Fermentation performance of strain 36aS1.10.4 in industrial lignocellulose hydrolysate fermentation media

As the original OPEFB hydrolysate (YPH1) had low sugar content and was readily fermentable (Fig. 4), additional sugar was supplemented to Hydrolysate I to observe the difference in fermentation performance by strains 36aS1 and 36aS1.10.4. Fermentation was conducted in YP medium containing sugar-enriched hydrolysate I (YPH1.1, Table 2). Under this condition, strain 36aS1 only consumed half amount of xylose in 72 h with the maximal specific xylose consumption rate of 0.30 g/g DW/h (Fig. 6a; Table 4). The final ethanol titre of 43 g/L was obtained with the maximal specific ethanol productivity of 0.91 g/g DW/h. On the other hand, the evolved strain 36aS1.10.4 almost consumed all xylose in 72 h with the maximal xylose consumption rate of 0.54 g/g DW/h (Fig. 6b; Table 4). This was 1.8-fold that of strain 36aS1. It is notable that strain 36aS1.10.4 exhibited not only higher xylose utilization rate but also higher glucose consumption rate than the parent strain, 36aS1 (2.82 versus 1.96 g/g DW/h). This demonstrated that the sugar utilization ability of strain 36aS1.10.4 in lignocellulose hydrolysate was improved after consecutive evolution in pentose media containing 6 g/L acetic acid.

In the end, fermentation performance of strain 36aS1.10.4 was evaluated in an original industrial wheat straw hydrolysate (YPH2, Table 2). In this case, strain 36aS1.10.4 was inoculated with an initial cell density of 2.5 g DW/L. About 98% of total sugars was converted and an ethanol titre of 54.11 g/L at 46 h was obtained (Fig. 6c). The maximal specific rates for glucose and xylose consumption were 2.61 and 0.54 g/g DW/h, respectively. Interestingly, the latter was higher than that obtained for glucose and xylose cofermentation in synthetic media (Table 3), further confirming that YP media are more favourable for sugar cofermentation. The maximal specific ethanol productivity was 1.38 g/g DW/h, which was higher than those in other reports (Table 4), however, consistent with that in our earlier report [32].

Among all the hydrolysate samples reported, wheat straw hydrolysate obtained from Inbicon (YPH2) exhibited the highest acetic acid content, 7.42 g/L (Table 4). Strain 36aS1.10.4 was able to ferment this hydrolysate with the

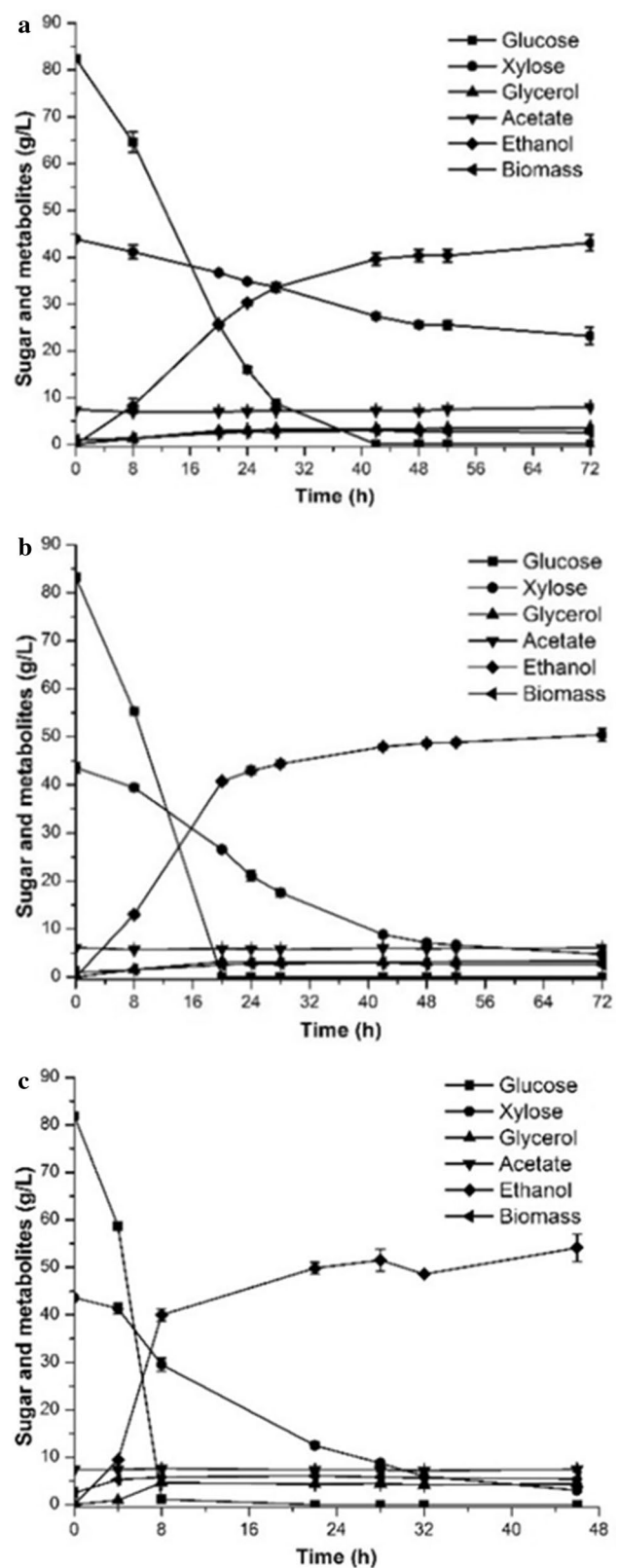


Fig. 6 Fermentation performance of yeast strains in lignocellulose hydrolysate fermentation media. **a** Strain 36aS1 in YPH1.1; **b** strain 36aS1.10.4 in YPH1.1; **c** strain 36aS1.10.4 in YPH2. The results represent the mean \pm standard deviation of duplicate independent experiments

Table 4 Comparison of batch fermentation performance of recombinant xylose-fermenting *S. cerevisiae* strains in biomass hydrolysate media

Strain name and xylose metabolizing pathway	Media	Initial sugar concentration (g/L)		Acetate (g/L)	Maximal sugar specific consumption rate (g/g DW/h)		Final ethanol volumetric productivity (g/L/h)	Maximal ethanol specific production rate (g/g DW/h)	Final ethanol mol yield (g/g)	Final ethanol titre (g/L)	References
		Glucose	Xylose		Glucose	Xylose					
36aS1, XI	YPH, OPEFB	83.17	43.56	6.07	1.96±0.20	0.30±0.15	0.60±0.02	0.91±0.17	0.30	43.07±1.77	This study
36aS1.10.4, XI	YPH, OPEFB	83.17	43.56	6.07	2.82±0.03	0.54±0.01	0.70±0.02	1.32±0.06	0.41	50.36±1.3	
	YPH, WS	82.66	43.96	7.42	2.61±0.03	0.54±0.01	1.18±0.06	1.38±0.05	0.44	54.11±2.85	
KE1-17, XR/XDH	MMH	38.5 ^a	13 ^a	6.3	1.36±0.09	NR	NR	0.58±0.03	0.43±0.00	NR	[15]
LF1, XI	YPUH, SEGS	86.60	39.09	4.52	NR	0.227	NR	0.334	0.413	NR	[16]
	YPUH, SPPR	54.94	23.79	0.00	NR	0.341	NR	0.529	0.416	NR	
GSI.11-26, XI	YPH, Arundo donax	59.34	22.18	6.80	NR	NR	0.43	NR	0.47±0.01	41	[17]
	YPH, Spruce	48.91	10.09	4.79	NR	NR	0.59	NR	0.43±0.00	32	
	YPH, WS/hay	68.22	28.22	0.70	NR	NR	1.02	NR	0.48±0.00	46	
STXQ, XI	YPH, OPEFB	41.81	30.00	5.67	3.08±0.02	0.33±0.02	0.39	1.37±0.03	0.42	28.4	[32]
JX123_noxE, XR/XDH	YPH, MS	100.0	30.5	NR	NR	NR	1.63	NR	0.433	55.5	[37]
Lg8-1, NR	YPH, WS	83	32	NR	NR	NR	NR	NR	0.46	55	[38]

The results in this study represent the mean ± standard deviation of duplicate independent experiments

MMH: minimal medium, and hydrolysate [15]; MS, *Miscanthus sacchariflorus*; NR, not reported; OPEFB, oil palm empty fruit bunch; WS, wheat straw; YPH, 10 g/L yeast extract, 20 g/L peptone, and hydrolysate; YPUH, 10 g/L yeast extract, 20 g/L peptone, 5 g/L urea, and hydrolysate

^aHexoses: glucose + mannose + galactoses; pentoses: xylose + arabinose

maximal specific xylose consumption rate of 0.54 g/g DW/h, which was among the highest compared to those in earlier reports (Table 4). Strain 36aS1.10.4 yielded an ethanol volumetric productivity of 1.18 g/L/h in YPH2, which was also among the highest (Table 4) except that reported by Lee and his colleagues (1.63 g/L/h) when an engineered industrial *S. cerevisiae* yeast strain was used [37]. The above analysis suggests that strain 36aS1.10.4 is promising in lignocellulose hydrolysate fermentation.

Formic acid, acetic acid, furfural, hydroxymethylfurfural (HMF), and biomass-derived phenolic compounds such as vanillin, syringaldehyde, 4-hydroxybenzaldehyde, and phenol are the main lignocellulosic biomass-derived inhibitory compounds. Both evolutionary engineering [17, 39, 40] and rational metabolic engineering [41] were successfully applied to enhance inhibitor tolerance and, therefore, hydrolysate capability of engineered yeast strains. In this study, the combination of adaptive evolution in complex media containing pentoses and acetic acid, and further screening in lignocellulose hydrolysate media allowed the rapid isolation of a stable hydrolysate-cofermenting recombinant yeast strain 36aS1.10.4.

Further fermentation in sugar-enriched industrial hydrolysate media (Table 2) demonstrated that strain 36aS1.10.4 exhibited much better performance than the unevolved strain 36aS1. (Fig. 6a, b; Table 4). This could be attributed to its evolution in pentose media containing acetic acid (Fig. 3) and its isolation from hydrolysate media (Fig. 4). In addition, glucose and xylose cofermentation was observed for strain 36aS1.10.4 and this could be due to its isolation in hydrolysate media based on its superior ethanol production capabilities. The exceptionally higher ethanol titre obtained by strain 36aS1.10.4 than those by the rest isolates indicates glucose and xylose cofermentation (Fig. 4b).

Notably, acetic acid content did not decrease in all hydrolysate fermentation experiments (Fig. 6). Adaptive evolution of strain 36aS1 in pentose media containing acetic acid generated pentose-utilizing strain library 36aS1.10 (Fig. 3) with enhanced hydrolysate fermentation capacity (Fig. 6a, b). However, such evolution process did not endow the strain with acetic acid utilization capability. Among the inhibitory compounds in lignocellulose hydrolysate, acetic acid is not the strongest inhibitor to glucose and xylose cofermentation [42]. However, it was the primary inhibitory compound present in lignocellulose hydrolysate (Table 2), in agreement with many earlier reports [14–17]. Adaptive evolution of strain 36aS1 in pentose media containing acetic acid successfully generated a hydrolysate-cofermenting strain 36aS1.10.4 (Fig. 4b). The above analysis suggests that the strain development methods adopted in this study are potential in generating yeast strains for cellulosic ethanol industries.

OPEFB is a potential lignocellulosic biomass available in Southeast Asia for biofuel production [1]. It has been extensively investigated for cellulosic ethanol production [43–46] with the highest ethanol titre of 83.6 g/L obtained through formiline pretreatment followed by simultaneous saccharification and fermentation [45]. For these studies [43–46], pentoses were almost removed in biomass pretreatment processes and primarily glucose was converted to ethanol using the wild-type *S. cerevisiae* strains. More recently, a diploid xylose-fermenting *S. cerevisiae* strain STXQ was successfully used to convert both glucose and xylose in a laboratory-generated OPEFB hydrolysate sample to ethanol and an ethanol titre of 28.4 g/L was obtained [32]. In this study, OPEFB hydrolysate (hydrolysate I) contained 6.07 g/L acetic acid comparable to those reported for other lignocellulose hydrolysate samples (Table 4) although its sugar content was low (Table 2). The parent strain 36aS1 was able to ferment OPEFB hydrolysate most likely through glucose and xylose cofermentation (Fig. 4). Both the parent strain 36aS1 and the evolved strain 36aS1.10.4 were able to ferment sugar-enriched OPEFB hydrolysate (YPH1.1). Ethanol titre of 50.36 g/L was obtained by strain 36aS1.10.4 with an ethanol yield of 0.41 g/g, both higher than those obtained by the parent strain 36aS1 (Table 4). This demonstrated the enhanced glucose and xylose cofermentation capabilities and potential of strain 36aS1.10.4 in OPEFB hydrolysate fermentation compared to strain 36aS1.

Conversion of wheat straw to ethanol has achieved tremendous advancement in recent years with the focus on high-gravity ethanol production. Through phosphoric acid plus hydrogen peroxide (PHP) pretreatment and high-solid loading separate hydrolysis and fermentation (SSF), up to 71.2 g/L ethanol titre was obtained with ethanol yield of 0.33–0.46 g/g consumed sugar [47, 48]. However, a commercial *S. cerevisiae* yeast strain from Angel Yeast Co., Ltd. (Yichang, China) was used and only glucose was fermented. Recently, recombinant xylose-fermenting yeast strains have been frequently used in conversion wheat straw hydrolysate to ethanol. Using a continuous membrane bioreactor, ethanol titre of 44 g/L was obtained with an ethanol yield of about 0.42 g/g [49]. In batch separate hydrolysis and cofermentation (SHCF) of alkaline preextracted and steam pretreated wheat straw, ethanol titre and yield of 54.5 g/L and 0.46 g/g were obtained, respectively [38]. Compared to SHCF, simultaneous saccharification and cofermentation (SSCF) is more favourable to high-titre ethanol production [35, 50, 51]. Through hybrid SSCF/SHCF processing of SO₂ pretreated wheat straw, ethanol titre and yield reached 40 g/L and 0.39 g/g, respectively [50]. By a temperature-profiled multifeed SSCF of wheat straw, ethanol titre of 65 g/L and yield of 0.36 g/g were obtained [51]. The highest ethanol titre of 71 g/L was obtained through SSCF using a flocculating xylose-fermenting yeast strain with ethanol yield of 0.45 g/g [35]. In this study, an ethanol titre of 54.11 g/L

and yield of 0.44 g/g were obtained for industrial wheat straw hydrolysate sample (YPH2) using strain 36aS1.10.4 (Table 4). These results are quite comparable to the best results obtained through SHCF reported so far [38]. They further confirm that the rapid strain development methods reported in this work is effective and strain 36aS1.10.4 is potential in industrial lignocellulosic ethanol production, in particular, when both cellulose and hemicellulose hydrolysates should be utilized. Strain 36aS1.10.4 may be potentially used for fermentation of various industrial lignocellulose hydrolysates and, therefore, can be the workhorse in lignocellulosic ethanol industries.

Conclusions

The combination of DNA assembly with repetitive genome integration using an integrative and replicative plasmid harbouring an autonomous replicative sequence allowed the pentose-utilizing gene cassettes assembled in vivo and integrated into the yeast genome. It resulted in a pentose-utilizing recombinant yeast strain with optimal combination and integration of pentose-metabolizing pathway genes. By consecutive transfer alternatively in YPX and YPA media containing 6 g/L acetic acid, a genetically stable yeast strain library, 36aS1.10, was obtained with enhanced growth in pentose media. Direct isolation on lignocellulose hydrolysate plates followed by screening in hydrolysate liquid medium generated the evolved strain 36aS1.10.4, which exhibited strong glucose and xylose cofermentation capacity. In the fermentation of an original wheat straw hydrolysate, 54.11 g/L ethanol was obtained with ethanol volumetric productivity of 1.18 g/L/h and ethanol yield of 0.44 g/g. These are quite comparable to the best results obtained so far for wheat straw hydrolysate through SHCF. We, therefore, conclude that strain development methods outlined in this study are efficient in rapid generation of a hydrolysate-cofermenting yeast strain. Strain *S. cerevisiae* 36aS1.10.4 can be a flexible workhorse for ethanol production from a variety of lignocellulose hydrolysates.

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