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A genome shuffling-generated *Saccharomyces cerevisiae* isolate that ferments xylose and glucose to produce high levels of ethanol

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Abstract Genome shuffling is an efficient approach for the rapid improvement of industrially important microbial phenotypes. This report describes optimized conditions for protoplast preparation, regeneration, inactivation, and fusion using the *Saccharomyces cerevisiae* W5 strain. Ethanol production was confirmed by TTC (triphenyl tetrazolium chloride) screening and high-performance liquid chromatography (HPLC). A genetically stable, high ethanol-producing strain that fermented xylose and glucose was obtained following three rounds of genome shuffling. After fermentation for 84 h, the high ethanol-producing *S. cerevisiae* GS3-10 strain (which utilized 69.48 and 100% of the xylose and glucose stores, respectively) produced 26.65 g/L ethanol, i.e., 47.08% higher than ethanol production by *S. cerevisiae* W5 (18.12 g/L). The utilization ratios of xylose and glucose were 69.48 and 100%, compared to 14.83 and 100% for W5, respectively. The ethanol yield was 0.40 g/g (ethanol/consumed glucose and xylose), i.e., 17.65% higher than the yield by *S. cerevisiae* W5 (0.34 g/g).

Keywords Genome shuffling · *Saccharomyces cerevisiae* · Xylose metabolism · Ethanol production

Introduction

Hemicelluloses represent a significant fraction of all woody plant material and represent a large unused reservoir of carbon sources that can be utilized for microbial growth. Xylose is the most abundant monosaccharide in lignocellulose hydrolysates after glucose. High ethanol yields from lignocellulosic residues are dependent on the efficient use of all available sugars, including glucose and xylose. The well-known fermentative yeast *Saccharomyces cerevisiae* is the preferred microorganism for ethanol production because it has high fermentation rates, high ethanol tolerance, high sugar tolerance, and is generally regarded as a safe microorganism (in the context of human infections). However, *S. cerevsiae* cannot ferment xylose [2]; therefore, over the last 20 years, this yeast has been the subject of various research efforts aimed at improving its ability to ferment xylose as a means of generating ethanol.

Xylose is first reduced to xylitol by xylose reductase (XR, EC 1.1.1.21), and then xylitol is oxidized to xylulose by xylitol dehydrogenase (XDH, EC 1.1.1.9; Fig. 1). A recombinant *S. cerevisiae* strain expressing XR and XDH was shown to ferment xylose into xylulose at low levels [1, 7, 14, 17]. Moreover, the growth of this industrial strain was extremely slow in the presence of xylose, and xylose fermentation efficacy was poor [19]. One explanation for the poor yields could be that the xylose metabolic pathways were blocked [8] and the redox between XR and XDH was not balanced [11, 26]. In addition, some studies demonstrated that xylose transport in *S. cerevisiae* was dependent on the glucose transport system [9].

Xylulose is phosphorylated by xylulokinase (XK, EC 2.7.1.17) [5, 19, 26] to xylulose-5-phosphate (X5P), which is then metabolized through the non-oxidative pentose phosphate pathway (PPP) and the glycolysis pathway [10] (Fig. 1). Slow xylulose consumption is attributed to low levels of endogenous XK, suggesting XK is a metabolic bottleneck to further metabolism of xylose. Overexpression of the *S. cerevisiae* xylulokinase gene may accelerate

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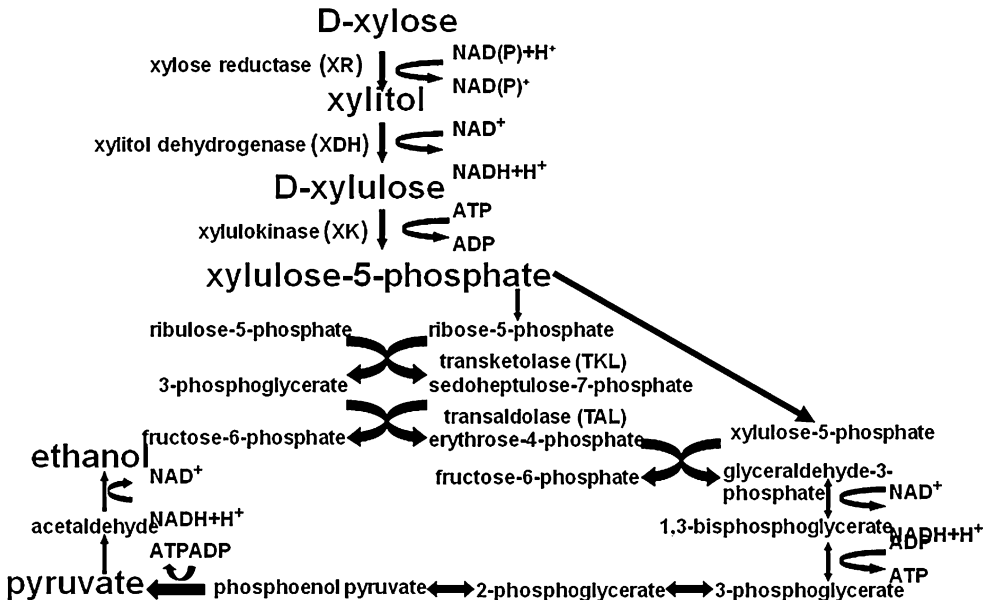

Fig. 1 Schematic representation of xylose metabolic pathway in metabolically engineered *Saccharomyces cerevisiae* strains

xylose metabolism, enhancing xylose utilization rates and ethanol yields. Most *S. cerevisiae* strains can not ferment xylose as effectively as glucose; therefore, bottlenecks in the xylose metabolic pathway associated with the XR, XDH, and XK genes need to be addressed by genetic engineering approaches in order to facilitate xylose fermentation by *S. cerevisiae* strains as a means of reaching the ethanol yields required for commercial bioethanol production [1, 14].

Our previous work [5, 18] described the construction of three homologous recombination plasmids that contained the XR (GenBank accession number FJ523203), XDH (GenBank accession number FJ040172), and XK (GenBank accession number FJ523204) genes, respectively. The XR and XDH genes were from *Candida shehatae* HDYXHT-01 (ACCC 20335), whereas the XK gene was from *S. cerevisiae* W5 itself. Using lithium acetate to carry out the transformations, we stably integrated plasmids possessing the XR, XDH, and XK genes separately into the *S. cerevisiae* chromosome genome and designated the recombinant strains YX-3, LX-4, and CXS-5, respectively.

In this study, the three recombinant strains YX-3, LX-4, and CXS-5 were used as starting strains and XR, XDH, and XK genes were combined into a single *S. cerevisiae* chromosome using the novel method of genome shuffling (Fig. 2), a technology designed for engineering phenotypes at the whole genome level [7]. This technique can be integrated with metabolic engineering to facilitate the creation of complex phenotypes, resulting in increased yields of metabolites. A respective strain possessing specific properties obtained by rational design can be subjected to genome shuffling to further improve product yields. The recombinant *S. cerevisiae* isolate generated by genome shuffling expressed three genes (XR, XDH, and XK) of the xylose metabolic pathway that together facilitated xylose fermentation that yielded ethanol. This is of significant importance in the context of the bioethanol conversion of lignocellulose materials into ethanol.

Materials and methods

Saccharomyces cerevisiae strains and growth conditions

S. cerevisiae W5, a diploid wild-type strain, was isolated from soils of Heilongjiang province, China, and maintained in our laboratory [5], *S. cerevisiae* YX-3, LX-4, and CXS-5 were derived from W5 and possessed, respectively, the XR, XDH, and XK genes. Three integrated plasmids pXRYX-3, pXDHLX-4, and pXKCXS-5 were constructed in our previous work and the XR gene was present as a single copy, whereas XDH and XK genes were utilized as multiply copies on their plasmids. All of the three plasmids were linearized and transformed into *S. cerevisiae* W5 separately. Three genetic stable strains, YX-3, LX-4, and CXS-5, were obtained and used as the starting strains in the following genome shuffling. All of the strains were grown in yeast peptone dextrose (YPD) liquid medium (w/v): 1% yeast extract, 2% peptone, and 2% glucose; or in yeast peptone xylose (YPX) liquid medium (w/v): 1% yeast extract, 2% peptone, and 2% xylose. In addition, strains were also grown in YPD solid medium containing 2% (w/v) agar, YPX solid medium containing 2% (w/v) agar, YPDS solid medium containing 2% (w/v) agar and 0.8 M sorbitol, or YPXS solid medium containing 2% (w/v) agar and 0.8 M sorbitol.

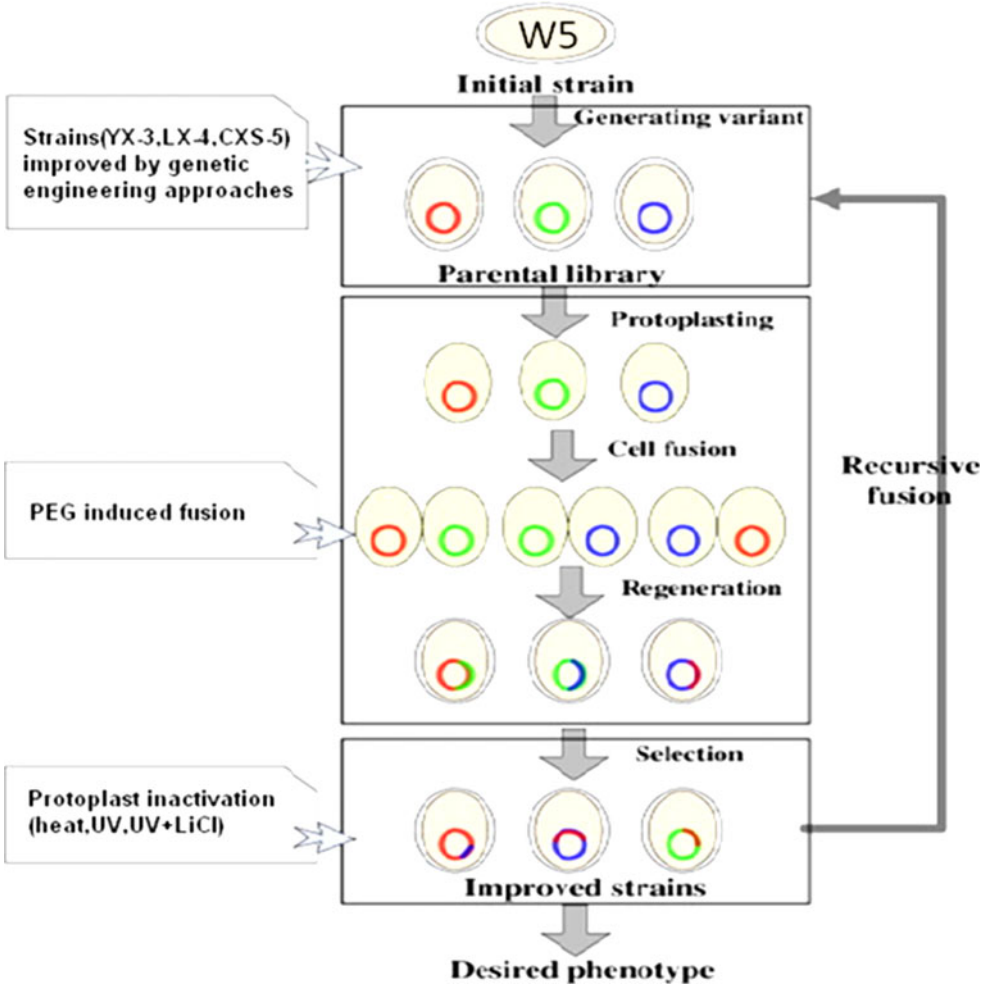

Fig. 2 The genome shuffling technique

TTC (triphenyl tetrazolium chloride) medium (w/v), which used to detect the yield of ethanol on Petri dishes, comprised two layers: the upper layer contains (w/v) 0.5% TTC and 1.5% agar (pH 5.0) and the bottom layer contained (w/v) 3% xylose, 0.5% yeast extract, 0.5% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.02% $CaCl_2$, 0.02% $MgSO_4{\cdot}7H_2O$, and 2% agar, pH 5.5.

Xylose and glucose co-fermentation medium consisted of (w/v; pH 5.5) 2.5% xylose, 5% glucose, 0.5% yeast extract, 0.5% KH_2PO_4, 0.2% $(NH4)_2SO_4$, 0.02% $CaCl_2$, 0.02% $MgSO_4{\cdot}7H_2O$, and 2% agar.

For the seed culture, one colony was inoculated into 20 mL YPX liquid medium and incubated at 30°C for 12 h; the products were then inoculated onto xylose and glucose co-fermentation medium at 5% (v/v) and fermented at 30°C with a shaking speed of 100 rpm for 84 h.

Reagents

Citric acid phosphate buffer (CPB) was prepared with 0.1 M citric acid and 0.2 M $Na_2HPO_4{\cdot}12H_2O$ and the pH adjusted to 6.0. The pretreatment agent comprised CPB with 0.2% (v/v) β-mercaptoethanol and 0.06 M EDTA-Na_2 and the protoplast preparation comprised CPB with 0.8 M sorbitol and 0.01 M EDTA-Na_2. Zymoliase was dissolved in CPB with 0.8 M sorbitol and sterile-filtered. PEG (polyethylene glycol) consisted of 30–40% PEG (w/v) in 0.8 M sorbitol and 0.01–0.05 M $CaCl_2$. All the reagents used in this study were of analytical grade.

Protoplast preparation and regeneration

Respective strains were incubated with shaking at 140 rpm for 12 h at 30°C in 20 mL YPD liquid medium in a 50-mL flask. The culture solution was then inoculated into 100 mL fresh YPD liquid medium in a 250-mL flask and cultured for 12 h. When the optical density (OD) reached 1.0 at 600 nm, cultures were harvested by centrifugation at 4,500 rpm for 15 min, washed twice with distilled water, and adjusted to a final concentration of 10^7 cells/mL. A 3 mL aliquot of pretreatment agent which contained 0.2% (v/v) β-mercaptoethanol and 0.06 M EDTA-Na_2 was added to the cell suspensions and incubated at 30°C for 20 min. After this incubation, cells were centrifuged and resuspended in

4 mL protoplast preparation reagent and 2 mL CPB containing 2% (w/v) zymoliase used for enzymatic digestion of the cell walls followed by incubation at 30°C for 15 min. The cells were centrifuged and resuspended in protoplast preparation agent [22]. The rates of protoplast formation and regeneration were obtained by determining colony counts using the following formulas: protoplast preparation (%) = $[(A - C)/A] \times 100\%$; protoplast regeneration (%) = $[(B - C)/(A - C)] \times 100\%$, where A refers to the total number of colonies counted on YPD medium before hydrolysis of cell wall by zymoliase, B represents the number of colonies counted on YPDS medium after hydrolysis of cell wall by zymoliase, and C represents the number of colonies counted on YPD medium after hydrolysis of cell wall by zymoliase.

Protoplast inactivation

Purified YX-3 protoplast suspensions were transferred into sterile test tubes and incubated in a 60°C water bath for 2, 7, 12, 17, 22, and 27 min to choose the optimal inactivation condition [28, 30]. Inactivation was confirmed by lack of growth in the YPDS medium.

The purified LX-4 protoplast suspensions were transferred to a sterile Petri dish (6 cm diameter) which was then placed under a preheated 30-W UV lamp at a vertical distance of 20 cm and irradiated for 10, 20, 30, 40, 45, and 50 min to choose the optimal inactivation condition [3, 24, 28]. The treated protoplasts were maintained in the dark for 2 h to avoid photoreactivation repair. Lack of growth in the YPDS medium was used to confirm the inactivation.

Activation in the presence of LiCl was carried out by adding LiCl solution to a purified CXS-5 protoplast suspension to a final concentration of 0.6% (w/v) and then irradiated for 5, 10, 15, 20, 25, and 30 min under a preheated 30-W UV lamp at a vertical distance of 20 cm to choose the optimal inactivation condition [28]. The treated protoplasts were kept in the dark for 2 h to avoid photoreactivation repair. Strains inactivated by UV plus LiCl were also confirmed by lack of growth in the YPDS medium.

Protoplast inactivation was calculated as (%) = $[1 - (A - B)/(C - D)] \times 100\%$, where A corresponds to the number of colonies observed growing on YPDS medium after inactivation, B corresponds to the number of colonies observed growing on YPD medium after inactivation, C represents the number of colonies observed growing on YPDS medium before inactivation, and D corresponds to the number of colonies observed on YPD medium before inactivation.

Protoplast fusion

Two inactivated protoplast suspensions were selected randomly at a concentration of approximately 1×10^7 protoplasts/mL. A 1-mL protoplast suspension was then blended and centrifuged at 3,000 rpm for 10 min. Protoplasts were then harvested and 2 mL PEG solution was preheated to 30°C and added to the protoplast suspensions. The mixed liquid was incubated in a 30°C water bath for 30 min and centrifuged at 3,000 rpm for 10 min. The supernatants were discarded and resuspended in 2 mL protoplast preparation agent. The mixed protoplasts were diluted by hypertonic solution and regenerated on YPXS medium. Protoplast fusion rates were calculated as follows: protoplast fusion = $[(A - B)/C] \times 100\%$, where A corresponds to the number of colonies observed growing on YPXS medium, B corresponds to the number of colonies observed on inactivated parent YPXS medium, and C corresponds to the number of parent colonies observed on YPDS medium.

Genome shuffling

Protoplasts from starting YX-3, LX-4, and CXS-5 strains were inactivated by either heating, UV, or UV plus LiCl, respectively. Inactivated protoplasts were fused randomly under suitable conditions and regenerated by plating on appropriate media. All regenerated colonies were collected and selected by TTC medium used as the preliminary screening method and correspond to the first round of genome shuffling. The selected colonies were referred to as the GS1 generation and used as new parental strains during the second genome shuffling round, the protoplasts preparation and the inactivation were the same. After three rounds, the target GS3 strain generation was obtained.

Genome shuffling control experiment

To demonstrate that the improved traits of the recombinant were caused by genome shuffling and not the repeated protoplast preparation, regeneration, and inactivation, which may have induced mutation easily [28], we designed the genome shuffling control experiment which consisted of protoplasts generated from starting colonies that were not fused but regenerated by plating on appropriate media. The regenerated strains were designated as the CE1 generation and were subjected to the next protoplast regeneration cycle. After three rounds, the target CE3 strain generation was obtained and compared to strains derived from genome shuffling.

Screening of recombinants

TTC was used as the color development reagent. This reagent could react with yeast metabolites and show a specific red color. The enzyme activity of respiration in yeast could be determined by the depth of the colony's color, namely the levels of ethanol production by the yeast. The

three starting strains YX-3, LX-4, and CXS-5 and the wild-type strain W5 could not stain red because they did not possess the ability to convert the xylose into ethanol. An alternative explanation for this observation may be that if the cells were under aerobic conditions and the flux of xylose was sufficiently low, no ethanol would be formed leading to a false negative. The upper layer of the TTC medium was poured over the bottom TTC medium layer. The appearance of red color implies the production of ethanol. Colonies that successfully underwent recombination stained dark red and were selected as parental strains for the next round of genome shuffling.

Secondary screening

Strains obtained by primary screening were activated in a YPX slope culture at 30°C and then transferred into 20 mL YPX liquid medium in a 50-mL flask and cultured at 30°C for 12 h. The fermentation broths were inoculated onto xylose and glucose co-fermentation medium at 5% (v/v) and fermented at 30°C with a shaking speed of 100 rpm for 84 h. The fermentation supernatants were then analyzed by high-performance liquid chromatography (HPLC).

HPLC analysis

The production of ethanol, xylose, glucose, and xylitol was determined by HPLC (Shimadzu LC-10ATvp) using a HPX-87H column (300 mm x 7.8 mm, Aminex HPX-87H ion exclusion column) at 65°C with a refractive index detector (RID-10A). The eluent used was 0.005 M H_2SO_4 at a flow rate of 0.8 mL/min. The analysis time was 18 min. The injection volume of the sample was 20 μL. The ethanol (Tianjin Guangfu Technology Development Co., China), xylose (Shanghai Boao Biotechnology Co., China), glucose (Tianjin kemiou Chemical reagents Co., China), and xylitol (Institute of Guangfu fine chemical industry of Tianjin) were used as standard materials.

Sample treatment

Fermentation broths were serially diluted 100-fold and 1-mL samples were added to 1.5-mL centrifuge tubes and centrifuged at 13,000 rpm for 10 min. Supernatants were used for HPLC analysis.

Enzyme assays

Cell extracts for the assays of xylose metabolic enzymes (XR, XDH, and XK) were prepared as follows: After cultivation in YPD medium for 48 h at 30°C, cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C and washed with distilled water three times. The soluble proteins from yeast were extracted by the YeastBuster™ protein extraction reagent (Novagen Co.) and the supernatants were analyzed for three enzyme activities. Protein concentration was measured according to the Bradford method [16]. XR activity was measured spectrophotometrically by monitoring nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm in a reaction mixture with the following composition [16]: 50 mM potassium phosphate buffer (pH 7.0), 20 mM xylose, and 0.15 mM NADPH as substrate. XDH activity was measured spectrophotometrically by monitoring the reduction of the oxidized form of nicotinamide adenine dinucleotide (NAD^+) at 340 nm in a reaction mixture with the following composition [16]: 0.5 M Tris–HCl buffer (pH 8.2), 5 mM xylitol, and 2.5 mM NAD^+ as substrate. XK activity was measured spectrophotometrically by monitoring the oxidation of the reduced form of nicotinamide adenine dinucleotide (NADH) by lactate dehydrogenase (LDH) at 340 nm in a reaction mixture of the following composition [4, 16]: 0.5 M Tris–HCl (pH 7.8), 50 mM $MgCl_2$, 10 mM PEP-K, 10 mM ATP, 2 mM NADH, 67.5 U/mL LDH, 40.3 U/mL PK (pyruvate kinase), and 10 mM xylulose. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol each of NADPH and NADH reduced or oxidized from the substrate per minute.

Genetic stability assessment of the recombinant strain

The strain that generated the highest ethanol output was cultured for five generations and cultured in the presence of xylose and glucose. If the fermentation medium (following HPLC analysis) contained similar levels of ethanol as the starting generation, the strain was considered stable and used for further analysis.

Results

Preparation and regeneration of protoplasts

Before protoplast fusion, a series of single factor tests were used to determine the following optimal conditions for protoplast preparation and regeneration of *S. cerevisiae* strain W5: selecting 12-h-old yeast cells and incubating them in the pretreatment reagent for 20 min followed by the addition of 2.0% (w/v) zymoliase to digest the *S. cerevisiae* cell wall under weak acid or weak alkali conditions. This was followed by incubations in a 10-mL test tube in the presence of 0.8 M sorbitol as an osmotic stabilizer. Suspensions were then stirred slowly for 15 min at 30°C. The rates of protoplast preparation and regeneration were 95.11 and 16.38%, respectively.

Table 1 Protoplast susceptibility to different inactivation treatments over time

Heat treatment (min)	2	7	12	17	22	27
YX-3 Lethal rate (%)	17.23 ± 0.20	98.94 ± 0.02	99.98 ± 0.01	99.99 ± 0.01	100 ± 0.00	100 ± 0.00
UV treatment (min)	10	20	30	40	45	50
LX-4 Lethal rate (%)	89.89 ± 0.12	99.98 ± 0.01	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
UV + LiCl treatment (min)	5	10	15	20	25	30
CXS-5 Lethal rate (%)	99.83 ± 0.08	99.95 ± 0.01	99.96 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00

Data are expressed as the mean values ± standard deviation of at least three independent experiments

As YX-3, LX-4, and CXS-5 strains were all *S. cerevisiae* W5 derivatives, these preparation and regeneration conditions were used for subsequent genome shuffling procedures.

Protoplast inactivation

Protoplasts were inactivated in three different ways: heating, UV, and a combination of UV plus LiCl. For heating inactivation, purified YX-3 protoplasts were incubated in a 60°C water bath for 22 min, resulting in a lethal rate of 100% (Table 1). For UV inactivation, an irradiation time of 30 min resulted in a 100% lethal rate and this time was used for inactivating LX-4 protoplasts (Table 1). For LiCl plus UV inactivation, the 0.6% (w/v) LiCl combined with a 20-min UV irradiation was used as the optimal inactivation condition for the CXS-5 protoplasts (Table 1).

Genome shuffling

Protoplast fusion conditions are critical to successful genome shuffling. The PEG and $CaCl_2$ concentrations, combined with the fusion time, can significantly affect the protoplast fusion efficiency. In this study, the orthogonal experimental design method was used to optimize fusion conditions. The designed table and experiment results of the orthogonal experimental design method are described in Table 2.

The *R* value refers to the range, which means the difference between maximum and minimum values. We can find the main important factors in the experiment by examining the *R* value, and this could help us find the best factor and level combination. From Table 2, the *R* value indicted that the PEG concentration was the most important factor in protoplast fusion, followed by the $CaCl_2$ concentration and fusion time. On the basis of the average of each factor, the optimal fusion conditions were 35% (w/v) PEG, 10 mM $CaCl_2$, and a 30-min fusion time.

Screening of high ethanol output strains generated by genome shuffling

As the YX-3, LX-4, and CXS-5 strains were derived from the *S. cerevisiae* W5 wild-type strain (derived using genetic engineering breeding methods), these strains have the same genetic background. In this study, the YX-3, LX-4, and CXS-5 strains were used as the starting strains for the first genome shuffling round. Protoplast preparation, regeneration, inactivation, and fusion were all carried out according to the optimal conditions described above. All regenerated colonies were selected by TTC screening, i.e., colonies staining dark red were selected as new parental strains for the next genome shuffling round. This process was repeated three times, i.e., GS1, GS2, and GS3. After three rounds of genome shuffling, GS3 had a higher yield of ethanol than GS2 and GS1, and finally 14 recombinants were obtained (Table 3) and GS3-10 was selected for additional analysis on the basis of its growth profile with the highest yield of ethanol (Table 3). After the preliminary screening in

Table 2 Results of orthogonal experimental conditions used for fusion

Level[a]	Factor			
	PEG concentration (%)	$CaCl_2$ concentration (mM)	*T* (min)	Fusion rate (%)
1	30	10	20	0.02923
2	30	30	30	0.07231
3	30	50	40	0.1
4	35	10	30	0.12308
5	35	30	40	0.06769
6	35	50	20	0.03539
7	40	10	40	0.06154
8	40	30	20	0.05846
9	40	50	30	0.09077
*T*1	0.201	0.213	0.123	–
*T*2	0.225	0.198	0.285	–
*T*3	0.21	0.225	0.228	–
*X*1	0.067	0.071	0.041	–
*X*2	0.075	0.066	0.095	–
*X*3	0.07	0.075	0.076	–
R	0.008	0.009	0.054	–

[a] *T* is the sum of the average of each factor, *X* is the average of each factor, *R* is the range (i.e., difference between maximum and minimum values)

Table 3 Fermentation results of screening recombinants after three rounds of genome shuffling

Strains	Remaining amount of xylose/total xylose (g/L)	Remaining amount of xylose/total glucose (g/L)	Production of xylitol (g/L)	Production of ethanol (g/L)
GS3-1	9.63/15.82	0/29.70	0.98	10.07
GS3-2	7.00/15.82	0/29.70	2.58	12.31
GS3-3	9.15/15.82	0/29.70	1.30	12.30
GS3-4	11.21/15.82	0/29.70	1.34	11.44
GS3-5	8.61/15.82	0/29.70	1.60	12.21
GS3-6	12.20/15.82	0/29.70	1.99	9.76
GS3-7	8.18/15.82	0/29.70	1.09	12.54
GS3-8	7.78/15.82	0/29.70	1.00	9.05
GS3-9	6.00/15.82	0/29.70	2.71	11.62
GS3-10	6.88/15.82	0/29.70	1.20	14.07
GS3-11	6.48/15.82	0/29.70	1.76	11.48
GS3-12	5.62/15.82	0/29.70	1.63	12.58
GS3-13	10.08/15.82	0/29.70	2.51	9.96
GS3-14	7.27/15.82	0/29.70	0.94	11.06
W5	10.23/15.82	0/29.70	1.62	9.94
YX-3	8.12/15.82	0/29.70	2.82	10.68
LX-4	10.43/15.82	0/29.70	0.88	10.42
CXS-5	10.10/15.82	0/29.70	1.22	10.35

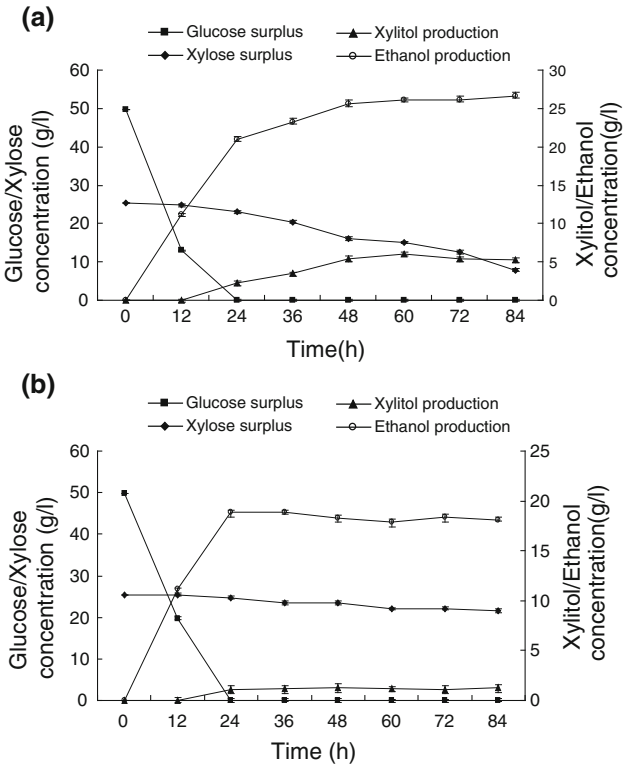

Fig. 3 Changes in the concentrations of residual glucose (*black squares*), residual xylose (*black diamonds*), xylitol production (*black triangles*), and ethanol production (*open circles*) in the presence of GS3-10 (**a**) or W5 (**b**) during fermentation in the presence of both xylose and glucose as carbon sources. Data are expressed as the mean ± standard deviation of three independent experiments

fermentation of 60 h the total xylose and glucose were 15.82 and 29.7 g/L, respectively; the GS3-10 was then cultured in YPX liquid medium and inoculated into the xylose and glucose co-fermentation medium and supernatants analyzed for ethanol production by HPLC (Fig. 3). A 84-h fermentation period was used and the W5 strain was used as a control.

Figure 3 illustrates the fermentation kinetics of GS3-10 and W5, including ethanol and xylitol production and glucose and xylose consumption. This analysis revealed significant differences between the two strains; specifically, strain GS3-10 produced significantly higher levels of ethanol compared with strain W5 (Fig. 3). Both strains consumed available glucose by 24 h that corresponded to each strain's highest ethanol output, i.e., 21.00 and 18.85 g/L for GS3-10 and W5, respectively. At later time points, GS3-10 continued to use xylose and produced ethanol and xylitol. As strain W5 did not possess the machinery to metabolize xylose effectively, the ethanol production curve was stable after 24 h. However, W5 can take up some xylose and produced very low xylitol yields (1.26 g/L). After a 84-h fermentation period, xylose utilization and xylitol production of GS3-10 were 17.67 and 5.23 g/L respectively, significantly higher than those of strain W5 (3.77 and 1.26 g/L, respectively). The ethanol yield of GS3-10 was 0.40 g/g (ethanol/consumed glucose and xylose), i.e., 17.65% more than that generated by *S. cerevisiae* W5 (0.34 g/g). However, GS3-10 was unable to completely ferment xylose, producing about 26.65 g/L ethanol and leaving 30.52% unused xylose after 84 h.

Table 4 shows that the GS3-10 and W5 could produce 19.85 g/L and 19.40 g/L ethanol, respectively, in conditions in which glucose was the sole carbon source. The outputs of ethanol from GS3-10 in the xylose and glucose co-fermentation medium were 26.65 g/L (increased by more than 34.26%), indicating that GS3-10 can utilize the xylose and convert it to ethanol. The inability to use the xylose meant that the production of W5 was stable, and 18.12 g/L ethanol was obtained at the end of the co-fermentation.

Genome shuffling control experiment

This study included a control experiment to assess the effects of the protoplast preparation and regeneration procedures on ethanol output. The obtained final colony was designated CE3-3. Culture in xylose and glucose co-fermentation medium for 84 h demonstrated that the significant improvement of the recombinants in producing ethanol

Table 4 Xylose and glucose fermentation by different strains

Strains	*A* (g/L)	*B* (g/L)	*C* (g/L)	*D* (g/L)	*E* (g/L)	*F* (g/L)	*G* (g/g)	*H* (g/L)
GS3-10	49.72 ± 0.15	25.43 ± 0.13	49.72 ± 0.15	17.67 ± 0.21	5.23 ± 0.10	26.65 ± 0.14	0.40 ± 0.02	19.85 ± 0.13
W5	49.72 ± 0.15	25.43 ± 0.13	49.72 ± 0.15	3.77 ± 0.13	1.26 ± 0.11	18.12 ± 0.18	0.34 ± 0.02	19.40 ± 0.19
CE3-3	49.72 ± 0.15	25.43 ± 0.13	49.72 ± 0.15	3.53 ± 0.13	1.14 ± 0.07	17.97 ± 0.20	0.34 ± 0.03	19.62 ± 0.15

Data are expressed as the mean values ± standard deviation of at least three independent experiments

A glucose in the mixed sugars, *B* xylose in the mixed sugars, *C* utilization of glucose in the mixed sugars, *D* utilization of xylose in the mixed sugars, *E* production of xylitol in the mixed sugars, *F* production of ethanol in the mixed sugars, *G* ethanol yield in the mixed sugars, *H* production of ethanol when using glucose as the sole carbon source

Table 5 XR, XDH, and XK activities in different cell extracts

Strains	Enzyme activities (U/mg)		
	NADPH-XR	NAD^+-XDH	NADH-XK
W5	3.17 ± 1.07	15.50 ± 1.75	90.87 ± 2.42
YX-3	3.54 ± 0.08	–	–
LX-4	–	25.36 ± 1.97	–
CXS-5	–	–	187.36 ± 2.05
GS3-10	4.07 ± 0.78	30.08 ± 1.96	137.82 ± 3.65

Data are expressed as the mean values ± standard deviation of at least three independent experiments

– data not detected in the experiment

was related to genome shuffling and that the repeated preparation and regeneration procedures that the protoplasts were subjected to had little effect on the observed ethanol-producing traits, suggesting that the recombinants with specific phenotypes were produced as a result of genome shuffling (Table 4).

Enzyme activities

In this study, we generated the xylose-fermenting yeast strain GS3-10 by co-expression of *xyl*1, *xyl*2, and *xks*1. The XR, XDH, and XK activities of different strains were measured (Table 5). The XR, XDH, and XK activities of *S. cerevisiae* GS3-10 were increased by more than 28.33, 94.08, and 51.68% compared to W5, indicating that the three kinds of genes in the recombinant had been effectively expressed, and also the technique of genome shuffling in *S. cerevisiae* had been successful. But the XK activity was a little low in the recombinant as in CXS-5; hence, this defect may be one of the bottlenecks for xylose fermentation in the recombinant.

PCR results of XR gene (*xyl*1) and XDH gene (*xyl*2)

The *xyl*1 and *xyl*2 genes were also amplified using the chromosome of GS3-10 as the template; the primers were *xyl*1-up (5′ ACTTCTAGATACATCCACAATGAGCCC) and *xyl*1-down (5′ TTCGGATCCTCTACGCAAAGAA AGCAG) for *xyl*1, and *xyl*2-up (5′ TGTTCTAGAATGACTG CWAACCCWTCMTTRGT) and *xyl*2-down (5′ TATCTC GAGYTAYTCWGGRCCRTCAATKARAC) for *xyl*2. The results showed that the two genes were amplified and indicated that the genome shuffling was successful (Fig. 4).

Genetic stability of the recombinant

The most effective ethanol-producing strain (GS3-10) was passaged five times and the ethanol production monitored by HPLC; about 1.34% failed the stability test after calculation. These data demonstrated that GS3-10 was stable over time because ethanol generation did not decrease over time, suggesting that this strain was genetically stable (Table 6).

Discussion

Although traditional breeding methods have succeeded in generating many industrial, ethanol-producing strains, it is a time-consuming and high-cost process. Genome shuffling is a technique that allows for the recombination of several genomes simultaneously at different sites without the necessity for detailed genomic information; therefore, multiple exchanges and multiple-gene recombination can occur rapidly and efficiently, resulting in the generation of a large number of strains that can then be tested for the desired phenotype [23, 24].

Genome shuffling is, in principle, applicable to the generation of strains with improved production pathways and phenotypes. It is straightforward to implement and well suited for industrial applications. Patnaik et al. [21] described the use of genome shuffling for improving the acid tolerance of a *Lactobacillus* strain, and Zhang et al. [27] successfully used genome shuffling to improve antibiotic production from a commercial *Streptomycete* isolate. Yu et al. [25] recently used genome shuffling to improve the glucose tolerance of *L. rhamnosus* while simultaneously enhancing this strain's L-lactic acid production. Additionally, the yields of other biochemical products, such as

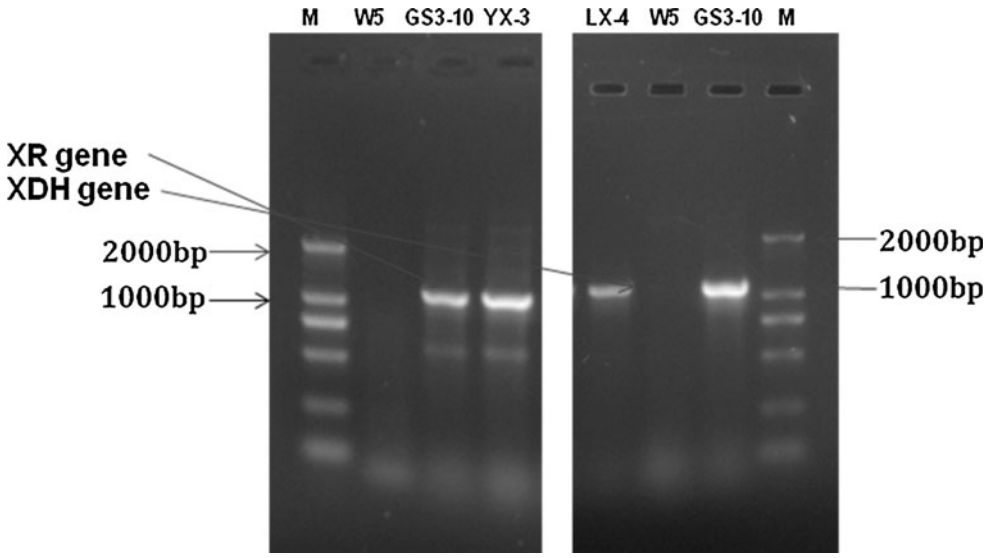

Fig. 4 PCR results of XR and XDH genes in different strains. *M* is the marker and *each lane* is marked with the strain name

Table 6 Genetic stability of recombinant GS3-10

Generation	1	2	3	4	5
Ethanol production (g/L)	27.50 ± 0.18	27.02 ± 0.29	26.54 ± 0.15	26.63 ± 0.21	27.34 ± 0.23

Data are expressed as the mean values ± standard deviation of at least three independent experiments

astaxanthin [29], taxol [28], ethanol [3, 6, 12], and bioinsecticide [13], were also successfully increased following genome shuffling modifications. In this report we demonstrated the effectiveness of genome shuffling to improve the xylose metabolic pathways in *S. cerevisiae* for the first time.

During classical genome shuffling, starting colonies are derived from wild-type strains subjected to traditional mutagenesis processes [28]. In this study, however, starting colonies YX-3, LX-4, and CXS-5 were derived from the genetic engineering *S. cerevisiae* W5 strain.

Genome shuffling can be used to improve many industrial microorganism phenotypes and is easy to carry out by using inactivated parental protoplasts [24, 28]. Generally speaking, if parental protoplasts are inactivated when testing only one method they may be difficult to regenerate. Therefore in this study, according to the principle of complementary protoplast damage, we tested three different parent protoplast inactivation methods before carrying out the fusion procedure. This approach reduced the time needed for screening fusion protoplasts and improved the efficacy of the procedure.

Although recombinant *S. cerevisiae* GS3-10 could convert xylose to ethanol using glucose as a carbon source (the major by-product was xylitol), the xylose consumption rates were not sufficient or efficient enough to sustain bioethanol production from lignocellulosic materials. An additional obstacle that needed to be overcome was the relatively low xylose consumption rates observed in these strains during xylose and glucose co-fermentation. One possible explanation [15] for the low xylose consumption rates observed during the co-fermentation process was that in the *S. cerevisiae*, xylose uptake efficiency may not have been as high as the glucose uptake capacity. Because no xylose-specific transporters have been described in *S. cerevisiae*, it appeared that xylose and glucose transport were carried out by the same pathway despite a xylose affinity that is 200-fold lower than that described for glucose [20]. An additional explanation [1, 11, 14] may be that the redox between XR and XDH was not in balance and/or that the downstream pentose phosphate xylose metabolism pathway was blocked. Regardless, the efficacy of recombinant *S. cerevisiae* to convert xylose into ethanol was not sufficient.

From Fig. 3, we know that the original W5 could also take up a little xylose (about 3.77 g/L, the xylose utilization was 14.83%) in the co-fermentation of glucose. We had also tried a xylose-only fermentation experiment in which we found that the W5 could not absorb xylose and no ethanol was produced, so we chose the W5 as candidate strain. From Table 5, we also detected that the W5 could show XR and XDH activities. The reasons for this may be that the methods used to assess enzyme activities were not very precise (UV spectrophotometry) and the soluble proteins from yeast extracted by the YeastBuster™ protein extraction reagent were very complex. Many other dehydrogenases and oxidases in the crude enzyme solution could also have

reactions with NADPH, NAD^+, and NADH, such that the resulting XR, XDH, and XK activities measured were not very specific. But these factors did not affect the relative comparison of enzyme activities of the various strains.

In conclusion, we report for the first time the use of genome shuffling as a means of improving the xylose metabolic pathways of *S. cerevisiae* using starting strains modified by genetic engineering breeding methodologies. Using this approach, we successfully generated a genetically stable strain capable of high ethanol production with the capacity to ferment xylose and glucose after only three rounds of genome shuffling, demonstrating that the *S. cerevisiae* xylose metabolic pathway could be engineered to convert lignocellulosic material into ethanol. The high ethanol-producing strain GS3-10, which fermented xylose and glucose with an efficacy of 69.48% and 100% compared to the 14.83% and 100% for W5, respectively, yielded 26.65 g/L ethanol, which was 47.08% greater than that of strain W5. Additional modifications will be needed before the development of robust *S. cerevisiae* strains with the capacity to ferment all sugars present in lignocellulosic hydrolysates into ethanol can be used commercially.

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