Saccharomyces Cerevisiae Strains Selected From Nature Significantly Increased the Production of 2-Phenylethanol Through Protoplast Fusion

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Research

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Abstract

Background: 2-Phenylethanol (2-PE) is an aromatic alcohol with rose fragrance, which is widely used as an additive in food, tobacco and daily chemical industries. Yeast is the main microorganism producing natural 2-PE, but it is limited by low yield and weak tolerance. Nature and fermented products is a resource treasury of yeasts with excellent traits. Screening strains with good phenotypic traits and conducting breeding by cell fusion for genetic pyramiding is an effective way to improve strains.

Results: In this study, 25 strains of 2-PE-producing yeasts were isolated from Chinese brewed samples. Three *Saccharomyces cerevisiae* strains with good traits in tolerance and 2-PE yield were screened out. The strain LSC-1 produces 2-PE of 3.41 g/L with an increase of 9.3% compared to the industrial strain CWY132. The strain NGER shows good tolerance to 2-PE at the concentration of 3.60 g/L in agar plate, and the thermotolerant strain S.C-1 shows growth ability at 41°C. Two rounds of protoplast fusion were performed with these three parent strains for pyramiding of traits. A fusion strain RH2-16 with high 2-PE yield and increased tolerance was obtained. Using 5 g/L L-phenylalanine as the precursor, RH2-16 produced 2-PE of 4.31 g/L through fermentation conversion and the molar conversion rate of L-Phe reached 115% in 36 h. Compared to the yield of the parental strain LSC-1 and the industrial strain CWY132, 2-PE in RH2-16 increased by 26.4% and 38.1%, respectively. Overexpression of the key enzyme genes *ARO8*, *ARO10*, and *ADH2* in the Ehrlich pathway in RH2-16 did not increase 2-PE production.

Conclusion: Diversified *S.cerevisiae* strains with different traits can be isolated from the brewing related samples. Protoplast fusion technology can effectively pyramid excellent genetic traits and breed yeast strains with significantly improved tolerance and 2-PE yield. Our research provided a breeding strategy for *S.cerevisiae* and a strain for industrial production of 2-PE. Overexpression of the key enzyme genes in 2-PE synthesis pathway does not necessarily improve increase production.

Background

2-Phenylethanol is an aromatic compound with fragrance widely used in the food and cosmetics industries in the world [1–3]. At present the global annual output of 2-PE is nearly 10,000 tons. 2-PE is mainly produced by chemical synthesis, but this method produces toxic by-products such as biphenyl [4, 5]. Another production method is the natural extraction from rose essential oil, but this method is inefficient and costly to carry out large-scale industrial production that affected by plant raw materials[6]. Recently the method of microbial conversion for natural 2-PE production has received extensive attention [1, 7–9]. The microorganisms that can produce 2-PE mainly include yeast, fungus and bacteria. Nowadays, the most researched are yeasts, such as *S.cerevisiae*[10, 11], *Pichia pastoris*[12], *Kluyveromyces lactis*[13] and *K.marxianus* [14]. It is reported that the fungus *Aspergillus oryzae* can synthesize 2-PE[15]; *Enterobacter* sp. CGMCC5087 produces 0.1 g/L 2-PE after 24 h of fermentation[7]. In the research and application of 2-PE producing yeasts, *S.cerevisiae* is the most commonly used one. A isolated heterodiploid *S.cerevisiae* strain showed a 2-PE yield of 3.58 g/L [11]. Compared with other microorganisms, *S.cerevisiae* is a model strain with clear genetic background, safe and edible and other

significant advantages. It also shows tolerance to many adverse environmental stresses and a high degree of adaptability to industrial environments. The large-scale industrial fermentation production technology of *S.cerevisiae* is relatively mature.

S.cerevisiae synthesizes 2-PE by de novo Shikimic acid pathway and Ehrlich pathway. When L-phenylalanine (L-Phe) is used as the sole nitrogen source in the medium, 2-PE is mainly synthesized through the Ehrlich pathway. L-Phe is catalyzed by aromatic amino acid aminotransferase I/II to generate phenylpyruvate, and then decarboxylated by phenylpyruvate decarboxylase to generate phenylacetaldehyde. Phenylacetaldehyde is reduced to 2-PE under the catalysis of alcohol dehydrogenase. This is currently the main process for the industrial production of 2-PE through microbial conversion[1]. Based on the Ehrlich pathway, how to increase the production of 2-PE has been extensively studied, including genetic engineering and optimization of bioconversion conditions. When three genes *ARO8*, *ARO9* and *ARO10* in Ehrlich pathway and the global transcription factor gene *ARO80* were overexpressed and acetaldehyde dehydrogenase gene *ALD3* in branch pathway was knocked out simultaneously, the 2-PE yield increased by nearly 5 times in *S.cerevisiae* strain W303-1B[16]. In addition, the production of 2-PE is also increased by enhancing the oxalate pathway. The *S.cerevisiae* strain overexpressing 3-deoxy-7-phosphoheptanonic acid synthase genes *ARO3* and *ARO4* and pyruvate kinase gene synthesized 13 mM 2-PE at a rate of 0.113 mol/mol [17]. A yeast strain *Pichia kudriavzevii* isolated from bagasse was optimized to reach a 2-PE yield of 27.2 mg/L [12].

Studies have shown that the tolerance of yeast to 2-PE is a key factor affecting yield. In addition, *S.cerevisiae* will produce more by-product ethanol when converting to produce 2-PE, and both will exert product inhibition effect simultaneously, which becomes the biggest obstacle to further increase the yield of 2-PE [18, 19]. By using continuous culture and In-Situ Product Recovery (ISPR) for product separation[2, 20–22], relieving the inhibitory effect of 2-PE on the growth of yeast, the output of 2-PE increased from 2.28 g/L to 5.14 g/L at the level of shake flask fermentation [23]. These studies indicated that the release of product inhibition or the improvement of the tolerance of *S.cerevisiae* to 2-PE is the bottleneck for the breakthrough of yield.

High concentrations of 2-PE affect many aspects of cells, including changes in morphology[24, 25], metabolic activity, gradual decrease in cell membrane permeability, intracellular ROS production and mitochondria damage[26]. The cell's tolerance to alcohols is complex and controlled by many factors, but the molecular mechanism is still unclear [27]. Using genetic engineering strategies to accurately manipulate the synthesis pathway of 2-PE might effectively increase the yield of 2-PE, but the improvement of tolerance is limited. In addition, in terms of natural 2-PE production requirements, the safety and naturalness of genetically engineered bacteria also cause a certain degree of concern. Therefore, it is necessary to explore new stable and efficient breeding strategies to improve the tolerance of *S.cerevisiae* to 2-PE and increase the yield accordingly. Yeast and its brewed food have accompanied human civilization for thousands of years. China's brewing industry has been developed since ancient times, and the diversity of yeast strains is very rich. For example, when preparing Moutai flavored wine, *S.cerevisiae* exists in an environment with multiple stress factors such as high temperature, high acid and

high ethanol concentration. During the process of making koji, the temperature can reach up to 60°C, the pH is around 2.0, and the ethanol can reach over 15%. The microbial flora that grows in this special brewing environment has unique physical and chemical characteristics. Therefore, yeasts with various excellent traits can be obtained through selective screening. For example, a thermotolerant strain obtained from multiple stress showed increased tolerance to 2-PE of 2.5 g/L, significantly higher than the nonthermotolerant strain of 1 g/L. Correspondingly, the 2-PE yield of the heat-resistant strain reached 4.5 g/L, which was 57% higher than that of the non-heat-resistant strain [28].

After obtaining multiple yeast strains with different excellent traits, it is necessary to integrate these different traits into one yeast strain. The commonly used strategy is protoplast fusion, through which a stable recombinant with parental genetic characteristics can be obtained through recombination of large fragments of genomic DNA. Therefore the purpose of improving traits can be effectively achieved without understanding the specific regulation mechanism of a trait [29–31]. In the process of protoplast fusion, parental inactivation is an effective and commonly used method for screening fusants, which means that the parents are each inactivated with different factor, and only the complementary regenerated fusants can grow. This method has been applied to improve the ethanol production performance of *S.cerevisiae*. For example, the fusion strain Q/L-F2 has both the fermentation performance and ethanol tolerance of the parents, and the tolerance of ethanol has reached 14% (v/v) [32]. In addition, for yeast strains of different species, protoplast fusion still has a significant effect. The fusion strain SP2-18 of *S.cerevisiae* and *P.pastoris* can assimilate xylose, and the ethanol yield is increased to 74.65 g/L compared to the parent's 33.12 g/L and 65.44 g/L [33]. The fusion strain R6 of *S.cerevisiae* and *Candida ethanol* showed the advantages of better fermentation activity, good taste and sensory than the parents in fermenting cider [34].

After obtaining better strains through cell fusion, gene editing technology can be used to implement more precise gene regulation and engineering strain construction to further increase the yield of 2-PE. CRISPR-Cas9 technology has been successfully applied to the genetic manipulation of *S. cerevisiae*. The delta locus in the *S.cerevisiae* genome is an often used target site for CRISPR-Cas9 integration with a higher repetition rate. DI-CRISPR (Delta Integrated CRISPR-Cas) was created and a total of 24 kb fragment was integrated into the delta sites and the number of copies was as high as 18[35].

Our previous research had shown that the *S.cerevisiae* industrial strain CWY132 produced 2-PE of 3.52 g/L under the optimized culture conditions [36, 37]. At present, the main bottleneck faced in breeding for strain to further increase 2-PE yield is to improve the tolerance. In this study, we excavated natural *S. cerevisiae* resources from China to screen strains with high yield and 2-PE tolerance, and then used protoplast fusion technology to carry out trait pyramiding to further improve 2-PE Yield. The strains obtained by cell fusion was further manipulated by using DI-CRISPR to explore the effect of overexpression of key enzymatic genes in the Ehrlich pathway on the yield of fusion strain 2-PE.

Results And Discussion

Isolation and identification of yeast strains

A total of 25 yeast strains were obtained from the samples collected in China (Table 1). *S. cerevisiae* is the main yeast among the isolated strains. Observation of yeast colonies showed that the colony morphology was generally round ridges, milky white, opaque, neat edges, and no wrinkles on the surface. The *Wickerhamomyces anomalus* colony has irregular edges and a large number of wrinkles on the surface. Through colony morphology analysis, microscopic cell observation and molecular identification, 19 strains of *S. cerevisiae*, 2 strains of *Wickerhamomyces anomalus*, 2 strains of *P. pastoris*, 1 strain of *Tolerula spores*, 1 strain of *Candida anglica* were identified. According to ITS sequence analysis, those with a similarity greater than 95% belong to the same yeast genus, and the genetic relationship of each strain was shown by drawing a phylogenetic tree. The *S. cerevisiae* CWY132 stored in this laboratory shows a close genetic relationship with the SC001, SC003 and SC009 isolated from Sichuan brewed samples. They were more closely related to *S. cerevisiae* JYC2558 in GenBank (Fig. 1). LSC-1 from CICC and SC008 isolated from Gansu homemade sourdough starters has high homology, while S.C-1 isolated from Zhejiang Quzhou rice wine is not closely related to S.C-2 from Zhejiang Shaoxing wine, the same province (Fig. 1).

Table 1
Source numbers and names of isolated yeast strains

source	Strain number	Strain name
Laboratory preservation	CWY132-NGER	<i>Saccharomyces cerevisiae</i>
	C-U	<i>Candida utilis</i>
	C-L	<i>Yarrowia lipolytica</i>
Purchased from CICC	LSC-1-LSC-2-LSC-3	<i>Saccharomyces cerevisiae</i>
Zhejiang Shaoxing Rice Wine Koji	S.C-1	<i>Saccharomyces cerevisiae</i>
Zhejiang Quzhou rice wine koji	S.C-2	<i>Saccharomyces cerevisiae</i>
Soil separation	SC001-SC002-SC003	<i>Saccharomyces cerevisiae</i>
Sichuan Liquor Brewing Koji	SC004-SC005-SC006-SC007	<i>Saccharomyces cerevisiae</i>
Gansu folk dough fermentation raw materials	SC008	<i>Saccharomyces cerevisiae</i>
	LQ001-LQ002	<i>Wickerhamomyces anomalus</i>
	LQ003	<i>Candida anglica</i>
	LQ004	<i>Torulaspora delbrueckii</i>
Downstream Fermented Products of Sichuan Soy Sauce Factory	SC009	<i>Saccharomyces cerevisiae</i>
Guangdong Biotech Company develops bacteria preparations	SC010	<i>Saccharomyces cerevisiae</i>
	LQ006	<i>Pichia pastoris</i>
Downstream fermentation products of Sichuan vinegar factory	LQ005	<i>Pichia pastoris</i>

Evaluation of the Performance of Isolated Strains

The yield of 2-PE, tolerance to 2-PE and high temperature of the 25 isolated yeast strains were tested and evaluated. The industrial *S.cerevisiae* strain CWY132 in our laboratory was used as a reference. The

results showed that the ability of different strains to convert L-Phe and synthesize 2-PE was significantly different. As shown in Table 2, there are 7 yeast strains with 2-PE yields between 1–2 g/L, and 4 yeast strains with yields below 1 g/L. The 2-PE synthesis capacity of *S.cerevisiae* is greater than other yeast species. Among them, the yields of 2-PE of the five *S.cerevisiae* strains LSC-1, SC002, SC003, SC004 and SC005 were stable above 3.4 g/L, which were at least 10% higher than the industrial strain CWY132 (Table 2). This result proved that *S.cerevisiae* has a natural advantage in the synthesis of 2-PE, compared with other unconventional yeasts. These results further confirmed that the production of 2-PE by yeasts depends on the yeast strains as described previously [13, 38].

Table 2
2-PE and ethanol production of isolated yeast strains

Strains	2-PE(g/L)	Molar conversion rate (%)	Ethanol(g/L)
CWY132	3.12 ± 0.03 ^a	85.1 ± 0.03	0.37 ± 0.11
NGER	2.02 ± 0.05	55.1 ± 0.05	0.54 ± 0.05
C-U	1.50 ± 0.01	41.0 ± 0.01	0.54 ± 0.09
C-L	0.52 ± 0.09	14.2 ± 0.09	0.48 ± 0.05
LSC-1	3.41 ± 0.05 ^b	95.0 ± 0.05	0.63 ± 0.07
LSC-2	1.39 ± 0.02	37.9 ± 0.02	0.43 ± 0.12
LSC-3	0.49 ± 0.03	13.4 ± 0.03	0.46 ± 0.05
S.C-1	1.77 ± 0.07	48.3 ± 0.07	0.64 ± 0.04
S.C-2	0	0	0
SC001	3.08 ± 0.11	84.03 ± 0.11	0.35 ± 0.01
SC002	3.41 ± 0.04 ^b	93.04 ± 0.04	0.39 ± 0.03
SC003	3.44 ± 0.09 ^b	93.86 ± 0.09	0.38 ± 0.03
SC004	3.46 ± 0.11 ^b	95.77 ± 0.11	0.62 ± 0.11
SC005	3.48 ± 0.05 ^b	96.04 ± 0.05	0.66 ± 0.08
SC006	3.32 ± 0.06	90.58 ± 0.06	0.40 ± 0.13
SC007	1.57 ± 0.08	42.84 ± 0.08	0.57 ± 0.12
SC008	3.33 ± 0.04	90.86 ± 0.04	0.37 ± 0.05
SC009	3.00 ± 0.09	81.85 ± 0.09	0.65 ± 0.06
SC010	3.13 ± 0.11	85.40 ± 0.11	0.39 ± 0.04
LQ006	1.87 ± 0.02	51.02 ± 0.02	0.87 ± 0.04
LQ001	2.62 ± 0.08	71.49 ± 0.08	0.61 ± 0.12
LQ002	2.26 ± 0.08	61.66 ± 0.08	0.58 ± 0.13
LQ003	1.62 ± 0.03	44.20 ± 0.03	0.41 ± 0.15
LQ004	0.15 ± 0.01	4.10 ± 0.01	0.58 ± 0.08
LQ005	0.23 ± 0.02	6.28 ± 0.02	0.92 ± 0.19
Values are mean ± standard error. Statistical significance was analysed through one-way ANOVA.			

With CWY132 as a control, the five high-yield *S.cerevisiae* strains LSC-1, SC002, SC003, SC004 and SC005 were tested to evaluate their growth performance. The results showed that there were no significant differences in the growth rates among these five strains, but their growth rate were all significantly higher than CWY132, and the maximum growth was also higher (Fig. 2). These results demonstrated that these strains with high yield of 2-PE also had good growth performance. As the growth rate of LSC-1 was higher than the other four strains, this strain was selected as the parental candidate strain for protoplast fusion.

Through gradient dilution and drop plate method, the strains tolerance to 2-PE was evaluated. Three strains of *S.cerevisiae* NGER, S.C-1 and LSC-1 were screened out, which showed good tolerance phenotype to 2-PE. When the concentration of 2-PE was lower than 3.00 g/L, the growth of these three yeasts and CWY132 were not significantly inhibited. But when the concentration was increased to 3.40 g/L, these three yeast strains grew significantly better than CWY132. When the 2-PE concentration in plate reached 3.60 g/L, only strain NGER showed growth at cell concentration of 10^7 /mL (Fig. 3a), indicating this strain has good traits underlying the ability to 2-PE tolerance. Our results are similar to those previously reported, that is, 4.0 g/L 2-PE can completely inhibit the growth of tested yeast cells [19].

The performance of these strains with good 2-PE tolerance at high temperature was also tested further. The results showed that at 40°C the growth of CWY132 was significantly inhibited, whereas the other three yeast strains still showed growth. When the temperature was increased to 41°C, only strain S.C-1 grew after incubation for 48h (Fig. 3b), indicating that S.C-1 resist the stress of high temperature very well.

Studies have shown that thermo-tolerance is a good indicator of other stress tolerance. For example, strains with higher temperature tolerance also have stronger resistance to oxidative and osmotic stress[39]. It was found that a thermo-resistant strain Ye9-612 had a constant growth rate at 30°C to 40°C, but the growth rate of the non-heat-resistant strain Ye9-596 (the offspring of the same parent) was severely affected[28]. The multiple stress resistance phenotype also exists in fungi and bacteria [40, 41].

In conclusion, using the 2-PE industrial strain CWY132 as a control, we obtained the LSC-1 strain with stronger 2-PE synthesis ability, the NGER strain with significantly improved tolerance to 2-PE, and the S.C-1 with better high temperature tolerance. These strains served as parent strains for subsequent cell fusion.

Optimization of Conditions for Protoplast Preparation

In protoplast preparation by enzymatic hydrolysis, the effect of enzyme concentration and treatment time on the preparation rate and regeneration rate of protoplasts was explored. We set the snailase concentration of 20, 30, 40, 50, 70, 100 and 140 mg/mL, respectively. The results showed that when treatment by snailase was 70 mg/mL for 100 min, the preparation rate was close to 75%~85%, and the

regeneration rate was close to 10 ~ 18% (Fig. 4a; Fig. 4b). Further, we improved the protoplast preparation by addition of driselase. At a concentration of 2%, the protoplast preparation rate of LSC-1 was close to 95% after 30 minutes of treatment with driselase (Fig. 4c).

Highly tolerant fusants produced by the first round of protoplast fusion

S. cerevisiae NGER and S.C-1 were used as the parental strains to construct the first round protoplast fusion through inactivating parental protoplasts to obtain the strains capable of enhancing 2-PE related characters. The inactivation rate was close to 100% at the condition of that NGER protoplasts were exposed to a water bath at 60 °C for 2 min and S.C-1 protoplasts were irradiated at a distance of 20 cm under a 25W UV lamp for 15 min (Fig. 4d, Fig. 4e). There are 20 fusants were obtained. One fusant RH1-4 showed similar growth performance to NGER but with increased 2-PE tolerance at a concentration of 3.40 g/L in drop plate (Fig. 5a). Next, we compared the growth performances of RH1-4, NGER, S.C-1 and CWY132 incubated at 40°C. The results showed that RH1-4 has the same thermal resistance as S.C-1 (Fig. 5b), demonstrating that the fusion strain RH1-4 has obtained both the traits of high temperature and 2-PE stress tolerance from parent NGER and S.C-1.

RH1-4 also showed increased 2-PE production of 2.11 g/L, increased by 11%~24% compared with two parental strains (Fig. 5c). The results showed that the stress resistance of the fusant RH1-4 was improved, thereby increasing the yield of 2-PE. The high tolerance of RH1-4 is very valuable for further improving the 2-PE synthesis ability in *S. cerevisiae*.

Construction of 2-PE high-yield fusants through the second round of protoplast fusion

S. cerevisiae RH1-4 and LSC-1 were used as parent strains for the second round of protoplast fusion to obtain a strain with high tolerance to 2-PE and increased yield. There are about 100 fusant strains were obtained and classified into grades A to C according to 2-PE tolerance, as follows: A: close to the level of LSC-1; B: between LSC-1 and RH1-4; C: close to the level of RH1-4 (Fig. 6a). Contrary to the expected result, only a small number of fusants in the C grade yielded 2-PE close to RH1-4 of 2.11 g/L, and most of them were lower than RH1-4, which was close to the yield of first round parent SC-1 of 1.67g/L (Fig. 6b). Although the tolerance of grade B fusants did not reach the expected level as RH1-4, their yields were relatively higher. Among them, one strain RH2-16 produced 2-PE of 4.05 g/L, increased by 18.8% compared with the parent LSC-1. The other selected strain RH2-26, also showed a high 2-PE yield of 3.96 g/L, an increase of 16.5%. (Fig. 6b). The results of transferring the excellent tolerance traits of RH1-4 to LSC-1 through the second round of fusion show that this method is very effective in further improving 2-PE yield. These results demonstrates that direct screening of yeast strains with multiple traits from nature and pyramid of traits through protoplast fusion is a practical method for yeast breeding. This method is more simple and efficient, compared with genome shuffling which needs mutant library construction and recursive fusion[42]. This approach would have a good application prospect in microbial breeding.

Identification of fusants and determination of their genetic stability

To test the genetic stability as well as to eliminate the effect of reverse mutation, fusants of interest were transferred at least 5 generations and the 2-PE and ethanol production capacity was detected and compared. The result demonstrated that the fusants showed good genetic stability. In order to confirm the fusants at the level of cell biology, the DNA content of the fusants and parent cells was determined. The results indicated that the DNA content of NGER, SC-1 and LSC-1 cells were 0.71, 0.67 and 1.08 μg per 10^7 cells, respectively. The DNA content of fusants RH1-4 and RH2-16 was higher, reaching 1.05 and 1.49 μg per 10^7 cells (Table 3). The DNA content of the fusants was greater than the DNA content of two parent strains, but was less than its sum, which was consistent with the characteristics of heterozygoma, confirming the preparation of the fusion strain. Fusants of interest were subcultured and the synthesis ability of 2-PE remained stable, the yield of RH2-16 reached the highest 4.31 g/L (Table 4). The comprehensive comparison of 2-PE yield, tolerance and other traits of parent and fusant strains are shown in Table 5.

Table 3
DNA content in fusants

Strains	DNA content $\mu\text{g}/10^7$
NGER	0.71 ± 0.09
S.C-1	0.67 ± 0.14
RH1-4	1.05 ± 0.11
LSC-1	1.08 ± 0.07
RH2-16	1.49 ± 0.13
RH2-26	1.56 ± 0.05

Table 4
Production of 2-PE of fusant in subculture

Strain number	generations	2-Phenylethanol(g/L)	Ethanol(v/v%)
RH1-4	1	2.47 ± 0.21	0.26 ± 0.12
	2	2.32 ± 0.09	0.31 ± 0.08
	3	2.35 ± 0.13	0.28 ± 0.01
	4	2.47 ± 0.07 ^a	0.36 ± 0.03
	5	2.47 ± 0.15	0.38 ± 0.03
LSC-1	1	3.39 ± 0.04	0.36 ± 0.17
	2	3.46 ± 0.07	0.45 ± 0.03
	3	3.41 ± 0.07	0.51 ± 0.05
	4	3.53 ± 0.03 ^b	0.65 ± 0.11
	5	3.43 ± 0.12	0.61 ± 0.13
RH2-16	1	3.98 ± 0.08	0.46 ± 0.2
	2	3.92 ± 0.09	0.61 ± 0.03
	3	4.31 ± 0.02 ^c	0.74 ± 0.06
	4	3.99 ± 0.06	0.77 ± 0.04
	5	4.01 ± 0.05	0.74 ± 0.13
RH2-26	1	3.88 ± 0.02	0.47 ± 0.01
	2	3.87 ± 0.12	0.53 ± 0.11
	3	3.83 ± 0.08	0.57 ± 0.12
	4	3.88 ± 0.01 ^d	0.55 ± 0.07
	5	3.75 ± 0.27	0.59 ± 0.02
Analysis of statistical significance was performed through one-way ANOVA.			

Table 5
Summary of phenotypes of parents and fusants

category		Strain number	Tolerance to 2-PE(g/L)	2-PE yield(g/L)	Maximum cell concentration that can grow at 40°C
The first round fusion	Parent 1	NGER	3.6	2.02	10 ⁵
	Parent 2	S.C-1	3.4	1.77	10 ⁴
	Fusants	RH1-4	3.6	2.11	10 ⁴
The second round fusion	(Parent 1)				
	Parent 2	LSC-1	3.4	3.48	10 ⁵
	Fusants	RH2-16	3.6	4.31	10 ⁵

Inokuma et al [43] performed transcriptome analysis on the diploid yeast strain with good stress tolerance and xylose utilization obtained by rearranging the spore genome, and found genes that affect the strain's tolerance to multiple stresses. It helped to understand the multiple tolerance mechanism of yeast to high temperature and acid. Therefore, the RH2-16 in this study with good traits should be an excellent candidate strain for analysis of character and research on mechanisms underlying 2-PE metabolic pathway regulation.

2-PE production of DI-CRISPR-edited strains

In order to further use genetic engineering technology to increase the 2-PE yield of fusion strain, we tested the effect of overexpression of 2-PE synthesis related genes by using DI-CRISPR with RH2-16 as the starting strain. The strains with high copy numbers of the genes in Ehrlich pathway, aromatic amino acid aminotransferase (*ARO8*), phenylpyruvate decarboxylase (*ARO10*) and alcohol dehydrogenase (*ADH2*), were successfully constructed.

A total of 7 strains expressing these genes combinations were obtained(Fig. 7), $\uparrow ARO8$, $\uparrow ARO10$, $\uparrow ADH2$, $\uparrow ARO8+\uparrow ARO10$, $\uparrow ARO8+\uparrow ADH2$, $\uparrow ARO10+\uparrow ADH2$ and $\uparrow ARO8+\uparrow ARO10+\uparrow ADH2$, and the yield of 2-PE of these strains were determined(Table 6). The 2-PE yield of the starting strain RH2-16 is 3.92 g/L. In the genetic manipulated strains, RH-ER-3 and RH-ER-1-3 showed yield of 3.91 g/L and 3.88 g/L respectively, close to the starting strain RH2-16. However, the yield of other strains decreased significantly. Among them, the RH-ER-2 strain was 3.49 g/L, a decrease of 12%. The results show that this method cannot effectively increase the production of 2-PE, and even reduces the yield. The most likely explanation for this result is that high concentration 2-PE is toxic to yeast cells. Therefore, improving the tolerance of 2-PE is the bottleneck in the breeding of *S. cerevisiae* to overcome the increase of 2-PE.

Table 6
2-PE yield of strains manipulated by DI-CRISPR

Number	Strains	Feature	2-PE yield(g/L)
	RH2-16	WT	3.92 + 0.08 ^a
1	RH-ER-1	↑ <i>ARO8</i>	3.71 + 0.12 ^c
2	RH-ER-2	↑ <i>ARO10</i>	3.49 + 0.13 ^d
3	RH-ER-3	↑ <i>ADH2</i>	3.91 + 0.08 ^{baa}
4	RH-ER-1-2	↑ <i>ARO8</i> +↑ <i>ARO10</i>	3.81 + 0.03 ^b
5	RH-ER-1-3	↑ <i>ARO8</i> +↑ <i>ADH2</i>	3.88 + 0.06 ^{ba}
6	RH-ER-2-3	↑ <i>ARO10</i> +↑ <i>ADH2</i>	3.56 + 0.11 ^{dc}
7	RH-ER-1-2-3	↑ <i>ARO8</i> +↑ <i>ARO10</i> +↑ <i>ADH2</i>	3.59 + 0.09 ^{dcdb}
Analysis of 2-PE production statistical significance was performed through one-way ANOVA.			

Conclusion

In order to obtain 2-PE high-yielding strains, a strategy of screening yeasts from natural and brewed foods and breeding through cell fusion was selected. Three excellent yeast strains, LSC-1 (good performance in both growth and 2-PE synthesis), NGER (tolerance to high content of 2-PE) and SC-1 (thermal-resistant and grows at 41°C) were screened out. After two rounds of protoplasts fusion, the traits underlying 2-PE yield enhance and tolerance have been successfully pyramided into a fusant strain RH2-26 with the highest 2-PE yield of 4.31 g/L. This strain shows comprehensive excellent traits, which is better than industrial strain CWY132 in both the growth and 2-PE production performances. This strain also shows good tolerance to 2-PE and high temperature, which is very suitable for the environmental conditions of large-scale industrial fermentation production. Moreover, we found genetic manipulation through DI-CRISPR on the Ehrlich pathway genes in the high-production-strain did not enhance 2-PE production. In short, we suggest that screening of *S.cerevisiae* strains with different traits from nature and breeding by protoplast fusion is an efficient strategy for yeast improvement.

Materials And Methods

Strains and media

S. cerevisiae strains CWY132 and NGER are stored in our laboratory. LSC-1, LSC-2, and LSC-3 were purchased from the China Center of Industrial Culture Collection (CICC). The remaining strains were isolated in this study. YPD medium (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 20 g/L agar)

was used for strain activation and isolation. Single colonies were picked into YPD liquid medium and cultured in a 30°C shaker at 200 rpm for 12 h; YPDS medium (protoplast regeneration medium): 1 mol/L sorbitol is added to YPD medium; 2-PE fermentation medium: 30 g/L glucose, 0.5 g/L MgSO_4 , 5 g/L K_2PO_4 , 1.5 g/L yeast extract, add 5 g/L L-Phe as precursor. All media were sterilized at 121°C for 15 minutes before use.

0.2 mol/L PB solution: 0.2 mol/L $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 87.7 mL and 0.2 mol/L $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 12.3 mL, mix well, pH 6.0; PBS solution: mix 50 mL of 0.2 mol/L PB solution and 450 mL of 1 mol/L sorbitol solution, pH 6.0; Solutions were sterilized at 121°C for 15 min. Preparation of cell-wall lysis pretreatment agent: take 0.2 mL β -mercaptoethanol and 2.233 g EDTA.2Na, dissolve in PBS solution, and dilute to 100 mL. 0.22 μm filter was used for bacteria remove. All reagents were purchased from Sangon Biotech Shanghai, China.

Isolation and identification of yeast strains

Samples were collected from breweries including Zhejiang Shaoxing wine, Zhejiang Quzhou rice wine, and Sichuan white wine, commercial fermentated broth, homemade sourdough starters in Gansu province, and Sichuan soy sauce and vinegar factory preparation residues.

Yeast strains were isolated as follows. 1.0 g of the sample was dissolved in 9 mL of sterilized water and mixed well. After gradient dilution, sample was spread on YPD medium plate and incubated at 30°C for 48 h. The yeast colonies characterized by spherical, milky white and opaque were further streaked to obtain single colony and confirmed by microscopic observation. The isolated strains were further subjected to molecular identification.

The genomic DNA of the yeast strain was extracted with Rapid Yeast Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China). The full sequences of ITS were amplified with primer pair Seq-F (5' - CGCCAGGGTTTTCCCAGTCACGAC-3') and Seq-R (5' - GAGCGGATAACAATTTCACACAGC-3'). PCR reaction program: pre-denaturation at 94°C for 5 min; denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, 30 cycles; final extension at 72°C for 5 min. PCR products were purified for sequencing after agarose gel electrophoresis. The full ITS sequences obtained by PCR were Blasted against GenBank nucleic acid database for similarity analysis. The species of the strains were determined according to whether the sequences have high similarity, and MEGA7.0 software was used to perform phylogenetic tree analysis.

Determination of growth curve

The growth curve of all strains was made by measuring the cell density with spectrophotometer. The activated single colony was picked into YPD liquid medium and cultured in a shaker at 30°C at 200 rpm for 12 h. Then the OD_{600} of the cultured yeast solution was measured and adjusted to a density of 1×10^7 cells/mL. The cell suspension was inoculated into the fresh YPD medium at a ratio of 10% (v/v), and the

OD₆₀₀ of the culture medium was measured every 2 h. Using the OD₆₀₀ as ordinate and culture time as abscissa, growth curves were drawn.

Determination of tolerance to 2-PE and high temperature

The tolerance of 2-PE was analyzed by dilution gradient plate method. The yeast concentration was adjusted to 10^7 cells/mL, and then diluted with sterile water to 10^6 , 10^5 , 10^4 , 10^3 cells/mL. 5 μ L of the serious diluted yeast solution was taken and dropped on the YPD plate with series concentrations of 2-PE (2.6, 2.8, 3.0, 3.2, 3.4, 3.6 g/L). After being placed to dry, put it in a 30°C incubator for 2–3 days. In the high temperature tolerance test, the plates were cultured at 37°C, 40°C and 41°C for 3 days to observe the growth of the strains.

Protoplast preparation, fusion, regeneration

The yeasts were picked into 5 mL fresh YPD liquid medium, and cultured in a 30°C shaker at 200 rpm for 12–14 h until logarithmic growth phase. 4 mL of the yeast suspension was taken and centrifuged at 6000 rpm for 5 min. The pellet was washed twice with PB buffer and 4 mL of pretreatment agent was added and shaken slowly at 60 rpm for 30 min in a shaker at 30°C. Cells were collected by centrifugation at 6000 rpm for 5 min and mixed with 4 mL enzyme cocktail consisting of 7% snailase(Sangon Biotech Shanghai, China) and 2% driselase(Sigma-Aldrich,USA) in PBS solution. The enzymatic hydrolysis was carried out for 2 h at 30°C with shaking at 60 rpm. Then the digested cell solution was centrifuged at 3000 rpm for 10 min to remove the enzyme solution. The protoplast was washed twice and resuspended in the PBS. The methods for determining the protoplast formation rate and regeneration rate are as follows. Take out the yeast suspension before enzymolysis and dilute it with sterile water, then spread it on the YPD plate, incubate at 30°C for 2 d, and record the number of colonies (A); Take the yeast suspension after lytic enzyme treatment, dilute with sterile water and spread it on the YPD plate, incubate at 30°C for 2 d, and record the number of unbroken colonies (B); Take the obtained protoplast suspension, spread it on the YPDS plate, incubate at 30°C for 2 d, and record the number of regenerated colonies (C); Calculate the formation rate and regeneration rate of protoplasts based on the values of A, B, and C. Protoplast formation rate=(A-B)/A $\times$ 100%. Regeneration rate of protoplast= (C-B)/(A-B) $\times$ 100%.

The method of parental inactivation is used for cell fusion and selection. Reliable fusants could be obtained only when the inactivation efficiency reached 95% or more. We observed and counted the prepared protoplast suspension on a hemocytometer, diluted it to 10^5 with PBS, add 4 mL of the protoplast suspension to a sterile plate. The plate suspension was placed under a 25W preheated ultraviolet lamp and irradiated at 20 cm for different time, then diluted with PBS solution and coated on the YPDS plate. The protoplast suspension without ultraviolet treatment was used as a control. The plates were incubated statically at 30°C and calculated the survival rate of protoplasts to determine the best UV mutagenesis time. Similarly, the protoplast suspension with a concentration of 10^5 was treated in a 60°C water bath for different time. The survival rate of protoplasts was calculated with no heat treatment as a control.

The concentration of the two parent protoplast suspensions was adjusted to 10^7 /mL, 0.5 mL of each was mixed in a 1.5 mL EP tube, centrifuged at 3000 rpm for 10 minutes, the supernatant was discarded, and 1 mL of 40% PEG6000 was added to suspend the suspension. Incubate at 30°C for 25 minutes. Protoplast suspensions were centrifuged at 3000 rpm for 10 min and collected cells were diluted with PBS solution to 10^5 /mL. 100 μ L was taken and spread on YPDS plate, and incubated at 30°C for 3 d. Through the screening method of parent inactivation, the colonies grown on the YPDS plate were preliminarily determined as fusants.

Fusants identification

Fusants were also characterized by measuring the genomic DNA content. It was believed that the fusion cell's genomic DNA content should be greater than that of any parent, but should be close to or less than the sum of the DNA content of the two parents. The following formula was used to quantify the DNA concentration: where C was the DNA concentration (g/mL), K_i was the dilution factor, and E was the DNA concentration where 1 UV absorption unit was equal to 50 μ g/mL.

$$C = OD_{260/280} \times K_i \times E / 1000$$

Overexpression of *ARO8*, *ARO10* and *ADH2* genes in the Ehrlich pathway by using DI-CRISPR

The ORF of key enzyme genes *ARO8*, *ARO10* and *ADH2* in the Ehrlich pathway was driven by the PGK promoter and terminated with CYC1 terminator to form a gene expression cassette, respectively. The target genes were designed to be integrated on the delta site of the *S.cerevisiae* RH2-16 genome through DI-CRISPR method as described by reference[35]. The three genes are permuted and combined to obtain 7 gene editing strains: $\uparrow ARO8$, $\uparrow ARO10$, $\uparrow ADH2$, $\uparrow ARO8 + \uparrow ARO10$, $\uparrow ARO8 + \uparrow ADH2$, $\uparrow ARO10 + \uparrow ADH2$, $\uparrow ARO8 + \uparrow ARO10 + \uparrow ADH2$. All the constructions were transformed into *S.cerevisiae* fusant strain with Li/Ac mediated method [38]. The transformants were molecular characterized by PCR with constructs-specific primer pairs(the forward primer is inside the ORF of target gene, the reverse primer is at the delta site of *S.cerevisiae* genome).

2-PE fermentation production and yield determination

A single colony was inoculated into 50 mL YPD liquid medium and cultured at 30°C with shaking at 200 rpm for 12–14 h to the logarithmic growth phase. The yeast cell concentration was adjusted to 10^7 cells/mL and the resulting cell suspension was added to 50 mL fermentation medium containing 5 g/L L-Phe at the inoculation ratio of 10%(v/v). The fermentation was carried out for 36 hours by incubation at 30°C, 200 rpm. Fermentation broth samples were taken to determine the concentration of 2-PE. The experiment was conducted three times independently, with at least three repetitions for each treatment.

2-PE assay by gas chromatographic analysis

2-PE production was assayed by gas chromatography(GC). Sample pretreatment: Fermentation broth was centrifuged and 200 uL of supernatant was taken as assay sample. Add 200 uL of internal standard solution (methyl isobutyl methanol 1 g/L aqueous solution) into sample, add 500 uL of ethyl acetate, shake and mix, centrifuge at 5000 rpm/min for 1 min, take the upper organic phase for GS measurement. Standard curve preparation: prepare 0.5, 1.0, 5.0, 10.0 g/L of 2-PE aqueous solution of standard concentration. GC: Shimadzu GC2014; FID detector; column Rtx-1 (30m*0.25mm*0.25um, Restek); Carrier gas: nitrogen inlet, 240°C; split ratio: 49.0; column flow: 1.45 mL/min; Purge flow: 3 mL/min. Detector: 250°C oven: 80°C for 1 min, 20°C/min to 200°C.

Declarations

- Ethics approval and consent to participate

Not applicable

- Consent for publication

Informed consent for publication was obtained from all participants.

- Availability of data and materials

All data generated or analyzed during this study are included in this article.

- Competing interests

No competing interests.

- Funding

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- Authors' contributions

Lucheng Lin, Carried out the protoplast fusion and screening of strains. Zhiwei Xu, Constructed the CRISPR/Cas9 system, carried out the gene editing. Weixia Wang, Protoplasts preparation. Kun Wang, Conducted 2-PE analysis. Tingheng Zhu, Guidance and design of the entire research project. All authors commented on the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

The phylogenetic tree of yeast strains was drawn using MEGA7.0 software. An unrooted phylogenetic tree was constructed by the neighbour-joining method, together with 1000 bootstrap replicates based on the ITS sequence alignments. GenBank accession numbers: Wickerhamomyces anomalous AZ80, MT652638; Candida anglica CBS 4262, NR163770; Pichia fermentans EE9B, MT645416; Torulaspora delbrueckii K1, KU308410; S.cerevisiae AUMC 13515, MH534922; S. cerevisiae JYC2558, MK044039; S.cerevisiae DQY7, KC621078.

Figure 2

Growth curve of yeast strains. Yeast cell growth was monitored by the increase in the optical density of the cultures at 600 nm (OD₆₀₀). The data were calculated from three biological replicates. The average deviation is between 5% and 10%.

Figure 3

Growth phenotypes of LSC-1, NGER and S.C-1 strains under different stresses. a Gradient diluted yeast cells were dropped on YPD agar supplemented with gradient concentration of 2-PE and incubate at 30°C for 36 h; b Gradient diluted yeast cells were dropped on YPD agar and incubated at 37°C, 40°C, and 41°C for 48 h. CWY132 was used as a control.

Figure 4

The optimization of conditions for protoplast preparation and fusion. a The effects of different concentrations of snailase on protoplast preparation and regeneration of LSC-1; b Effect of 7% snailase treatment duration on the preparation and regeneration of LSC-1 protoplasts; c Effect of 2% driselase treatment duration on the preparation and regeneration of LSC-1 protoplasts; d The lethality rate of NGER after water bath heat treatment at 60 °C; e The lethality rate of S.C-1 after UV treatment.

between 5% and 10%. The asterisk indicate that compared with S.C-1, the 2-PE yield of each strain is significantly different at the level of $p < 0.05$.

Figure 6

Screening of 2-PE high-yielding fusants in the second round of protoplast fusion. a Fusants strains and RH1-4 were tested under heat stress at 40°C. b The 2-PE production of fusants strains were measured, and strains with different 2-PE yields were classified into grades A to C according to 2-PE tolerance.

Figure 7

Molecular characterization of strains integrated the Aro8, Aro10 or ADH2 and their combinations at delta sites by PCR with constructs-specific primer pairs. WT, wild type. 1-7 represents 7 strains corresponding to the strains in table 6 respectively.