Construction of CRISPR/CAS9 system for industrial *Saccharomyces cerevisiae* strain and genetic manipulation effect on 2-phenylethanol pathway

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Research

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Abstract

Background

The market demand for natural 2-phenylethanol (2-PE) continues to increase. *Saccharomyces cerevisiae* can synthesize 2-PE through the Ehrlich pathway. There are few studies on the improvement of the diploid industrial strains of *S. cerevisiae* by gene editing technology. There is no report on the comparison of genetic manipulation effect among *S. cerevisiae* strains with different 2-PE yield background, and the study on knockout of 2-PE downstream product synthesis gene and its effect on the yield of 2-PE have not been found.

Results

The CRISPR/CAS9 system with high efficiency for diploid *S. cerevisiae* CWY132 strain for industrial production of 2-PE was constructed. When the length of the homology arm of donor DNA is increased from 60bp to 500bp, the efficiency of gene editing increased from 0–100%. Using CRISPR/CAS9 technology, the branched acetaldehyde dehydrogenase genes *ALD2* and *ALD3* and the terminal acetyltransferase gene *ATF1* in the Ehrlich pathway of *S. cerevisiae* strains with different 2-PE yields were knocked out. The results showed that in the high-yielding CWY-132 strain, the 2-PE yield decreased from 3.50 g/L to 1.65 g/L when double *ALD2* and *ALD3* were knocked out, a decrease of 52.8%. When *ATF1* was knocked out, the yield of 2-PE decreased to 0.83 g/L, a decrease of 76.2%; In the low-yielding strain PK-2C, the yield of 2-PE increased from 0.21 g/L to 1.20 g/L when *ALD2* was knocked out, an increase of 471%. When *ATF1* was knocked out, the yield of 2-PE increased to 0.45g/L, an increase of 114%. The results show that the same genetic manipulation strategy for strains with different 2-PE yields backgrounds produces significantly different or even opposite effects. In addition, we found that the insufficient supply of NADH in cells can significantly affect the production of 2-PE, and the tolerance of cells to 2-PE is also a key factor that limits the further increase of 2-PE production in high-yielding strain.

Conclusions

This study shows that the length of the Donor DNA homology arm is a key factor affecting the efficiency of CRISPR/CAS9 gene editing in industrial diploid *S. cerevisiae* strains. Our result also shows that it is not feasible to increase the 2-PE production in high-yielding strains by blocking the branch pathway in the Ehrlich pathway. Breakthrough in the 2-PE yield of the high-yielding strains requires improved strains' tolerance to 2-PE and increase the cellular NADH level.

Background

2-Phenylethanol (2-PE) is an aromatic alcohol with the fragrance of roses, which is naturally present in roses and fermented foods[1]. 2-PE contains a unique fragrance and is highly sought after by people. It is

widely added in food, tobacco, cosmetics and daily chemical products[2–4]. 2-PE can also be used as a substrate for the production of its derivatives, such as another valuable flavor compound, ethyl phenylacetate[5]. At present, the methods for producing 2-PE in industry are mainly chemical synthesis and natural methods[6]. The chemical method not only has toxic substrates, but also contains by-products that are difficult to remove, so it is difficult to be widely used. The food industry is particularly prominent, which makes people more inclined to use natural 2-PE[7]. The natural production of 2-PE can be directly extracted from plants or transformed with microorganisms[8]. The price of chemically synthesized 2-PE in the market is about 5\$/kg, while the natural one is up to 1000\$/kg[9, 10]. Due to the insufficient supply of raw materials and low production efficiency, the direct extraction method cannot meet industrial production [11]. The production of natural 2-PE is mainly focused on microbial transformation.

Among the microorganisms currently known to produce 2-PE, they mainly include *S. cerevisiae*, *Escherichia coli*, *Meyerozyma guilliermondii*, *Pichia pastoris*, *Candida glycerinogenes* and *Yarrowia lipolytica* etc.[12]. Among them, *S. cerevisiae* is an internationally recognized safety model microorganism for both industrial processes and scientific research[13], it is the most widely studied strain for 2-PE production [4, 14, 15].

The metabolic pathway of 2-PE production in *S. cerevisiae* has been studied very clearly. One is the shikimic acid pathway of de novo synthesis of 2-PE from glucose[16], the second is the Ehrlich pathway through biotransformation of L-phenylalanine to 2-PE[12, 17]. The synthetic 2-PE of the former is much lower than that by the Ehrlich pathway[18, 19]. In the Ehrlich pathway, first L-Phe enters the cell under the action of amino acid permease (such as Gap1, Agp1 and Tat1)[20–22], and then it is transformed into 2-PE (Fig. 1): L-Phe is converted into phenylpyruvate under the action of transaminase, which are aromatic transaminase I (Aro8) and aromatic transaminase II (Aro9), which are encoded by the genes *ARO8* and *ARO9*; Phenylpyruvate is catalyzed by phenylpyruvate decarboxylase to remove a carboxyl group to form phenylacetaldehyde[5]. There may be 5 genes encoding phenylpyruvate decarboxylase in *S. cerevisiae*, including *PDC1*, *PDC5*, *PDC6*, *ARO10* and *THI3*[23, 24], when L-Phe is used as the sole nitrogen source, it is mainly expressed by *ARO10*[25]; Finally, phenylacetaldehyde produces 2-PE under the action of alcohol dehydrogenase (Adh1, Adh2, Adh3, Adh4 and Adh5) [5, 19, 26]. Overexpression of *ARO8* and *ARO10* in *S. cerevisiae* S288C obtained 2-PE yield of 2.61 g/L after 60 h fed-batch fermentation, which was 36.8% higher than that of the wild type[13].

There are precursors and energy shunts caused by two branch pathways during the synthesis of 2-PE. One is phenylacetaldehyde, the precursor of 2-PE, can be oxidized to phenylacetic acid under the action of acetaldehyde dehydrogenase[1]; The second is that the product 2-PE can be further converted into 2-phenylacetoethyl ester under the action of acetyltransferase[12, 27] (Fig. 1). In the oxidation process of phenylacetaldehyde, *ALD3* and *ALD2* genes play a key role[1, 28], and acetyltransferase is encoded by *ATF1*. Bosu Kim et al. Knocked out *ALD3* and *ALD2* of *S. cerevisiae* W303, and when 83 mg/L L-Phe was used as substrate, the final 2-PE yield increased by about 35% compared with that of wild type[1]. Overexpression of *AAP9*, *ARO9* and *ARO10* and knockout *ALD2* and *ALD3* in *C. glycerinogenes* resulted in

an increase of 2-PE production by 38.9% to 5g/L[29]. However, the blocking of the consumption pathway of 2-PE catalyzed by *ATF1* has not been reported yet.

CRISPR/CAS9 gene editing technology has been widely used in genetic engineering of microorganisms including *S.cerevisiae* for advantages of high efficiency and the ability to edit multiple genes at the same time[30, 31]. Compared with haploid laboratory yeast strains, diploid industrial *S.cerevisiae* strains are more robust and characterised by high levels of genomic diversity, which makes genetic engineering difficult[30]. Although CRISPR/CAS9 gene editing technology has been applied to laboratory yeast[32, 33] and industrial yeast[30], the editing efficiency in industrial yeast is still at a low level. Recent studies on 2-PE production by *S. cerevisiae*, YS58 strain[22], S288c strain[13] and W303 strain[1] are mostly haploid experimental strains, and there are few studies on diploid strains for industrial production. Therefore, it is necessary to explore to improve the gene editing efficiency of industrial yeast in order to carry out genetic engineering of strains.

2-PE can increase the fluidity of cell membranes, reduce the uptake of glucose and amino acids, and inhibit the growth of *S. cerevisiae* through induction of insufficient respiration[34], and its toxicity to cells becomes the main bottleneck to further increase its production. Generally, diploid strains of *S.cerevisiae* used for industrial production are more stress-resistant than laboratory strains and have excellent fermentation performance[35, 36]. Therefore, on the basis of industrial production strains with a certain 2-PE tolerance, the use of gene editing technology for precise modification of metabolic pathways is an effective strategy to further increase yield. In addition, the level of NADH in the cell reflects the redox level in the cell, which plays an important role in the growth and metabolism of the cell[37, 38], so the regeneration of the reducing power in the cell has also become an important method for regulating cell metabolism.

In the previous research of our group, we obtained 2-PE-producing diploid *S. cerevisiae* CWY-132 and realized industrial production. Through the optimization of medium composition, culture conditions and fermentation conversion process, the 2-PE level reached 3.98 g/L in shake flask[39] and 4.1 g/L in the small-scale fermentation tank[40]. This study established and optimized the CRISPR/CAS9 gene editing system of CWY-132 with 100% efficiency when manipulate Ehrlich pathway genes. This system was used for comparative study of the performances of genes manipulations related in 2-PE pathway in different production strains, and the effect of *ATF1* gene knockout was reported for the first time. In addition, the effects of cell tolerance to 2-PE and the supply of NADH on 2-PE production were also discussed.

Results And Discussion

Construction of CRISPR/CAS9 system in industrial diploid *S.cerevisiae*

The CRISPR/CAS9 gene editing system of *S.cerevisiae* has been successfully established and applied, but the editing efficiency in industrial diploid strains is still not high. Stovicek et al. constructed the CRISPR/CAS9 system in industrial yeast, but the editing efficiency was only 65%~78%[30]. Therefore, it is necessary to greatly improve the gene editing efficiency of industrial *S.cerevisiae* strains.

The CRISPR/CAS9 system mainly includes three parts: sgRNA, Cas9 protein, and Donor DNA. The PAM site on the target gene is used as a guide mark. The sgRNA sequence can bind to the first 20bp of the PAM site, and then guide the Cas9 protein to cut this site. Then homologous recombination repair is performed through Donor DNA to achieve targeted editing of the target gene. In this paper, a high-efficiency CRISPR/CAS9 system was constructed in the industrial strain CWY132 of 2-PE, and it was found that the construction method of gRNA plasmid and the length of the Donor DNA homology arm affect the editing efficiency. Two strategies were used to construct gRNA plasmids(Fig. 2a). One was to amplify the gRNA plasmid backbone into three fragments containing homologous arms to make it homologous recombined into a new plasmid(program 1). Second, the complete gRNA plasmid was constructed in vitro by DPN I-mediated reverse PCR(program 2). The results show that the gRNA plasmid construction method shown in program 2 can greatly increase the transformation efficiency. The number of transformants is increased from zero to about hundreds in each plate. When constructing Donor DNA, *ATF1* and *ALD3* were used as the target genes to be knocked out, and homology arms of 60 bp, 100 bp, 500bp, and 1000 bp were set respectively. The results show that when the length of the homology arm was 60 bp, the target gene editing efficiency is 0, and when the length reached 500bp, the editing efficiency can reach 100% (Fig. 2b). In addition, the effect of different PAM sites on the system efficiency was also studied. When the homology arm length of Donor DNA is 500 bp, its knockout rate of *ATF1* reached 100% at all the selected PAM sites.

In this research, we successfully constructed the CRISPR/CAS9 system in industrial diploid *S.cerevisiae*. The transformation efficiency was improved by optimized plasmid construction method, and the gene editing efficiency was significantly improved to and reached to 100% by increasing the length of the Donor DNA homology arm, and we also found that the PAM site does not affect the editing efficiency. This system with high gene editing efficiency could provide technical support for genetic manipulation in the industrial *S.cerevisiae* strain.

The effect of knocking out ALD2, ALD3, ATF1 on the 2-PE and ethanol production of different strains

2-PE synthesis was shunted to branch pathways in *S.cerevisiae* (Fig. 1). The 2-PE-producing industrial diploid *S.cerevisiae* strain CWY-132 and the laboratory haploid strain PK2-C were used to analyze the function of the branch pathway acetaldehyde dehydrogenase gene *ALD2* and *ALD3* and acetyltransferase gene *ATF1* of the 2-PE metabolic pathway. By using CRISPR/CAS9 technology, the above genes were single knocked out, double knocked out and overexpressed to see their changes in 2-PE and ethanol production. The knockout efficiency of these genes has reached 100%, further verifying the high efficiency of this gene editing system we established.

The 2-PE and ethanol yields of wild-type and gene-edited mutant strains were tested to study the functions and interaction effects of different genes. 5g/L L-Phe was used as a substrate for fermentation culture, and the supernatant was taken after 36 h of continuous fermentation to detect its 2-PE and ethanol content. The results showed that when inhibiting the branching pathway of acetaldehyde dehydrogenase, in the CWY-132 strain, the 2-PE yields of *ALD2Δ* and *ALD3Δ* strains were 3.02 g/L and

2.93 g/L, respectively, which were compared with 3.5 g/L of the wild type, it dropped by 14%. The 2-PE yield of the *ALD2ΔALD3Δ* strain was 1.65 g/L, which was about 52.8% lower than that of the wild type (Fig. 3a); In the haploid laboratory strain PK2-C, the 2-PE yields of *ALD2Δ* and *ALD3Δ* strains were 1.20g/L and 0.34g/L, respectively, which increased by about 471% and 62% respectively compared with the wild type (Fig. 3b). When inhibiting the downstream acetyltransferase gene *ATF1* of 2-PE synthesis, the 2-PE yields of *ATF1Δ* and *ATF1ΔALD3Δ* of CWY-132 strain were 0.83g/L and 0.85g/L, respectively, compared with the wild type, it is reduced by about 76% (Fig. 3a). The 2-PE yield of the *ATF1Δ* strain of PK2-C was 0.45 g/L, which was 114% higher than the wild-type yield of 0.21 g/L (Fig. 3b).

2-PE is toxic to *S.cerevisiae* cells, and the combined action of ethanol can enhance the inhibition effect on the growth[34]. The ethanol production of each strain was also tested and found that in CWY-132, the *ALD2Δ* and *ALD3Δ* strains were close to the wild type, and the *ALD2ΔALD3Δ* strain increased by about 45% compared with the wild type. In *ATF1Δ* and *ATF1ΔALD3Δ* strains, the ethanol production increased by 128% and 146% ,respectively, compared with the wild-type strain (Fig. 3a); Among the PK2-C strains, the ethanol production in *ALD2Δ* and *ALD3Δ* strains are close to the wild type, and the *ATF1Δ* strain is about 30% higher than the wild type (Fig. 3b). In the CWY-132 strains with overexpression of *ALD3* or *ATF1*, the production of 2-PE and ethanol decreased slightly (Fig. 3c).

These results demonstrate that the blocking of branch pathways in the 2-PE high-yielding industrial strain CWY-132 failed to increase the 2-PE production, on the contrary, the yield decreased. *ATF1Δ* and the double mutant strains *ALD2ΔALD3Δ* and *ATF1ΔALD3Δ* decreased more significantly. To investigate these unexpected results, the low-yielding strain PK2-C was used for further study. The results showed that the yield of 2-PE increased when these genes was knocked out, which was consistent with the results of reported studies. For example, Bosu Kim et al. found that the 2-PE yield of haploid laboratory *S.cerevisiae* strain W303 that knocked out *ALD3* and *ALD2* was 35% higher than that of wild type[1]. These results prove that in the regulation of 2-PE biosynthesis, by blocking the branch pathway and downstream product synthase, the effect is different in strains with different yield backgrounds.

The knock-out effect on the synthase acetyltransferase of the downstream substance of 2-PE is more obvious. In the CWY-132 strain of *ATF1* gene deletion, its colony on the plate is smaller and grow more slowly in liquid media when compared with the wild-type strain (Fig. 4a 4b), and the 2-PE production is greatly reduced, indicating that *ATF1* involved in yeast growth and plays a crucial role in regulation of 2-PE catalytic conversion. Studies by other groups have shown that the acetyltransferase encoded by *S.cerevisiae* *ATF1* is a key enzyme in acetate synthesis[41],and deletion or overexpression of *ATF1* will significantly affect the production of alcohols and acetates[42, 43].

The synthesis level of alcohols in *S.cerevisiae* is related, and the yeast performs coordinated regulation. The results of this study show that in different strains of relatively high-yield of 2-PE, the increase or decrease of 2-PE production is often accompanied by corresponding changes in ethanol, and the trend is generally opposite. For example, in the CWY-132 strain, the 2-PE dropped significantly after the *ATF1* gene was knocked out, while the ethanol increased significantly. The *ALD2ΔALD3Δ* strain also has a similar

situation. However, this phenomenon did not occur in the low-yield haploid strain PK-2C, indicating that yeast cells have global regulation of the total alcohols stress substances in the cell to reduce cytotoxicity.

The effect of initial L-Phe concentration, correlation between 2-PE and ethanol, and NADH on 2-PE production

With CWY-132 as the starting strain, the initial addition amount of L-Phe was set at 0.1g/L, 1g/L, 3g/L and 5g/L. The 2-PE production of different engineered yeast strains under L-Phe concentrations was studied. The result showed that the 2-PE production of *ALD2Δ*, *ALD3Δ* and *ALD2ΔALD3Δ* strains increased by 20%, 23% and 29% respectively under the substrate of 1g/L L-Phe (Fig. 5a). When L-Phe concentration was increased to 3g/L, the 2-PE decreased compared with the wild type (Fig. 5a). In particular, the *ALD2ΔALD3Δ* strain reduced the 2-PE production by nearly 50% under the 5g/L L-Phe substrate (Fig. 5a). However, the 2-PE production of *ATF1Δ* and *ATF1ΔALD3Δ* strains at the addition of 1g/L, 3g/L and 5g/L L-Phe substrate has been at a very low level (Fig. 5a).

It can be seen from the metabolic pathway (Fig. 1) that there is a certain connection between the ethanol and the 2-PE metabolic pathway. Ethanol can be converted into ethyl acetate catalyzed by acetyltransferase, and acetaldehyde can be converted into acetic acid by acetaldehyde dehydrogenase. Therefore, when these two enzymes are knocked out, the ethanol content is greatly increased. The results of L-Phe addition study further verified that the production of 2-PE and ethanol are always in a negative correlation (Fig. 5b 5c). On the basis of the *ALD2ΔALD3Δ* strain, the pyruvate decarboxylase PDC1 in the ethanol metabolism pathway was further knocked out, and the result found that ethanol production decreased significantly, while 2-PE production increased by 50% (Fig. 3a). These results further indicate that 2-PE is in competition with ethanol synthesis, and the increase in ethanol production will lead to a decrease in 2-PE production. The initial 2-PE yield of strain CWY-132 reached 3.4 g/L, and the tolerance of this strain to 2-PE on the plate was also 3.4 g/L (Fig. 6a). The dual toxicity of ethanol and 2-PE to the strain further damages the viability of the strain, which in turn affects the yield of 2-PE.

Study has shown that glutathione (GSH) can enhance the tolerance of *Candida glycerinogenes* to 2-PE[44]. In order to verify that increasing the 2-PE tolerance of *S. cerevisiae* can increase the yield, the addition of 0.1 mM GSH to the culture media increased the tolerance of CWY-132 to 2-PE from 3.4 g/L to 3.6 g/L (Fig. 6a). Adding 0.1 mM GSH during the fermentation process can increase the production of 2-PE by about 10% in the WT, *ALD2Δ*, and *ALD3Δ* of CWY-132 (Fig. 6b). The result suggests that the toxic effect of 2-PE is a key limiting factor affecting the further increase of 2-PE production.

The growth of CWY-132 mutants and wild type was compared. The growth curve measurement and YPD plate streaking culture results showed that the growth of the *ATF1Δ* strain and the *ALD3ΔATF1Δ* strain was more weakened compared with the wild type, and the biomass was significantly reduced. The growth of *ALD2Δ* or *ALD3Δ* strain showed no significant changes, but the biomass of *ALD2ΔALD3Δ* strain decreased (Fig. 4a 4b). The growth and biomass of PK2-C mutant strains did not change (Fig. 4c 4d). These results indicate that blocking the branch pathways in the 2-PE synthesis pathway, especially the

terminal shunt pathway, has an inhibitory effect on the growth of industrial diploid *S. cerevisiae* with high production of 2-PE.

The redox level reflects some metabolic activities related to cell growth and biosynthesis, and controls cell metabolism. The levels of NAD^+ and NADH are key indicators of redox status[38]. When the production of ethanol increases, it can oxidize NADH in the cell and break the balance of NADH/ NAD^+ in the cell [37, 45], resulting in slow cell growth and further affecting the production of 2-PE. Measurement of the NADH content in different strains constructed, we found that the NADH content of the *ALD2 Δ ALD3 Δ* , *ATF1 Δ* and *ATF1 Δ ALD3 Δ* strains all decreased by 10%-15% compared with the wild type (Fig. 6c). After 2g/L NAD^+ was added to the fermentation process, the 2-PE yield increased by 10% (Fig. 6b). Therefore we speculated that the metabolism of ethanol and 2-PE in *S. cerevisiae* strains have something in common. The blocking of the 2-PE synthesis branch pathway leads to an increase in ethanol production, which in turn leads to an imbalance between NADH/ NAD^+ in the cell. Dysregulation causes weakened cell growth, which in turn reduces 2-PE production.

These results indicate that strains with different yield backgrounds should be optimized for the amount of substrate L-Phe. For strains that require a higher concentration of L-Phe, the 2-PE yield cannot be increased or even decreased after knocking out the shunted genes. The results also indicate that ethanol and 2-PE exert dual toxicity to *S. cerevisiae*, which suggests that the overcome of the low tolerance of strain to 2-PE is a key problem. Construction of tolerance improved strains could be an effective strategy in further increasing the production of 2-PE. In addition, redox state should also be considered to improve the supply of NAD^+ .

Conclusions

In this study, a CRISPR/CAS9 gene editing system was constructed in the diploid *S. cerevisiae* that produces 2-PE, and the editing efficiency reached 100% after optimization. When using this system to knock out the branch pathway of the Ehrlich pathway related genes of the 2-PE synthesis of different strains, it is found that the effect is quite different in different strains. The production of 2-PE decreased in the industrial strains, while increased in the low-yielding haploid laboratory strains due to the blocking of the branching pathway. These results suggest that the future application of gene editing strategies to improve the production of *S. cerevisiae* 2-PE should fully consider the strain background. In addition, the dual toxicity of 2-PE and ethanol to *S. cerevisiae* is an important obstacle to the continued synthesis of 2-PE. Enhancing the strain's tolerance to 2-PE is effective strategy in future for 2-PE strain improvement and production. Moreover, it is also necessary to comprehensively consider the supply of the cell NADH/ NAD^+ in catalytic conversion for the 2-PE synthesis. This study deepened the understanding of the regulation of 2-PE synthesis by *S. cerevisiae*, and provided strategies and references for further development of new strategies for high-yield strain breeding.

Materials And Methods

Yeast strains and culture conditions

The *S. cerevisiae* strains used in this study are shown in Table 1. *S. cerevisiae* CWY-132 was a industrial strain. Yeast strains were cultured at a constant temperature of 30°C in a complete medium YPD (1% yeast extract, 2% peptone, 2% glucose, if making a solid medium, add 2% agar powder). The overnight cultured seed culture solution was inoculated into the fermentation medium (5g/L L-phenylalanine ,30g/L glucose, 0.5g/L magnesium sulfate, 5g/L potassium dihydrogen phosphate, 1.5g/L yeast extract) at cell densities of 10^7 cells/mL, and the filling amount was 50mL/250mL, and the fermentation was continued for 36 h at 30°C and 200 rpm.

E. coli cells were grown in Luria–Bertani medium (0.5% yeast extract, 1% tryptone and 1% NaCl) supplemented with 100 µg/mL ampicillin at 37°C to select positive transformants.

2-PE tolerance determination

The tolerance of 2-PE was analyzed by dilution gradient plate method. Dilute the exponentially growing *S.cerevisiae* cells to 10^7 cells/ml, and then spot 5 µL of 10-fold serial cell dilution on solid YPD plates containing 3.2 g/L, 3.4 g/L, and 3.6 g/L 2-PE. Cultivate at a constant temperature of 30°C.

Construction of CRISPR/CAS9 system

All plasmids used in this study are shown in Table 1, and primers are shown in Table 2. The Cas9 plasmid was transformed into *S. cerevisiae* CWY-132 and PK2-C respectively, and then the transformants containing the Cas9 plasmid were re-used as the starting strain for the next step of gene manipulation. The PAM site is obtained through the website <http://yeastriction.tnw.tudelft.nl> . The gRNA plasmids used in this study were obtained by inverse PCR (Fig. 2a). Take the construction of gRNA plasmids when *ALD3* is knocked out as an example. Using the original gRNA plasmids as templates, PCR was performed with primers ALD3 g-F and ALD3 g-R. The resulting PCR reaction solution was subjected to template elimination treatment with *Dpn* I enzyme, and then transformed into *E. coli* Dh5α after DNA purification, and cultured in selective medium overnight. The obtained plasmid was confirmed by sequencing and used for transformation. The other gRNA plasmids are obtained in the same way. When constructing DONOR DNA, homology arm lengths of 60 bp, 100 bp, 500 bp, and 1000 bp were set respectively. The fragments of 60 bp and 100 bp homology arm length were chemically synthesized, and the homology arm lengths of 500 bp and 1000 bp were obtained by overlap extension PCR. Take the 500bp Donor DNA homology arm of *ALD3* gene as an example. First, the wild-type *S.cerevisiae* genome was used as a template, and the two homologous arm fragments on the left and right were amplified with primer pairs ALD3 L500-F, ALD3 L-R and ALD3 R-F, ALD3 R500-R, respectively. The fragment obtained is recovered and purified by gel and used as a template again. The primers ALD3 L500-F and ALD3 R500-R are used for another round of overlap extension PCR to connect to complete Donor DNA (Fig. 7a). Subsequently, the constructed donor DNA and gRNA plasmid were co-transformed into *S.cerevisiae* strains containing Cas9, positive transformants on dual antibiotic plates containing hygromycin and G418 were selected. The

gene deletion or integration in transformants was determined by PCR. The general operation flow of the CRISPR/CAS9 system is shown in Fig 7b.

Yeast transformation

The plasmids and DNA fragments were transformed into yeast cells by the PEG/lithium acetate method[46]. Transformed yeasts were cultured in YPD medium supplemented with 40 µg/mL G418 sulfate and 80 µg/mL hygromycin for selection.

Analytical method for 2-PE

Take out 1mL of fermentation broth, 10000 r/min, centrifuge for 10 minutes, take the supernatant liquid and analyze by gas chromatography(GC). Sample pretreatment: add 200 uL internal standard solution (methyl isobutyl methanol 1 g/L aqueous solution) to the supernatant, add 500uL ethyl acetate, shake and mix, centrifuge at 5000 rpm/min for 1 min, take the upper organic phase perform testing. Standard curve preparation: prepare 0.5, 1.0, 5.0, 10.0 g/L of 2-phenylethanol aqueous solution of standard concentration, and treat as above. The determination conditions for analyzing the concentration of 2-PE in the fermentation broth by GC are: Shimadzu GC2014 gas chromatograph; FID detector; column Rtx-1 (30m*0.25mm*0.25um, Restek); carrier gas is nitrogen; split flow; The split ratio is 49.0; the column flow rate is 1.45 mL/min; the purge flow rate is 3 mL/min; the temperature of detector is 250°C; the oven is maintained at 80°C for 1 min, and then increased to 200°C by 20°C/min; the sample volume is 1 uL.

NADH detection

The double antibody sandwich method was used to determine the level of microbial nicotinamide adenine dinucleotide (NADH) in *S.cerevisiae*. Collect the cells through centrifuge and discard the supernatant. The ratio of extract solution (volume) to cell number (10^4 cells) was 500:1. The cells are broken by ultrasound (ice bath, 200W, ultrasound 3s, repeat 30 times at an interval of 10s), centrifuge at 8000g for 10 min at 4°C, take the supernatant, and put it on ice for testing. Coat the microwell plate with purified microbial NADH antibody to make solid phase antibody, and add NADH to the micro-wells of the coated monoclonal antibody in sequence, and then combined with HRP-labeled NADH antibody to form an antibody-antigen-enzyme-labeled antibody complex. After thorough washing, the substrate TMB is added for color development. TMB is converted into blue under the catalysis of HRP enzyme and into the final yellow under the action of acid. The color intensity is positively correlated with the microbial nicotinamide adenine dinucleotide (NADH) in the sample. The absorbance (OD value) was measured with a microplate reader at a wavelength of 450 nm, and the concentration of microbial nicotinamide adenine dinucleotide (NADH) in the sample was calculated from the standard curve.

Plasmid elimination

In this study, the elimination of gRNA plasmid and Cas9 plasmid was achieved by passage. The initial yeast was cultured overnight in 5 mL YPD liquid without any antibiotics, and then passaged every 12h.

After fifth Generation, the plasmid elimination rate can reach more than 90%.

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.

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- Authors' contributions

Zhiwei Xu, Constructed the CRISPR/Cas9 system, carried out the gene editing. Zhe Chen, provided the CRISPR/Cas9 system gene editing guide and design. Lucheng Lin, carried out the 2-PE fermentation. Kun Wang, conducted 2-PE analysis. Jie Sun and Tingheng Zhu, conducted the whole study project and results analysis. All authors commented on the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Strains and plasmids used in this study

Strains and plasmids	Relevant genotype	Origin
<i>Saccharomyces cerevisiae</i>		
CWY-132	Diploid yeast strain	This lab
CWY-133	Diploid yeast strain, <i>ALD2Δ</i>	This study
CWY-134	Diploid yeast strain, <i>ALD3Δ</i>	This study
CWY-135	Diploid yeast strain, <i>ALD2Δ</i> , <i>ALD3Δ</i>	This study
CWY-136	Diploid yeast strain, <i>ATF1Δ</i>	This study
CWY-137	Diploid yeast strain, <i>ATF1Δ</i> , <i>ALD3Δ</i>	This study
CWY-138	Diploid yeast strain, <i>PDC1Δ</i>	This study
CWY-139	Diploid yeast strain, <i>ALD2Δ</i> , <i>ALD3Δ</i> <i>PDC1Δ</i>	This study
CWY-140	Diploid yeast strain <i>Overexpression ALD3</i>	This study
CWY-141	Diploid yeast strain <i>Overexpression ATF1</i>	This study
PK2-C	Haploid yeast strain <i>MATα</i>	This lab
PK2-C2	<i>MATα</i> , <i>ALD2Δ</i>	This study
PK2-C3	<i>MATα</i> , <i>ALD3Δ</i>	This study
PK2-C4	<i>MATα</i> , <i>ATF1Δ</i>	This study
<i>Escherichia coli</i>		
DH5α	Φ80 lacZΔM15 ΔlacU169 recA1 endA1 hsdR17 supE44 thi-1 gyrA relA1	This lab
Plasmids	KanR;f1 ori with P _{TEF1} ,P _{T3} ,P _{AmpR} ; <i>AmpR</i> ;P _{T3} - <i>CAS9</i> -T _{CYC1} ;	
Cas9	HygR ^r 2μori with P _{TEF1} ,P _{T3} ,P _{AmpR} <i>AmpR</i> ; P _{SNR52} -sgRNA-T _{SUP4}	This lab
gRNA	gRNA plasmid carrying <i>ALD2</i> sgRNA	This lab
ALD2- gRNA	gRNA plasmid carrying <i>ALD3</i> sgRNA	This study
ALD3- gRNA	gRNA plasmid carrying <i>ATF1</i> sgRNA	This study
ATF1- gRNA	gRNA plasmid carrying <i>PDC1</i> sgRNA	This study
PDC1- gRNA		

Table 2. Primers used in this study

Primer name	Sequence 5'-3'
Primers for plasmid construction	
ALD3 g-F	AGAACTTACAAGATACTATGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
ALD3 g-R	CATAGTATCTTGTAAGTTCTGATCATTATCTTTCACTGCGGAGA
ALD2 g-F	TTCAAATGCCAAAGGTGATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
ALD2 g-R	AATCACCTTTGGCATTGGAAGATCATTATCTTTCACTGCGGAGA
ATF1 g1-F	CAAATATTCTATATGCGATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
ATF1 g1-R	AATCGCATATAGAATATTTGGATCATTATCTTTCACTGCGGAGA
ATF1 g2-F	CTCCGTTTTTACATGTTTGTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
ATF1 g2-R	ACAAACATGTAAAAACGGAGGATCATTATCTTTCACTGCGGAGA
ATF1 g3-F	TCAAACCATTGAACTTCGAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
ATF1 g3-R	TTCGAAGTTCAATGGTTTGAGATCATTATCTTTCACTGCGGAGA
ATF1 g4-F	AAGTCTATCACCTTTTCGATGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
ATF1 g4-R	ATCGAAAAGGTGATAGACTTGATCATTATCTTTCACTGCGGAGA
ATF1 g5-F	CATTCTGAATCATCTCTTTTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
ATF1 g5-R	GAAAGAGATGATTCAGAATGGATCATTATCTTTCACTGCGGAGA
PDC1 g-F	GGAAGTCATTGACACCATCTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
PDC1 g-R	AGATGGTGCAATGACTTCCGATCATTATCTTTCACTGCGGAGA
Primers for Donor DNA construction	
ALD3 L1000-F	AGGATGAGATATTTGGCCCGGTTGT
ALD3 L500-F	AGAGCTATAAACTCAGTGAGATTTA
ALD3 L-R	AACATTTATATAACTATGTAACGAATTTTCTTTTGGCTAATTTTCTAAAT
ALD3 R-F	ATTTAGAAAATTAGCCAAAAGAAAATTCGTTACATAGTTATATAAATGTT
ALD3 R500-R	TCCAAAACATACATTCTTCAGTATC
ALD3 R1000-R	CACATGAAAATTTTTAAAGGTATGG
ALD2 L-F	GCTACCTCTTAATGTGTCACAAG
ALD2 L-R	CTTACATAATGATAACTATCACATTTTCTTTTGGCTTATTTTCACG
ALD2 R-F	CGTGAAAATAAGCCAAAAGAAAATGTGATAGTTATCATTATGTAAG
ALD2 R-R	TTACTAATTCCTTAACCCTTAGG
ATF1 L500-F	CTGACACCCGGATAATTAAGAAGTG
ATF1 L1000-F	TGGTATAATCAAGCGTATTTAGAAG
ATF1 L-R	ATATCAGTCAAGCATCATGTGAGATGAGAGCTGATAAATTGATGGTATTT
ATF1 R-F	AAATACCATCAATTTATCAGCTCTCATCTCACATGATGCTTGACTGATAT
ATF1 R500-R	GCATTTGTTTGTAGCTTTGCATTG
ATF1 R1000-R	ATAGCATGCTCCTATTATAGATGTG
PDC1 L-F	GTATGCTCTTCTGACTTTTCGTG
PDC1 L-R	AACTAATAATTAGAGATTAAATCGCTTTTGATTGATTTGACTGTGTTA
PDC1 R-F	TAACACAGTCAAATCAATCAAAAGCGATTTAATCTCTAATTATTAGTT
PDC1 R-R	GAAATCAGCTTGTGGGTATTGTTC

Figures

Figure 1

The 2-PE metabolic pathway and ethanol metabolic pathway in *Saccharomyces cerevisiae*. The 2-PE metabolic pathway includes the Ehrlich pathway and the de novo shikimic acid pathway. L-Phe, L-phenylalanine; PPA, phenylpyruvate; PAAL, phenylacetaldehyde; 2-PE, 2-phenylethanol; PAA, phenylacetic acid; 2-PEAc, ethyl phenylacetate; EMP, glycolysis pathway; PYR, pyruvate; AH, acetaldehyde; HAc, acetic acid; EtOH, ethanol; EA, ethyl acetate; 2-KG, 2-ketoglutarate; L-Glu, L-glutamic acid; Aro8/9, Amino transferase I/II; Aro10, phenylpyruvate decarboxylase; ADH1-5, alcohol dehydrogenase 1-5; Ald2/3, acetaldehyde dehydrogenase 2/3; Atf1, acetyltransferase; PDC, Pyruvate decarboxylase

Figure 2

a Two methods of gRNA plasmid construction, the left is the scheme one, the right is the scheme two. b The influence of the length of the DONOR DNA homology arm on the editing efficiency of the CRISPR/CAS9 gene editing system. The data represents the average of three independent experiments, and the deviation of the average is between 5% and 10%. The asterisk indicates a significant difference when $p < 0.05$, which is based on the analysis of variance statistical test.

Figure 3

2-PE and ethanol production of wild-type strains and engineered strains. a The 2-PE production and ethanol production of wild-type (CWY-132) and ALD2Δ, ALD3Δ, ALD2ΔALD3Δ, ALD2ΔALD3ΔPDC1Δ, ATF1Δ, ATF1ΔALD3Δ strains. b The 2-PE production ethanol production of wild type (PK2-C) and ALD2Δ, ALD3Δ and ATF1Δ strains. c 2-PE production and ethanol production of wild-type (CWY-132) and over-expressing ATF1 and over-expressing ALD3 strains. The data represents the average of three independent experiments, and the deviation of the average is between 5% and 10%. The asterisk indicates that when $p < 0.05$, the 2-PE and ethanol production of wild-type and mutant strains are significantly different, which is based on the statistical test of variance analysis.

Figure 4

Growth of wild-type strains and engineered strains. a The growth curve of wild-type (CWY-132) and ALD2Δ, ALD3Δ, ALD2ΔALD3Δ, ATF1Δ, ATF1ΔALD3Δ strains on YPD medium. b The streaking results of wild-type (CWY-132) and ALD2Δ, ALD3Δ, ALD2ΔALD3Δ, ATF1Δ, ATF1ΔALD3Δ strains on the YPD plate. c Growth curve of wild type (PK2-C) and ALD2Δ, ALD3Δ, ATF1Δ strains in YPD liquid medium. d The streaking results of wild type (PK2-C)ΔALD2Δ, ALD3Δ, ATF1Δ strains on the YPD plate.

Figure 5

The 2-PE production and ethanol production of each strain under different initial L-Phe concentrations. a 2-PE production and ethanol production of wild-type (CWY-132) and Δ ALD3, ALD2 Δ , ALD2 Δ ALD3 Δ , overexpression ALD3, ATF1 Δ Δ ALD3 Δ , overexpression ATF1 strains at L-Phe concentrations of 0.1g/L, 1g/L, 3g/L, and 5g/L, respectively. b.c The line chart of ethanol production and 2-PE production of wild-type (CWY-132) and ALD3 Δ , ALD2 Δ , ALD2 Δ ALD3 Δ , overexpression ALD3, ATF1 Δ , ATF1 Δ ALD3 Δ , and overexpression ATF1 strains under different initial L-Phe concentrations. The data represents the average of three independent experiments, and the deviation of the average is between 5% and 10%. The asterisk indicates that when $p < 0.05$, the 2-PE and ethanol production of wild-type and mutant strains are significantly different, which is based on the statistical test of variance analysis.

Figure 6

a The drip plate results of CWY-132 wild-type strain on 2-PE plates containing 3.2g/L, 3.4g/L, 3.6g/L, and the drip plate results when 5mM GSH was added. b 2-PE production of wild type (CWY-132) and *ALD2Δ*, *ALD3Δ*, *ALD2ΔALD3Δ*, *ATF1Δ*, *ATF1ΔALD3Δ* strains when added with 2g/L NADH, 2g/L NAD⁺, 0.1mM GSH. c NADH content of wild Type (CWY-132) and *ALD2Δ*, *ALD3Δ*, *ALD2ΔALD3Δ*, *ATF1Δ*, *ATF1ΔALD3Δ*, *PDC1Δ*, *ALD2ΔALD3ΔPDC1Δ* strains. The data represents the average of three

independent experiments, and the deviation of the average is between 5% and 10%. The asterisk indicates a significant difference when $p < 0.05$, which is based on the analysis of variance statistical test.

Figure 7

a The basic principle of DONOR DNA construction. b The basic operation flow of CRISPR/CAS9 gene editing technology in *Saccharomyces cerevisiae*