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ORIGINAL ARTICLE

Metabolic engineering of *Saccharomyces cerevisiae* for accumulating pyruvic acid

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Abstract Pyruvate decarboxylase (PDC), a key enzyme in alcoholic fermentation in *Saccharomyces cerevisiae*, can degrade pyruvic acid to further convert acetaldehyde into ethanol. The main structural genes encoding PDC are *PDC1* and *PDC5*. In this study, metabolic engineering principles were used to block the further metabolism of pyruvic acid; *Saccharomyces cerevisiae* Y2-1 with *PDC1* disruption and Y2-15 with both *PDC1* and *PDC5* disruption were obtained using the LiAc/SS carrier DNA/PEG method. The specific PDC activity of mutant *S. cerevisiae* Y2-1 decreased by 31 % compared to that of the parent strain Y2, while specific PDC activity was barely detectable in mutant *S. cerevisiae* Y2-15. Moreover, the mutant Y2-1 with *PDC1* disruption displayed no obvious effect on the rate of growth in the yeast nitrogen base with glucose (YNBG) medium, but the growth rate of *S. cerevisiae* Y2-15 was significantly lower than that of the parent strain Y2. Finally, through optimization of the fermentation medium, the accumulation of pyruvic acid by Y2-15 increased to 24.65 g/L over a period of 96 h, 16.86-fold higher than with the parental strain Y2 by shake flask cultivation.

Keywords *Saccharomyces cerevisiae* · Pyruvic acid · *PDC1* · *PDC5* · Gene disruption · Metabolic engineering

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Introduction

Saccharomyces cerevisiae is an important model microorganism with a wide range of research applications (Liu and Yu 2002), especially now that its complete genome sequence is known and 80 % of the gene function has been identified (Wood et al. 2002). However, *S. cerevisiae* has also been used by molecular biologists as a host for producing therapeutic proteins (Gellissen et al. 1992) and chemicals with commercial value by means of metabolic engineering (Porro et al. 1995; Ostergaard et al. 2002; Overkamp et al. 2002).

Traditionally, *S. cerevisiae* has been used for the rapid conversion of sugars to ethanol. Pyruvic acid, an important intermediate metabolite, is associated with many metabolic pathways in microorganisms. With the advent of modern molecular biotechnology, many pyruvic acid high-yield strains have been engineered using various biotechnological methods (Tomar et al. 2003; Zelić et al. 2003; Causey et al. 2004). Moreover, optimal process control strategies have greatly contributed to improved pyruvate production (Li et al. 2002; Zelić et al. 2004). In order to obtain a high-yield pyruvic acid strain and increase its productivity, it is necessary to block the metabolism of pyruvic acid converting to other metabolic products by *S. cerevisiae*.

Pyruvate decarboxylase (PDC) is a key enzyme in alcoholic fermentation by *S. cerevisiae*, since it cleaves pyruvic acid, the end product of glycolysis, to form CO_2 and acetaldehyde (Barnett 1976). The structure of PDC comprises four subunits (*PDC1*, *PDC2*, *PDC5*, and *PDC6*); for *S. cerevisiae*, *PDC1* and *PDC2* have been shown to affect pyruvate decarboxylase activity (Schmitt and Zimmermann 1982). Surprisingly, the *PDC1* deletion mutation conferred a different phenotype from that of the *PDC1*-8 mutation described by Schmitt and Zimmermann (1982). The *PDC1* deletion mutants had about 50 % of the pyruvate decarboxylase activity when glucose

was used as the carbon source for fermentation. This activity was most likely due to a second structural gene (*PDC5*) encoding pyruvate decarboxylase that was very similar to *PDC1*, since the *PDC1* deletion mutants still had a full-length mRNA hybridizing to *PDC1* probes in Northern blot analysis (Schaaff et al. 1989).

While there are three structural genes (*PDC1*, *PDC5*, and *PDC6*) that encode PDC, the main genes are *PDC1* and *PDC5* (Pronk et al. 1996). In this study, the disruption of *PDC1* alone and *PDC1* and *PDC5* together was performed using metabolic engineering principles in order to increase the accumulation of pyruvic acid by blocking its further metabolism. Growth characteristics, the production of pyruvic acid, and PDC activity were investigated in medium for *S. cerevisiae* Y2 and the mutants Y2-1 and Y2-15.

Materials and methods

Strains, plasmids, reagents, and culture conditions

Strains, plasmids, and primers used in this study are listed in Tables 1 and 2. *Escherichia coli* DH5α was used for plasmid construction. The parent strain *Saccharomyces cerevisiae* Y2 was obtained from the Tianjin Key Laboratory of Industrial Microbiology, Tianjin, China. Recombinant *E. coli* strains were grown at 37 °C in lysogeny broth (LB) medium with 100 mg/L ampicillin. *S. cerevisiae* Y2 was grown in yeast-extract peptone glucose (YEPG) medium (10 g/L yeast extract, 20 g/L tryptone, 20 g/L glucose, pH 5.5) at 30 °C. *S. cerevisiae* Y2-1 was grown in YEPG medium with Geneticin® (800 μg/L) and sodium acetate (2 g/L) at 30 °C, and *S. cerevisiae* Y2-15 was grown in YNBG medium (6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose, pH 5.5) supplemented with Geneticin® (800 μg/L) and sodium acetate (2 g/L) at 30 °C, except for pyruvate synthesis. All chemicals were of analytical grade. G418 (Geneticin®) sulfate was purchased from Sigma-Aldrich (Shanghai, China). The agarose gel extraction kit, yeast genome extraction kit, plasmid extraction Kit, and the 1 kb DNA Marker and DNA Marker III were purchased from Tiangen Biotech (Beijing) Co., Ltd. (Beijing, China). The DIG-High Prime DNA Labeling and Detection Starter Kit I was purchased from Roche Diagnostics GmbH, Mannheim, Germany. All restriction endonucleases and Taq DNA polymerase used in this study were purchased from Takara Bio.

Pyruvic acid fermentation conditions

The inoculum medium, with an initial pH of 5.5, was composed of 30 g/L glucose, 10 g/L peptone, 5 g/L yeast extract, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L sodium acetate, and 0.5 % (v/v) corn steep liquor. The fermentation medium, with a initial pH of 5.5, was composed of 80 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 2.5 g/L sodium acetate, 1 % (v/v) corn steep liquor, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 mL metal ions liquor (2 g/L $CaCl_2 \cdot 2H_2O$, 2 g/L $FeSO_4 \cdot 7H_2O$, 5 g/L $ZnCl_2$, 0.2 g/L $MnCl_2 \cdot 4H_2O$, 0.05 g/L $CuSO_4 \cdot 5H_2O$), and 4 % $CaCO_3$. The initial pH was adjusted to 5.5, with 6 mol/L HCl. $CaCO_3$ was used as a pH buffer, which was subjected to dry-heat sterilization at 160 °C for 30 min before it was added to the medium. The seed culture was cultivated in

Table 1 Strains and plasmids used in the study

Strains or plasmids	Related characteristics	Source
Strain		
E. coli DH5α	*supE44*△*lacU169*(*ψ80lacZ*△*M15*) *hsdR17recA1endA1gyrA96thi-1relA1*	Tianjin University of Science and Technology
S. cerevisiae Y2	*his4*	Tianjin University of Science and Technology
S. cerevisiae Y2-1	*his4 PDC1*::*kan*^{r}	This work
S. cerevisiae Y2-15	*his4 PDC1*::*kan*^{r} *PDC5*::*his4*	This work
Plasmid		
pGEM-T		Nankai University
pPIC-3.5 K		Nankai University
pMD19-T		TAKARA
PT-1		This work
T-K		This work
TP1K		This work
PT-5		This work
T-H		This work
TP5H		This work

Table 2 Primers used in the study

Primer	Sequence (5′→3′) (underlined sections indicate restriction endonuclease sites)	Design principles and requirements
Pad1U	AATGCGGCCGCATGTCTGAAATTACTTTGG	Upstream primer of the *PDC1* ORF with *Not*I site
Pad1D	TAAGCGGCCGCTTAAATCGCTTATTGCTTAGC	Downstream primer of the *PDC1* ORF with *Not*I site
Kan1	AATAGATCTGCTCTCCCTTATGCGACTCCTG	Upstream primer of the *Kan*^{r} gene with *Bgl*II site
Kan2	TGGAATTCACTTGAAGTCGGACAGTGAGTG	Downstream primer of the *Kan*^{r} gene with *Eco*RI site
Pad5U	AATGCGGCCGCATGTCTGAAATAACCTTAGG	Upstream primer of the *PDC5* ORF with *Not*I site
Pad5D	TAAGCGGCCGCTTATTGTTTAGCGTTAGTAGCG	Downstream primer of the *PDC5* ORF with *Not*I site
His1	ATCAAGCTTAGAATTGGTTAATTGGTTG	Upstream primer of the *His4* gene with *Hin*dIII site
His2	TCGAATTCTAATGCGGTAGTTTATCAC	Downstream primer of the *His4* gene with *Eco*RI site
KanU	GTCAGCAACACCTTCTTCACGAG	Upstream primer of the *PDC1* disruption cassette, part of the *PDC1* sequence
Pdc1D	GTTGATACCGAAAGCGGAGGTAC	Downstream primer of the *PDC1* disruption cassette, part of the *Kan*^{r} sequence
PU1	GCCAAGTCAACTGTAACACCG	Upstream primer of the *PDC5* disruption cassette, part of the *His4* sequence
PU2	CAGCAGCATAGGGAAACACG	Downstream primer of the *PDC5* disruption cassette, part of the *PDC5* sequence

flasks at 30 °C and shaken at 200 rpm for 26 h, and 10 % (v/v) of the seed culture was then inoculated into a 500-mL flask containing 50 mL fermentation medium. Fermentation of pyruvic acid was carried out in flasks at 30 °C and shaken at 200 rpm for 96 h. All experiments were performed in triplicate.

Construction of the *PDC1* disruption cassette

The *PDC1* gene was amplified using the primers Pad1U and Pad1D with genomic DNA of *S. cerevisiae* Y2 as a template to obtain a DNA fragment 1,701 base pairs (bp) in length (fragment I, Fig. 1a) containing both the 5′and 3′ *Not*I sites. The genomic DNA of *S. cerevisiae* was prepared using the glass-bead method (Burke et al. 2002). This DNA fragment was digested with *Not*I and then sub-cloned to plasmid pGEM-T to obtain plasmid PT-1. The *kan*^{r} gene was amplified using primers Kan1 and Kan2 with the plasmid pPIC-3.5 k as a template to obtain a 1,616-bp DNA fragment (fragment II, Fig. 1a) containing a 5′ *Bgl*II and a 3′ *Eco*RI site. This DNA fragment was digested with *Bgl*II and *Eco*RI and then sub-cloned to plasmid pMD19-T to generate the plasmid T-K. The two plasmids PT-1 and T-K were digested by *Bgl*II and *Eco*RI, respectively, to obtain a 3,886-bp and a 1,593-bp fragment. These two fragments were then ligated using T4 DNA ligase to obtain plasmid TP1K containing a 2,479-bp *PDC1* disruption cassette (fragment III, Fig. 1a).

Construction of the *PDC5* disruption cassette

The *PDC5* gene was amplified using primers Pad5U and Pad5D with the genomic DNA of *S. cerevisiae* Y2 to obtain an 1,801-bp DNA fragment (fragment I, Fig. 2a) containing both the 5′ and 3′ *Not*I sites (underlined sections of primers in Table 2). The DNA fragment was digested with *Not*I and then sub-cloned to plasmid pGEM-T to obtain plasmid PT-5. The *His4* gene was amplified using primers His1 and His2 with plasmid pPIC-3.5 k as a template to obtain a 3,329-bp fragment (fragment II, Fig. 2a) containing a 5′ *Hin*dIII and a 3′ *Eco*RI site. This fragment was digested with *Hin*dIII and *Eco*RI and was then sub-cloned to plasmid pMD19-T to obtain the plasmid T-H. The two plasmids PT-5 and T-H were then digested by *Hin*dIII and *Eco*RI to obtain fragments 3, 696 bp and 3,329 bp in length, respectively. The two fragments were ligated using T4 DNA ligase to produce plasmid TP5H containing the 4,333-bp *PDC5* disruption cassette.

Disruption of *PDC1* and morphology of transformants

The plasmid TP1K was digested with *Not*I to obtain the disruption cassette DNA fragment (P1K), and P1K was then transformed to *S. cerevisiae* Y2 using the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl 2007). After incubation at 30 °C for 1–2 h, the cells were spread on YEPG plates with G418 (800 μg/L) and sodium acetate (2 g/L). Plates were incubated at 30 °C for 2–3 days until colonies appeared. The colonies were replica-plated onto new YEPG plates containing G418, and transformants were then selected based on morphology and growth characteristics. For confirmation, the *PDC1* disruption cassette sequence in the transformant genomes was amplified using the primers Pad1U and Pad1D with the genomic DNA of Y2-1 as a template, and the *Kan* gene marker in the transformants was further amplified using

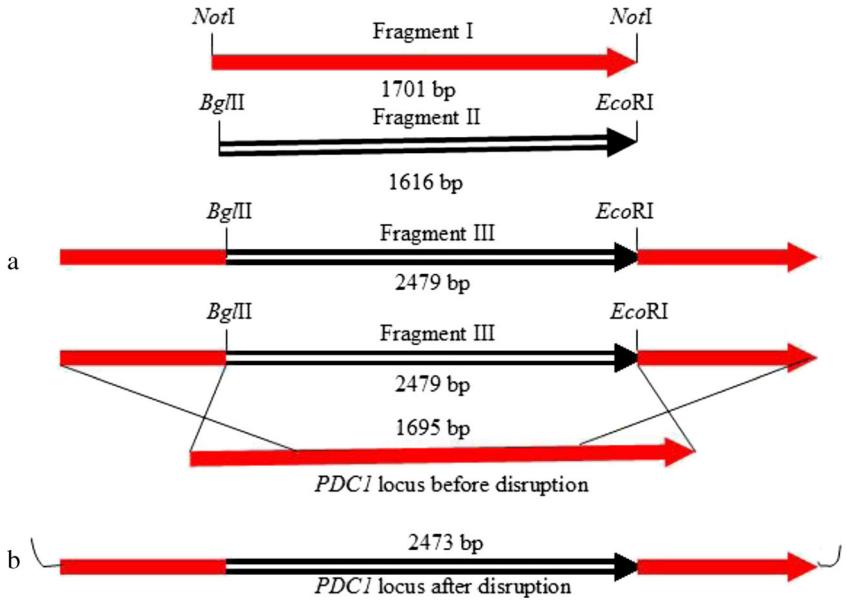

Fig. 1 Schematic representation of the DNA fragments for gene disruption and the disruption of *PDC1* by homologous recombinant. **a** Structure of DNA fragments I–III. Fragment I denotes PCR product of *S. cerevisiae PDC1*; Fragment II denotes PCR product of *Kan*^{r}; Fragment III denotes PCR product containing *S. cerevisiae PDC1*-F1, *Kan*^{r}, and *S. cerevisiae PDC1*-F2 (*from left to right*); **b** *PDC1* gene disruption by homologous recombination

primers Kan1 and Kan2 with genomic DNA of Y2-1 as template.

Disruption of *PDC5* and morphology of transformants

The plasmid TP5H was digested with *Not*I to obtain the disruption cassette DNA fragment P5H, and P5H was then transformed to *S. cerevisiae* Y2-1 using the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl 2007). After incubation at 30 °C for 1–2 h, the cells were spread on YNBG plates with G418 (800 μg/L) and sodium acetate (2 g/L). Plates were incubated at 30 °C for 2–3 days until colonies appeared. The colonies were replica-plated onto new YNBG plates supplemented with G418 and sodium acetate, and transformants were then selected based on morphology and growth characteristics. For verification, the *PDC5* disruption cassette sequence in the transformant genomes was amplified using the primers Pad5U and Pad5D with the genomic DNA of Y2-15 as a template, and the *His* gene marker in the transformants was further amplified using primers His1 and His2 with genomic DNA of Y2-15 as a template.

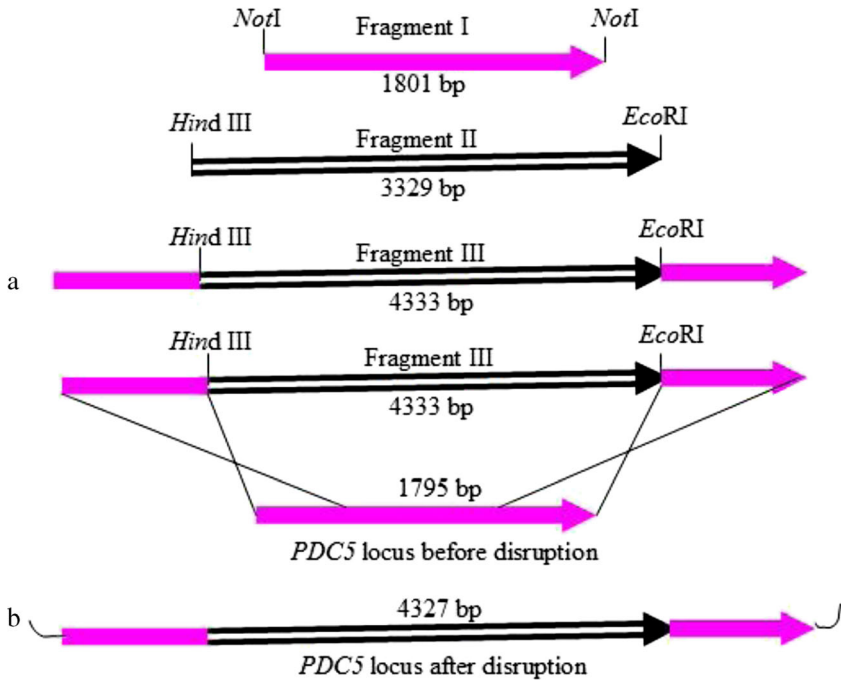

Fig. 2 Schematic representation of DNA fragments for gene disruption and disruption of *PDC5* by homologous recombination. **a** Structure of DNA fragments I–III. Fragment I denotes the PCR product of *S. cerevisiae PDC5*; Fragment II denotes the PCR product of *His4*; Fragment III denotes the PCR product containing *S. cerevisiae PDC5*-F1, *His4*, and *S. cerevisiae PDC5*-F2 (*from left to right*). **b** *PDC5* gene disruption by homologous recombination

Southern blot analysis

The genomic DNA of *S. cerevisiae* was extracted from cells growing in YNBG medium according to a yeast genomic DNA extraction protocol. Southern hybridization was performed using the DIG-High Prime DNA Labeling and Detection Starter Kit I to confirm homologous recombination events. For analysis of the mutants of Y2-1 and Y2-15, the genomic DNA of *S. cerevisiae* Y2-1 was digested with *Bgl*II and *Eco*RI, respectively. Probe I (487 bp), which underwent PCR amplification with the primers KanU and Pdc1D, was used to confirm the *PDC1* disruption. The genomic DNA of *S. cerevisiae* Y2-15 was digested with *Hin*dIII and *Eco*RI. Probe II (572 bp), which was amplified by PCR with the primers PU1 and PU2, was used to verify the *PDC5* disruption. The Southern blotting procedure was performed according to manufacturer instructions.

Specific PDC activity assays

The three strains were pre-grown for 2 days in YNBG medium to stationary phase. Aliquots of 1 mL from these cultures were inoculated into 50 mL of fermentation medium and shaken for 12 h at 30 °C (Candy et al. 1991). Cells were taken and then washed twice using 10 mM Na_3PO_4 (with 2 mM EDTA) buffer (pH 6.0), and centrifuged for 10 min at 4 °C. The precipitate was resuspended in 20 mL 10 mM Na_3PO_4 (with 2 mM $MgCl_2$) buffer (pH 6.0), oscillated with glass beads for 10 min at 4 °C and centrifuged for 10 min at 4 °C, and the supernatant was then collected to determine the concentration of protein and PDC-specific activity (Bradford 1976; Ciriacy and Breitenbach 1979). Protein concentrations of the supernatants were calculated by comparison to the linear relationship between standard protein products and absorbance values (595 nm). The specific PDC activity was measured using the method reported by Gao et al. (2011), with slight modifications, as follows: the experimental group included reagents that were added to the colorimetric tube: 2.7 mL 200 mM citric acid buffer, 0.1 mL 1 M sodium pyruvate, 0.05 mL 6.4 mM β-NADH, 0.05 mL 200 U/mL alcohol dehydrogenase, 0.1 mL supernatant, pH 6.0. The control included only the following reagents: 2.8 mL 200 mM citric acid buffer, 0.1 mL 1 M sodium pyruvate, 0.1 mL supernatant, pH 6.0. The PDC-specific activity was measured at 340 nm per minute using the TU-18 spectrophotometer (Beijing, China) at 25 °C. One unit of enzyme activity was defined as the conversion of l.0 μM pyruvate to acetaldehyde per minute under conditions of pH 6.0 at 25 °C.

Growth characteristics

Saccharomyces cerevisiae strains were pre-grown in fresh YNBG medium for 2 days to stationary phase, and a 5-mL culture was then transferred to a 500-mL flask containing 50 mL of fresh YNBG medium and shaken for 24 h at 30 °C. Viable cells of these cultures were spread on YNBG plates at tenfold dilution every 2 h, and the number of viable cells was determined. All experiments were performed in triplicate.

Analytical methods

The fermentation broth was centrifuged at 12,000×*g* for 10 min, and the supernatant was used to identify metabolites. The concentration of pyruvic acid in the fermentation broth was determined using high-performance liquid chromatography (HPLC) with an organic acid column (catalog #125-0140, Bio-Rad Laboratories). The mobile phase was a 0.5-mM H_2SO_4 aqueous solution at a flow rate of 0.6 mL/min and injection volume of 25 μL. The wavelength of the UV detector was set at 210 nm, and the column temperature was controlled at 50 °C. Glucose was determined using an SBA-40C biosensor (Biology Institute of Shandong, Academy of Sciences, Shandong, China). Yeast cell growth (biomass) was measured turbidimetrically at 660 nm (OD_{660}) after the culture broths were diluted 50-fold with water. The optical density value was converted to dry cell weight (DCW) using the calibration equation (1 OD_{660}=0.3 g DCW/L). Crude extracts were prepared using the method described by Ciriacy and Breitenbach (1979), and protein concentrations were determined according to Bradford (1976) using bovine serum albumin (Sigma-Aldrich) as the standard. Basic molecular biology techniques were performed according to manufacturer instructions or standard procedures (Sambrook et al. 1989).

Results and discussion

Construction of the *PDC1* disruption cassette

The steps involved in the construction of TP1K were described above in their entirety. The size of the final plasmid TP5H containing the *PDC1* disruption cassette was 5,479 bp. Verification of the plasmid was performed by double-digestion with *Eco*RI and *Bgl*II, which was expected to produce two DNA fragments, one approximately 1.6 kb and the other approximately 3.9 kb. Electrophoresis analysis showed two bands in the 1 % agarose gel, as expected, demonstrating that the plasmid was successfully constructed (Fig. 3a). In addition, we expected that digestion of the plasmid TP1K with *Not*I would produce the *PDC1* disruption cassette (P1K), and electrophoresis analysis revealed DNA bands of approximately 2.5 kb 3.0 kb DNA (Fig. 3b), providing further proof of successful plasmid construction.

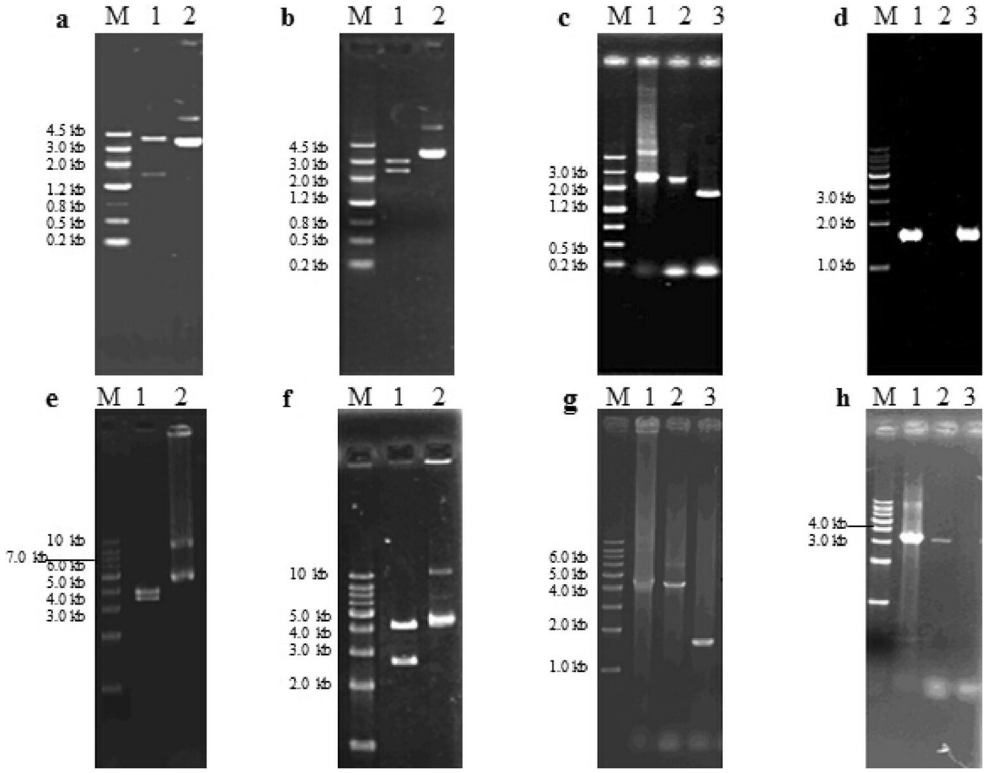

Fig. 3 Construction of the deletion cassette and verification of the strains Y2-1 (Δ*PDC1*) and Y2-15 (Δ*PDC1* Δ*PDC5*). **a** Analysis of TP1K digested by *Eco*RI and *Bgl*II. M: DNA Marker III; 1: TP1K digested by *Eco*RI and *Bgl*II; 2: TP1K. **b** Analysis of TP1K digested by *Not*I. M: 1-kb DNA Marker; 1: TP1K digested by *Not*I; 2: TP1K. **c** Electrophoresis analysis of PCR products of Y2-1. M: DNA Marker III; 1, 2, and 3 are the PCR products of TP1K, Y2-1, and Y2, respectively. **d** Electrophoresis analysis of PCR products of *Kan*^{r}. M: 1 kb DNA Marker; 1, 2, and 3 are the PCR products of TP1K, Y2-1, and Y2, respectively. **e** Analysis of TP5H digested by *Eco*RI and *Hin*dIII. M: 1 kb DNA Marker; 1:TP5H digested by *Eco*RI and *Hin*dIII; 2: TP5H. **f** Analysis of TP5H digested by *Not*I. M: 1 kb DNA Marker; 1: TP5H digested by *Not*I; 2: TP5H. **g** Electrophoresis analysis of PCR products of Y2-15. M: 1 kb DNA Marker; 1, 2, and 3 are the PCR products of TP5H, Y2-15, and Y2, respectively. **h** Electrophoresis analysis of PCR products of *His*4. M: 1 kb DNA Marker; 1, 2, and 3 are the PCR products of TP5H,Y2-15 and Y2, respectively

Disruption of *PDC1* in *S. cerevisiae* Y2

The mutant Y2-1 with *PDC1* disruption was described above. If the *PDC1* of Y2 is successfully disrupted, the PCR products using the primers Pad1U and Pad1D with the Y2-1 genome as a template should produce an approximately 2.5-kb DNA band in 1 % agarose gel (Fig. 3c), and the PCR products using primers Kan1 and Kan2 with the Y2-1 genome as a template should produce an approximately 1.6-kb DNA band in 1 % agarose gel (Fig. 3d). We can successfully obtain the mutant Y2-1 with *PDC1* disruption from Fig. 3c and d.

Construction of the *PDC5* disruption cassette

The complete steps for the construction of TP5H were described above. The size of the final plasmid TP1K containing the *PDC5* disruption cassette was 7,025 bp. Verification of the plasmid was performed by digesting it with *Hin*dIII and *Eco*RI, which was expected to produce a 3,696-bp DNA fragment and a 3,329-bp DNA fragment. Electrophoresis analysis revealed the presence of these two bands in 1 % agarose gel, confirming successful construction of the plasmid (Fig. 3e). Furthermore, digestion of the plasmid TP1K with *Not*I was expected to produce the *PDC5* disruption cassette (P5H), and electrophoresis analysis revealed an approximately 4.3-kb DNA band and an approximately 2.7-kb DNA band in 1 % agarose gel (Fig. 3f), providing further proof that the plasmid was successfully constructed.

Disruption of *PDC5* in mutant Y2-1

The process for producing a mutant Y2-15 with both *PDC1* and *PDC5* disruption was described above. According to the results, if the *PDC5* gene of Y2-1 is successfully disrupted, the PCR products using primers Pad5U and Pad5D with Y2-15 genome as a template should produce an approximately 4.3-kb DNA band in 1 % agarose gel (Fig. 3g), and the PCR products using primers His1 and His2 with the genomic DNA of Y2-15 as a template should produce an approximately 3.3-

kb DNA band in 1 % agarose gel (Fig. 3h). We are thus able to obtain the mutant Y2-15 with both *PDC1* and *PDC5* disruption (Fig. 3g and h).

Southern blot analysis of mutants Y2-1 and Y2-15

As Y2-1 was a mutant with *PDC1* disruption, the use of probe I and probe II (described in "Methods") for Southern blotting was expected to produce one band. Likewise, because Y2-15 was a mutant with disruption of both *PDC1* and *PDC5*, we anticipated that using probe I and probe II for Southern blotting would produce two bands. The results of the Southern blot, as shown in Fig. 4, confirm our expectations, demonstrating that *PDC1* and *PDC5* were successfully disrupted.

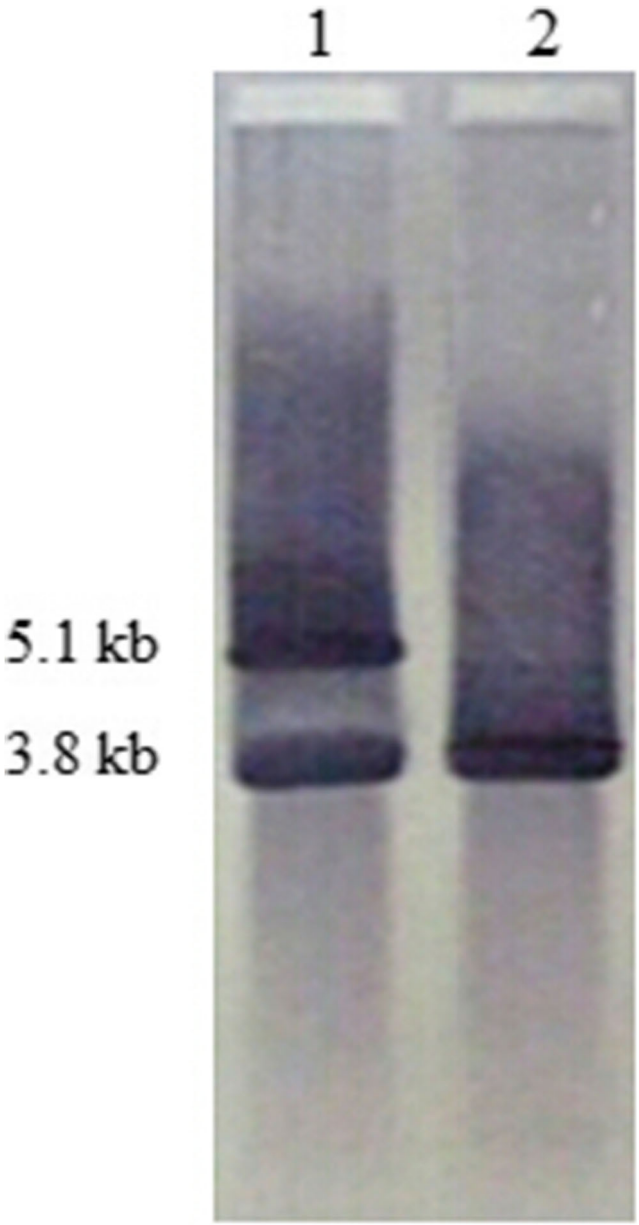

Fig. 4 Southern blot analysis of Y2-1 (column 2) and Y2-15 (column 1)

Growth curves and transformant genetic stability

The growth behavior of the *PDC* disruption strains in YNBG medium was characterized by analysis of the growth curves, as described in Fig. 5. The results revealed little effect on the growth of the mutant strain Y2-1, while the growth rate of the mutant Y2-15 was notably reduced with both *PDC1* and *PDC5* disruption, which may be due to the breakdown of glucose that occurs in the tricarboxylic acid cycle. As pyruvate is the end product of glycolysis, some respiratory enzymes in the mitochondria were significantly reduced, such as NADH oxidase (Hohmann and Cederberg 1990; Hohmann 1991). When degradation of pyruvate via PDC does not occur, some metabolic products are insufficient to improve the growth for *S. cerevisiae* Y2-15, and therefore it is necessary to optimize the fermentation medium to improve the accumulation of pyruvic acid, and the optimum fermentation conditions have been displayed in media (YNBG). By sub-cultivating 10 generations on YNBG plates, the transformants Y2-1 and Y2-15 can maintain stable inheritance with G418 resistance (data no shown).

PDC activity analysis

Disruption of *PDC* should produce a change in PDC activity. For verification, we determined the PDC activity according to the earlier description. The results demonstrated that, as expected, the PDC activity of the

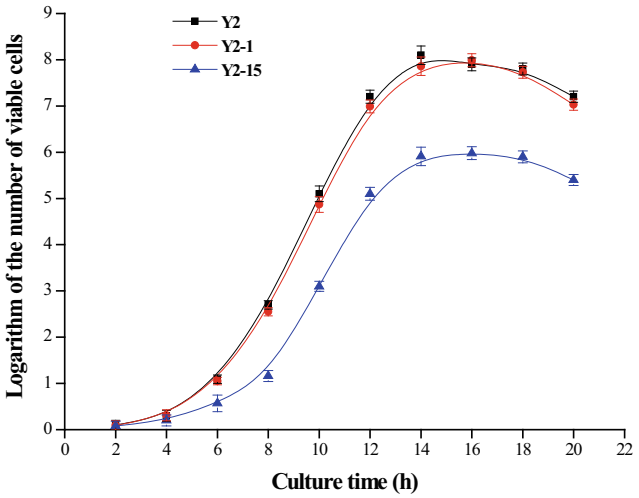

Fig. 5 Growth curves of the parent strain Y2 and the mutants Y2-1 and Y2-15. Strains were pre-grown for 2 days in fresh YNBG medium to stationary phase, and 5 mL cultures were placed into 500 mL flasks containing 50 mL of fresh YNBG medium and shaken at 200 rpm for 24 h at 30 °C

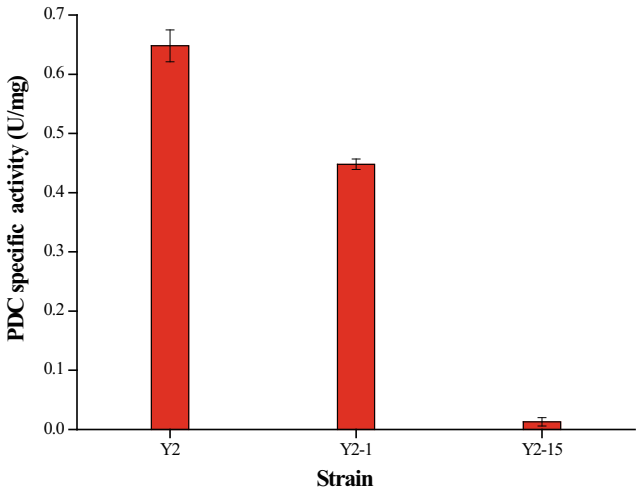

Fig. 6 Determination of PDC activity of *S. cerevisiae*. Strains were pre-grown for 2 days in fresh YNBG medium to stationary phase, and 5-mL cultures were placed into 500 mL flasks containing 50 mL of fresh fermentation medium and shaken at 200 rpm for 12 h at 30 °C

mutants Y2-1 and Y2-15 decreased by 31 and 98 % compared with that of the parent strain Y2 (Fig. 6). The result was just as we expected, since the mutant Y2-1 with *PDC1* disruption exhibited no great change for pyruvate decarboxylase activity. Strikingly, the presence of *PDC5* is very important to maintain PDC activity in *S. cerevisiae* Y2. The deletion of *PDC1* displayed the existence of at least one additional and functional structural gene for pyruvate decarboxylase in *S. cerevisiae* Y2. Since the mutant with both *PDC1* and *PDC5* disruption displayed minimal detectable PDC activity, we suggest that *PDC1* and *PDC5* are the only mainly structural genes in *S. cerevisiae* Y2. The results indicated that *PDC1* encodes a small part of PDC activity, and that *PDC5* is required to achieve full PDC activity (Fig. 6), which is consistent with the literature (Hohmann 1991).

Shake flask fermentation assays of *S. cerevisiae* Y2 and the mutants Y2-1 and Y2-15

Saccharomyces cerevisiae Y2 and the mutants Y2-1 and Y2-15 were individually cultivated in flasks at 30 °C and shaken at 200 rpm for 96 h. The shake flask fermentation results showed pyruvic acid yields of 3.51 g/L and 24.65 g/L for mutants Y2-1 and Y2-15, respectively (increases of 154 and 1,686 % compared to *S. cerevisiae* Y2) (Table 3). However, the growth of the parent strain Y2 was faster than that of the Y2-1 and Y2-15 mutants. In addition, the residual glucose concentrations in the fermentation broth were higher for the Y2-1 and Y2-15 mutants than for *S. cerevisiae* Y2. The single deletion of *PDC1* and the double deletion of *PDC1* and *PDC5*, however, did not change the phenotype in comparison to *S. cerevisiae* Y2, although the utilization of glucose was obviously altered when *PDC1* and *PDC5* were wholly deleted. The residual glucose at the end of fermentation was higher for Y2-15 than for Y2 or Y2-1. This may be due to the strong effect that the suppression of metabolic flow from pyruvic acid to acetaldehyde has on the redox balance, and as the glycolysis-derived NADH was not thoroughly consumed, the glucose consumption rate declined (Ishida et al. 2006). Since acetyl coenzyme A (acetyl-CoA) is an important requirement for mitochondria and ATP synthesis (Van Maris et al. 2004), the 1686 % increase in pyruvic acid yield in mutant Y2-15 indicates that the absence of *PDC1* and *PDC5* plays a key role in the accumulation of pyruvic acid. Compared to *Torulopsis glabrata*, however, the pyruvic acid yield by *S. cerevisiae* is still low (Li et al. 2002), and further research will be necessary to produce an *S. cerevisiae* strain capable of accumulating higher levels of pyruvic acid, thus improving productivity. First, there are enzymes in addition to PDC, such as pyruvate carboxylase (PYC) and pyruvate dehydrogenase (PDH), that catalyze pyruvic acid (Pronk et al. 1996), and disruption of certain genes encoding PYC and PDH could further block the metabolism of pyruvic acid.

Table 3 Pyruvic acid yields and theoretical yields of strains Y2, Y2-1, and Y2-15 in fermentation broth

Strain	Pyruvic acid (g/L)	OD_{600}	Residual glucose (g/L)	Theoretical yield [pyruvic acid/total glucose (%)]
Y2	1.38±0.04	0.97±0.01	2.34±0.12	1.7 %
Y2-1	3.51±0.07	0.77±0.02	5.72±0.10	4.3 %
Y2-15	24.65±0.05	0.65±0.01	8.89±0.09	30.8 %
Torulopsis glabrata (Li et al. 2002)	46.36±0.05			48.3 %

Note: The inoculum culture (10 % *v/v*) was then inoculated into 500 mL flasks containing 50 mL of fermentation medium. Fermentation of pyruvic acid was carried out in flasks at 30 °C and shaken at 200 rpm for 96 h. The concentration of pyruvic acid in the fermentation broth was determined by HPLC with an organic acid column (Bio-Rad). The mobile phase was a 0.5-mM H_2SO_4 aqueous solution at a flow rate of 0.6 mL/min and injection volume of 25 μL. The wavelength of the UV detector was set at 210 nm, and the column temperature was controlled at 50 °C. Glucose was determined using a biosensor (SBA-40C). Yeast cell growth (biomass) was measured turbidimetrically at 660 nm (OD_{660}) after the culture broths was diluted 50-fold with water. All experiments were performed in triplicate.

Second, the microorganism for producing pyruvic acid has traditionally been *T. glabrata*, which is chiefly a multi-vitamin autotrophic strain (Li et al. 2002), and would provide another avenue for improving the accumulation of pyruvic acid. Third, in our study, the disruption of *PDC1* and *PDC5* resulted in a much lower growth rate for Y2-15 than Y2, and thus we should explore ways to improve the growth of Y2-15, especially involving the Embden-Meyerhof-Parnas (EMP) glycolytic pathway (Stockland and San Clemente 1969). Fourth, in-depth study of fermentation conditions, particularly with regard to fermentation tanks conditions, will provide a solid base from which to cultivate the industrial production of pyruvate by *S. cerevisiae*.

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