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Knock Down of Erythrulose Kinase (*eyk1*) Leads to the Enhancement of Erythritol Production in *Yarrowia lipolytica* Mutant Strain YE4-2

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Abstract: Erythritol is a nearly zero-calorie sweetener, which is in great demand in food and health care industry. Our previous work obtained a *Yarrowia lipolytica* mutant strain YE4-2 with high erythritol yield. However, its conversion rate of glucose needs further improvement. Erythrulose kinase (*eyk1*) participates in the early stages of erythritol catabolism; the existence of *eyk1* prevents the accumulation of erythritol. In this study, the genetic transformation system of *Y. lipolytica* YE4-2 was firstly established and the Cre/loxp homologous recombination knockout system was constructed. By using the Cre/loxp system, *eyk1* gene was deleted. According to the PCR verification of *eyk1* gene, it was preliminarily determined that one allele of *eyk1* in this recombinant strain was knocked out. The recombination strain showed an average conversion rate of 48.51%, which was 49.26% higher than that of YE4-2 and the erythritol productivity is increased by 62.26%, which would be a significant improvement in the industrial scale.

Keywords: Erythritol, *Yarrowia lipolytica*, Genetic Transformation System, Cre/loxp, *eyk1*

Introduction

Erythritol is a substance widely existed in nature and it is a kind of sweetener with almost zero calories. The molecular energy value of erythritol is 1.67 kJ/g, which is the lowest among food-grade polyose sweeteners found so far, only about 1/10 of that in sucrose (sucrose 16.72 kJ/g) (Bernt *et al*., 1996). According to literature reports, after the 24 h reaction time of once ingested erythritol in the body, about 80% of erythritol is excreted through urine and the remaining 20% is absorbed by the large intestine. The small amount of erythritol entering the large intestine is also difficult to be used by bacteria fermentation. Therefore, the actual energy value of erythritol is only 0.84 kJ/g, which is called “zero” calorific value sweetener (Kawanabe *et al*., 1992). In addition, erythritol also has the advantages of high tolerance in human body and small influence on blood insulin levels, so it is widely used in medicines and calorie-free food additive, bringing gospel to diabetes and obesity patients.

Erythritol is commonly generated from glucose via fermentation processes using osmophilic yeasts (Moon *et al*., 2010). It was found that the yeast used to produce erythritol included *Torula sp*. (Dou *et al*., 2013), *Yarrowia lipolytica* (Rymowicz *et al*., 2009), *Pichia sp*. (Lee *et al*., 2002), *Candida sp*. (Moon *et al*., 2010) and so on. The sugar tolerance, fermentation speed, efficiency and by-product ratio are important indexes for breeding high yield erythritol strains. *Yarrowia lipolytica* is able to utilize many carbon sources such as glycerol, glucose, fatty acids and alkanes (Papanikolaou *et al*., 2002). It could also use complex carbon sources, such as agricultural waste, corn steep liquor, to produce erythritol.

A strain *Yarrowia lipolytica* Y-22 (Pei *et al*., 2015) with high yield of erythritol and quite few byproducts of glycerol, ribitol was obtained in our lab by the flask fermentation of high-osmotic resistant yeasts from

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different sources. Its fermentation conversion rate can reach about 50% at different scale fermentation levels. Taking Y-22 as the starting strain and applying conventional NTG mutagenesis technique, a mutant strain YE4-2 with 20% higher erythritol yield was screened on the high-sugar mutagenesis medium after 1h treatment at the NTG dose of 150 mg/L (Han *et al*., 2015). The mutant strain produced 192 g/L erythritol by 4 d fermentation on 350 m^3 fermentation tank, which was 20% higher than the original strain and the conversion rate was 56%. However, the conversion rate is still far from the theoretical conversion rate. Therefore, our work attempts to modify the erythritol metabolic pathway on molecular level, so as to further increase the erythritol accumulation. The metabolic pathways of *Yarrowia lipolytica* on glucose or glycerol are shown in Fig. 1. When erythritol is synthesized from glycerol, the latter is initially catalyzed by glycerol kinase and then glycerdehydrogenase to produce Dihydroxyacetone Phosphate (DHAP). DHAP enters into the pentose phosphate pathway and is converted into erythrose-4-phosphate by a Transketolase (TK). While when glucose is applied as carbon source, it enters the glycolysis pathway under the action of Hexokinase (HK) and the intermediate glucose 6-phosphate enters the pentose phosphate pathway (HMP) under the action of Glucose-6-Phosphate Dehydrogenase (G6PD). The latter is converted into erythrose-4-phosphate by a series of Transketolase (TKL) and Transaldolase (TAL). Then the erythrose-4-phosphate from both the glycerol and glucose is dephosphorylated by an Erythrose-4P Phosphatase (E4PP) to erythrose and then reduced to erythritol by an Erythrose Reductase (ER). However, erythritol is not the terminal product of the metabolism; *Y. lipolytica* can also use it as carbon source. Vandermies *et al*. (2017) reported that the gene *eyk1* encodes Erythrulose Kinase (EK), which is involved in the early stage of erythritol catabolism (shown in the red dotted frame). It can phosphorylate erythriol's direct downstream product erythritose and generate erythritose phosphate. The latter can continue to produce methyl aldehyde (formaldenhyde) and Dihydroxyacetone Phosphate (DHAP), which goes to glycolysis or TCA pathway. Thus, the catabolism of erythritol causes a certain negative effect on the accumulation of erythritol. Our hypothesis is the knocking down of the erythrulose kinase gene (*eyk1*) could eliminate the erythritol split-flow, therefore increase its accumulation. In this study, the genetic transformation system of *Yarrowia lipolytica* was established and on this basis, the Cre/loxp recombinant system was used to knockout *eyk1* gene of *Yarrowia lipolytica*, thereby inhibiting the catabolism of erythritol and improving the yield.

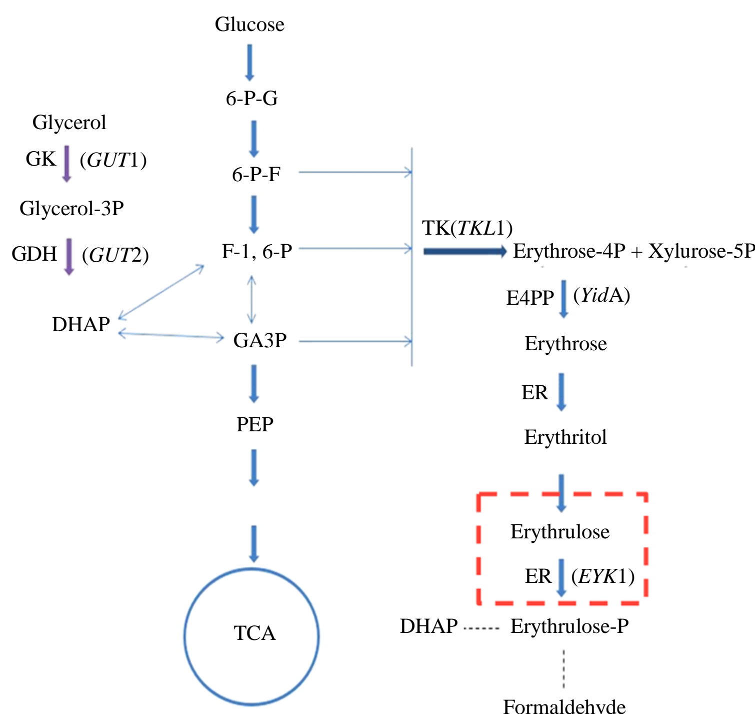

Fig. 1: Metabolic of erythritol in yeast; (GK: Glycerol Kinase; GDH: Glycerdehydrogenase; DHAP: Dihydroxyacetone Phosphate; 6-P-G: Glucose 6-Phosphate; 6-P-F: Fructose-6-Phosphate; F-1, 6-P: Fructose-1, 6-Diphosphate; GA3P: 3-Phosphoglyceraldehyde; PEP: Phosphoenolpyruvate; PA: Pyruvic Acid; TCA: Tricarboxylic Acid Cycle; TK: Transketolase; E4PP: Erythrose-4P Phosphatase; ER: Erythrose Reductase; EK: Erythrulose Kinase)

Material and Methods

Strains and Culture Conditions

Y. lipolytica Y-22 and *Y. lipolytica* YE4-2 were preserved in our lab. The mutant strain *Y. lipolytica* YE4-2 used in this study were derived from the wild-type *Y. lipolytica* Y-22 (Pei *et al.*, 2015). The *Y. lipolytica* strains were grown in Yeast extract Peptone Dextrose (YPD) medium containing 10 g yeast extract, 20 g tryptone and 20 g glucose in one liter Milli-Q water and for solid media, 20 g/L agar was added. Shake-flask cultures were performed in triplicate in a rotary shaker (DLHR-Q200, HDL APPARATUS) at 30°C and 200 rpm. After 72 h of preculturing in 35 mL of YPD liquid medium, cells were collected by centrifuging under 5600 g at 4°C (centrifuge, 5804R, Eppendorf) and were transferred to a 250 mL-flask containing 35 mL of fermentation medium (25% glucose, 0.5% NH_4CIT, 0.5% yeast extract, 0.025% $MgSO_4$, 0.025% KH_2PO_4 and 0.005% $CuSO_4$) for fermentation at 30°C and 200 rpm. Then the 96 h of fermentation cultures were collected for further analysis.

Screen of Antibody Resistant for Y. lipolytica

Y. lipolytica cells were collected by centrifugation of liquid culture at 4°C, 5600 g for 5 min, the sediment were diluted to 1×10^6 cells/ml after washing with sterile physiological saline. 100 µL (1×10^5 cells) of the dilution was coated to YPD plates containing different antibiotics in triplicate. The concentration gradient of antibiotics were set to 0, 400, 600, 700, 800, 1000 mg/L for geneticin G418 (Shenggong, China) and zeocin (Invitrogen, USA); 0, 20, 60, 150, 200, 300 mg/L for phleomycin (Invitrogen, USA) and 0, 200, 400, 600, 700, 800, 1000 mg/L for hygromycin B (Invitrogen, USA). The coated plates were placed in the incubator at 30°C for 7 days and the inhibitory effects of different concentrations of antibiotics on the strains were observed.

Selection of Electrical Shock Conditions for Y. lipolytica

The expression vector pPICZαA carrying zeocin/ phleomycin resistance gene was digested with restriction endonuclease Sac I to prepare a linearized plasmid. Different plasmid concentrations, shocking voltages and shocking time on the conversion efficiency to *Y. lipolytica* were tested. The linearized plasmid pPICZαA (5, 6, 7, 8, 9, 10 ug) was added to 80 uL *Y. lipolytica* YE4-2 competent cells for electroporation, the conversion voltage was 1500 V, 1800 V, 2000 V, 2200 V and the shocking time was 4, 5, 6 ms. After the electric shock, 1 mL of pre-cooled sorbitol was immediately added to the system and cells were placed at a 30°C shaker at 100 rpm/min for 1 h. Then 200 uL of the rejuvenated cells were coated to the YPD solid plate containing 300 mg/L of phleomycin. After incubation two days at 30°C, the transformants number was recorded and transformants were selected for PCR positive identification to determine transformation efficiency. The primers zeo-F/R used in PCR verification are shown in the appendix. *Construction of selective vector for Y. lipolytica*.

The sequence of pPICZαA was obtained from the NCBI website, primers were designed (see appendix for primers) according to the sequence information. The zeocin resistance gene expression cassette pTEF-pEM7-zeocin-TT (*ppz*) was amplified by PCR and ligated to the pCloneEZ-TOPO vector (Taihe Biotechnology, Beijing, China). The new plasmid was named as pCloneEZ::*ppz* and was transformed to *E. coli* DH5α. Positive transformants were screened on zeocin-resistant plates. Then pCloneEZ::*ppz* was extracted from positive transformants and digested with Xba I and Bgl II. The *ppz* fragment was cloned into the same enzyme digested (Xba I and Bgl) pUG6 vector to obtain *ppz* selectable plasmid pUG::*ppz*.

The *hpt* II gene in the pCAMBIA1300 plasmid was cloned by the same method and digested with Nco I and Cla I and then cloned into pUG6 vector to obtain *hpt* selectable plasmid pUG::*hpt*. All restriction enzymes were purchased from FastDigest Thermo Scientific™ (USA). The correctness of the resulting constructs was verified by DNA sequencing.

Construction of eyk1 Deleted Mutant based on Cre/loxp Recombination System

The *eyk1* Disruption Cassettes (DC) were constructed according to a cloning-free strategy derived from Cre-lox method (Fickers *et al.*, 2003). The mechanism of Cre/loxp system can be simply divided into the following steps (Vandermies *et al.*, 2017). (1) Constructing a vector carrying two palindromic structures, loxp, between which, a resistance screening marker gene can be attached; (2) a primer carrying a 40-80 bp target gene flank sequence and loxp sequence were applied to amplify the knockout component. (3) The linearized knockout module was transformed into a recipient cell by electroporation transformation and integrated into the genome by homologous recombination to obtain a transformant. (4) Linearized plasmid carrying the Cre recombinase was transferred to the transformant obtained in the previous step and then genes between loxp sites were associated, recombined and disintegrated under the effect of Cre (Dolinski *et al.*, 1997; Hirschman *et al.*, 2006). Since the two loxp sites are asymmetric and directional, it thus mediated three different recombination reactions (Vandermies *et al.*, 2017): (1) when the direction is opposite, the segment between the two sites is inverted; (2) when the direction is syntropy, the segment between the two sites is deleted; (3) when the two sites are on different molecules, the translocation would occur, as Fig. 2.

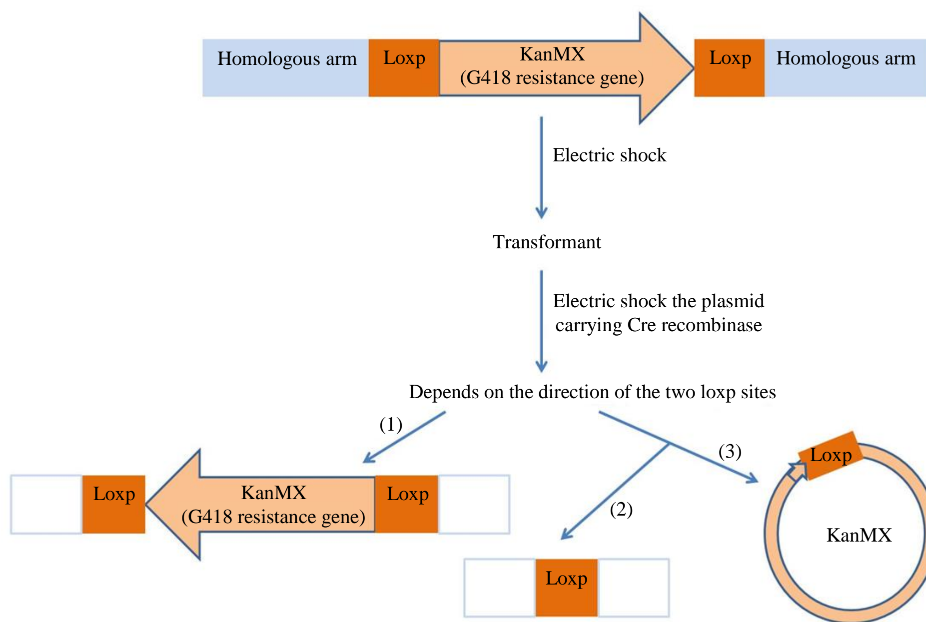

Fig. 2: The mechanism of Cre/loxp system

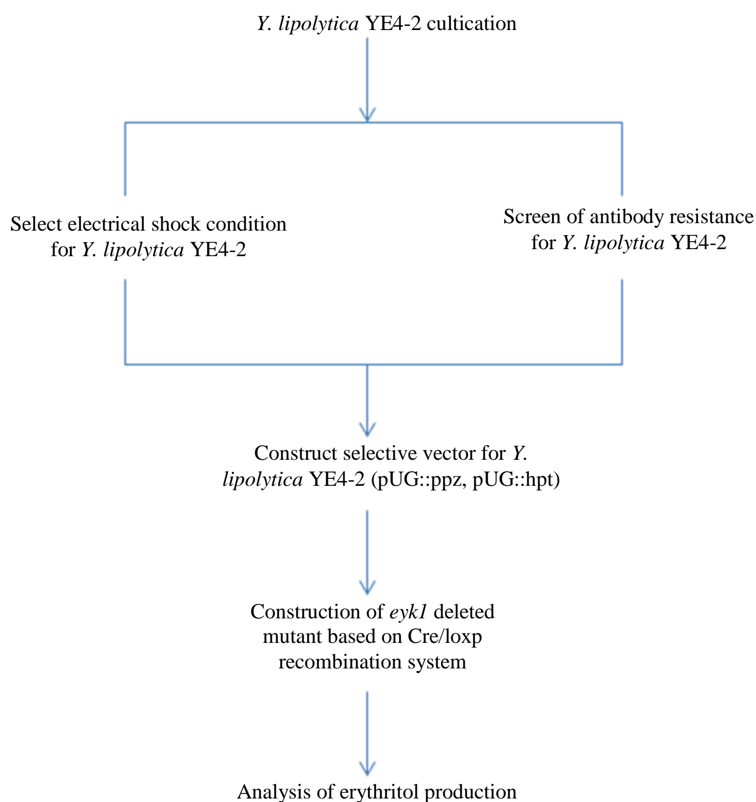

Fig. 3: Overall process map in this research

In this study, the primer EL-F/R was designed based on the *Y. lipolytica eyk1* gene and the pUG6::*hpt* II plasmid loxP-*hpt* II -loxP. The 5' ends of EL-F and EL-R have 60 bp homologous bases with the *Y. lipolytica eyk1* gene flankings, respectively. The 3' ends of EL-F and EL-R have 19 bases homologous with the loxP-*hpt* II-

loxP flanking sequences of plasmid pUG6. The pUG6-*hpt* II vector was used as the template and the knockout component containing *eyk1* up- -loxp-*hpt* II-loxp-*eyk1* down was amplified using EL-F/R primers. Primers sequences are shown in the appendix.

Then the knockout module (5 ug) was mixed with 80 uL *Y. lipolytica* YE4-2 competent cells and electroshocked at 2000 V for 6 ms. 1 mL of pre-cooled sorbitol was quickly added and shaken slowly for 2 h at 30°C. Then cells were applied to the YPD plate containing 800 mg/L hygromycin B and cultured at 30°C for 2 days. The transformed yeast was verified by PCR to identify whether the sequence of interest was correctly knocked out or integrated.

Quantifying Biomass, Glucose Concentration and Erythritol Concentration

Biomass was determined gravimetrically after the cells had been washed and dried at 105°C for 24 h ($W_{\text{dry biomass}} = W_{\text{clean tube with dry cells}} - W_{\text{the clean tube}}$). Biomass yield was defined as the dry weight of biomass (W) per liter fermentation broth (g/L). Glucose and erythritol concentrations were determined using an HPLC (Agilent 1200 series; Agilent Technologies) equipped with a refractive index detector and an Aminex HPX-87H ion exclusion column (300×7.8 mm; Bio-Rad). Elution was performed using 15 mM trifluoroacetic acid as the mobile phase at a flow rate of 0.5 mL/min and a temperature of 65°C (Jovanović *et al*., 2014). Their concentrations (g/L) were determined according to the standard curves established in this study. Glucose utilization rate = (glucose concentration before fermentation - glucose concentration after the fermentation)/glucose concentration before fermentation ×100%. Product yield (g/L) = erythritol concentration after fermentation- erythritol concentration before fermentation. Erythritol productivity (g/L/h) = product yield/fermentation time. Conversion rate (%) = product yield (g/L)/glucose utilized (g/L) ×100% (Germec *et al*., 2019).

Finally, an overall process map was drawn to clearly show the research steps (Fig. 3).

Results and Discussion

Screen of Antibody Resistant of Y. lipolytica

The most commonly used resistance screening markers for genetic engineering of industrial scale applied yeast are Geneticin (G418), zeocin, phleomycin, hygromycin B etc., (Fu, 2013; Vickers *et al*., 2013; Amelina *et al*., 2016). In order to achieve genetic modification of *Y. lipolytica* YE4-2, it is necessary to find a proper resistance screening marker. In this experiment, four commonly used yeast screening antibiotics were selected for the resistance screening test of the starting strain *Y. lipolytica* Y-22 and the mutant strain *Y. lipolytica* YE4-2. Since the same microorganism has a certain tolerance range for different antibiotics. This range is inconsistent for different antibiotics; in addition, different microorganisms have different tolerances for the same antibiotic. Therefore, we first searched the literature to find the range of sensitivity of *Y. lipolytica* to different antibiotics and then through gradient experiments, we finally determined its reasonable use concentration, so as to achieve the purpose of screening strains. According to the method in 2.2, 1×10^5 cell solutions were applied to YPD plates with different type and concentration of antibodies.

The growth on geneticin G418 was observed as shown in Table 1. It shows that even at a high concentration (1000 mg/L) of geneticin G418, *Y. lipolytica* can still grow and the inhibitory effect is not obvious. The growth of the cells on YPD plates with different concentrations of zeocin is shown in Tab.2. When zeocin reached 400 mg/L, the yeast was inhibited from growing within 48 h, however colonies were observed after 48 h. When the concentration reached 800 mg/L, the inhibitory effect could be extended to 72 h. When zeocin concentration was 1000 mg/L, only a few single colonies appeared after 96 h.

It was reported that phleomycin, which is the same family as zeocin and can also be degraded by the resistance gene *shble*, has a good inhibitory effect on zeocin-insensitive yeast (Chen *et al*., 2008; Falciatore *et al*., 1999). Cell dilution was applied to YPD plates with different concentrations of phleomycin according to the method of 2.2. After incubation at 30°C for a period of time, the growth was observed as shown in Table 3. When the concentration of phleomycin reaches 300 mg/L, it has obvious inhibitory effect on *Y. lipolytica*. However, the drawback is that phleomycin is relatively expensive, 1 mL of 20 mg/ml phleomycin requires 700 RMB, thus the cost will be high when applying phleomycin in such a high concentration.

The growth of the cells on YPD plates with different concentrations of hygromycin B is shown in Table 4. It can be seen that when hygromycin B reaches 200 mg/L or more (200-400 mg/L), it has an inhibitory effect on *Y. lipolytica*. However, after the incubation time increased to 72 h, colonies appear. When the antibiotic concentration was increased to 800 mg/L, *Y. lipolytica* YE4-2 could not grow, while for the starting strain Y-22, there are sporadic colonies growing after 120 h of culture. According to the characteristics of antibiotics, it is considered that the antibiotics may gradually break down. Therefore, hygromycin B can be used as a screening resistance for *Y. lipolytica* and the screening concentration is set at 800 mg/L.

Table 1: G418 resistant screening

			Culture time and growth condition		
No.	Antibody	Final concentration	24 h	48 h	72 h
①	G418	0 mg/L	+++	++++	+++++
②	G418	400 mg/L	+	++	+++
③	G418	600 mg/L	+	++	+++
④	G418	800 mg/L	+	++	++
⑤	G418	1000 mg/L	+	++	++

(Note: + Small colonies and low density; ++ Medium size and dense colonies; +++ Overgrown the entire plate, small colonies; ++++ Overgrown the entire plate, medium colonies; +++++ Overgrown the entire plate, large colonies; None: no clonies grow. The same note for the Tables 2, 3 and 4)

Table 2: Zeocin resistant screening

			Culture time and growth condition			
No.	Antibody	Final concentration	24 h	48 h	72 h	96 h
①	Zeocin	0mg/L	++	+++	++++	+++++
②	Zeocin	400 mg/L	None	+	++	++++
③	Zeocin	600 mg/L	None	+	++	+++
④	Zeocin	800 mg/L	None	None	+	++
⑤	Zeocin	1000 mg/L	None	None	None	+

(Note: see Table 1)

Table 3: Phleomycin resistant screening

			Culture time and growth condition		
No.	Antibody	Final concentration	24 h	48 h	72 h
①	Phleomycin	0mg/L	+++	++++	+++++
②	Phleomycin	20 mg/L	+	++	+++
③	Phleomycin	60 mg/L	+	++	+++
④	Phleomycin	150 mg/L	None	+	++
⑤	Phleomycin	200 mg/L	None	None	+
⑥	Phleomycin	300 mg/L	None	None	None

(Note: see Table 1)

Table 4: Hygromycin B resistant screening

		Culture time and growth condition				
Strain	Hygromycin B final concentration	24 h	48 h	72 h	96 h	120 h
Y-22	0mg/L	+	++	+++	+++	++++
YE4-2		+	++	+++	+++	++++
Y-22	200 mg/L	None	None	+	+	+++
YE4-2		None	None	+	+	++
Y-22	400 mg/L	None	None	+	+	+++
YE4-2		None	None	+	+	++
Y-22	600mg/L	None	None	None	+	+++
YE4-2		None	None	None	+	++
Y-22	700 mg/L	None	None	None	+	+++
YE4-2		None	None	None	+-	++
Y-22	800 mg/L	None	None	None	None	+-
YE4-2		None	None	None	None	None
Y-22	1000mg/L	None	None	None	None	None
YE4-2		None	None	None	None	None

(Note: see Table 1)

In summary, even under high concentration of geneticin G418, the inhibition effect to *Y. lipolytica* is not obvious; in the presence of zeocin, when the antibiotic concentration reaches 1000 mg/L, it has a good inhibitory effect within 96 h; in the presence of phleomycin, when the antibiotic concentration reaches

300 mg/L, it has a good inhibitory effect within 72 h; Zeocin and phleomycin are the same classes of antibiotics that can be inhibited by the same resistance gene (*shble*). Therefore, 1000 mg/L zeocin and 300 mg/L phleomycin could be selected as screening antibiotic and the effective screening time was 72 h. Hygromycin B resistance has an obvious inhibitory effect when the concentration is above 800 mg/L and has a good screening effect within 120 h. Therefore, hygromycin B was selected as a screening antibiotic with a screening concentration of 800 mg/L and an effective screening time of 96 h.

Optimization of Electric Shock Conditions of Y. lipolytica

Y. lipolytica is an unconventional high-osmotic resistant yeast, its cell wall is relatively thick. However, most of the electroporation methods for *Y. lipolytica* directly apply those used in common yeasts such as *Pichia pastoris* and *Saccharomyces cerevisiae* which are not so efficient (Fraczek *et al.*, 2018). In this experiment, the electroporation transformation conditions of *Y. lipolytica* were explored to find better transformation parameters.

Here, different concentrations of pPICZαA (carrying zeocin/phleomycin resistance gene *shble*) were transformed under different shock voltage and time. After the transformation, *Y. lipolytica* was applied to the YPD plate containing 300 mg/L of phleomycin and cultured at 30°C for two days. The number of single colonies was recorded and the transformants were subjected to PCR for verification. Results show that the most monoclonal can be obtained when 5 ug plasmids /80 uL competent cell were transformed under 2000 V for 6 ms and the transformants grow well; The transformants under this electric shock condition were identified by PCR and the positive conversion rate was 80%. Therefore, plasmid 5 ug/80 uL, at 2000 V, for 6 ms were selected as the conditions for electrotransformation. The electrotransformation method uses transient high-voltage electric pulses to create temporary pores on the cell surface, which can form small pores on the surface of the cell membrane (Saito *et al.*, 2006). At the same time, due to the electric shock action, the cell membrane is fused and finally a large pore is formed on the cell membrane for easy DNA entry. But there are many factors that affect the efficiency of electric conversion, such as electric shock voltage, pulse time, DNA content and so on. The results show that the electrical conversion efficiency is proportional to the content of exogenous DNA within a certain range, but excessive addition of exogenous DNA will cause the conversion efficiency to decrease. This result is basically consistent with literature reports (Kimoto *et al.*, 2003). In addition, under high shock voltage, the cell wall and plasma membrane can be instantaneously broken, while under low field strength, the accumulated charge must reach a certain strength before it can act on the plasma membrane to form pores. Therefore, the combination of proper pulse electric shock voltage and pulse time can improve the efficiency of electric conversion (Kimoto *et al.*, 1997).

Construction of the Y. lipolytica Selective Vector

The pUG6 vector contains a Cre-loxp recombination system and is commonly used for yeast gene knockout (Twaruschek *et al.*, 2018; Wei *et al.*, 2018). Since *Y. lipolytica* is sensitive to zeocin and hygromycin B, the pUG6 vector was first modified to achieve zeocin and hygromycin B resistance, respectively. The zeocin resistance gene was obtained by PCR from the plasmid pPICZαA and the hygromycin B resistance gene was obtained by PCR from the plasmid pCAMBIA1300. The resistance gene fragment and the pUG6 were cleaved and ligated according to the method 2.4 to construct the *Y. lipolytica* selective vector pUG6::*ppz* and pUG6::*hpt* II respectively (Fig. 4).

The results of transformation of pUG6::*ppz* to *E.coli* are shown in Fig. 5a. The migration rates of extracted plasmids No. 1-12 were slower than that of pUG6 (No. 13), which proved that the plasmids of 1-12 were all recombinant plasmids. The results of double enzyme digestion showed that the 4004 bp vector band (PUG6) and the 1174 bp *ppz* fragment could be successfully cleaved from the recombinant plasmid (Fig. 5b). Further plasmid sequencing also verified this (data not shown). Therefore, it was confirmed that the *ppz* fragment was successfully constructed on pUG6. At the same time, the *E. coli* transformants of the pUG6::*ppz* plasmid can be grown on a plate containing 50 mg/L zeocin, indicating that the pUG6::*ppz* vector has been successfully constructed. Thus it could be used for the next gene knockout assay.

For the transformant of pUG6:: *hpt* II, the hygromycin B gene were verified by PCR (Fig. 6a) and restriction enzyme digestion (Fig. 6b). From PCR verification, plasmids 3, 9, 10 and 11 were identified as positive transformant and enzyme digestion further proved it contains a 1041 bp *hpt* II and 3890 bp linear vector. The results showed that *hpt* II was successfully constructed on the pUG6 vector.

Disruption of eyk1 Gene in Y. lipolytica YE4-2

According to the method in 2.5, the knockdown component eyk^{up} -loxp-*hpt* II-loxp-eyk^{down} (2684 bp) was amplified using the recombinant plasmid pUG6::*hpt* II as template and EL-F/R (carrying *eyk1* flank sequences and loxp sequences) as primers. The liner

knockdown component was transformed into *Y. lipolytica* YE4-2 and after two days of culturing, the transformants were collected to screen *eyk1* disruption strains. Hygromycin B resistance gene based *hpt*-F/R was applied as primer and the results are shown in Fig. 7a. From transformants 1, 3 and 4, a band (1041 bp) equal in size to the positive control strip was cloned. It was initially determined that the hygromycin B resistance gene was successfully transferred into yeast genome DNA.

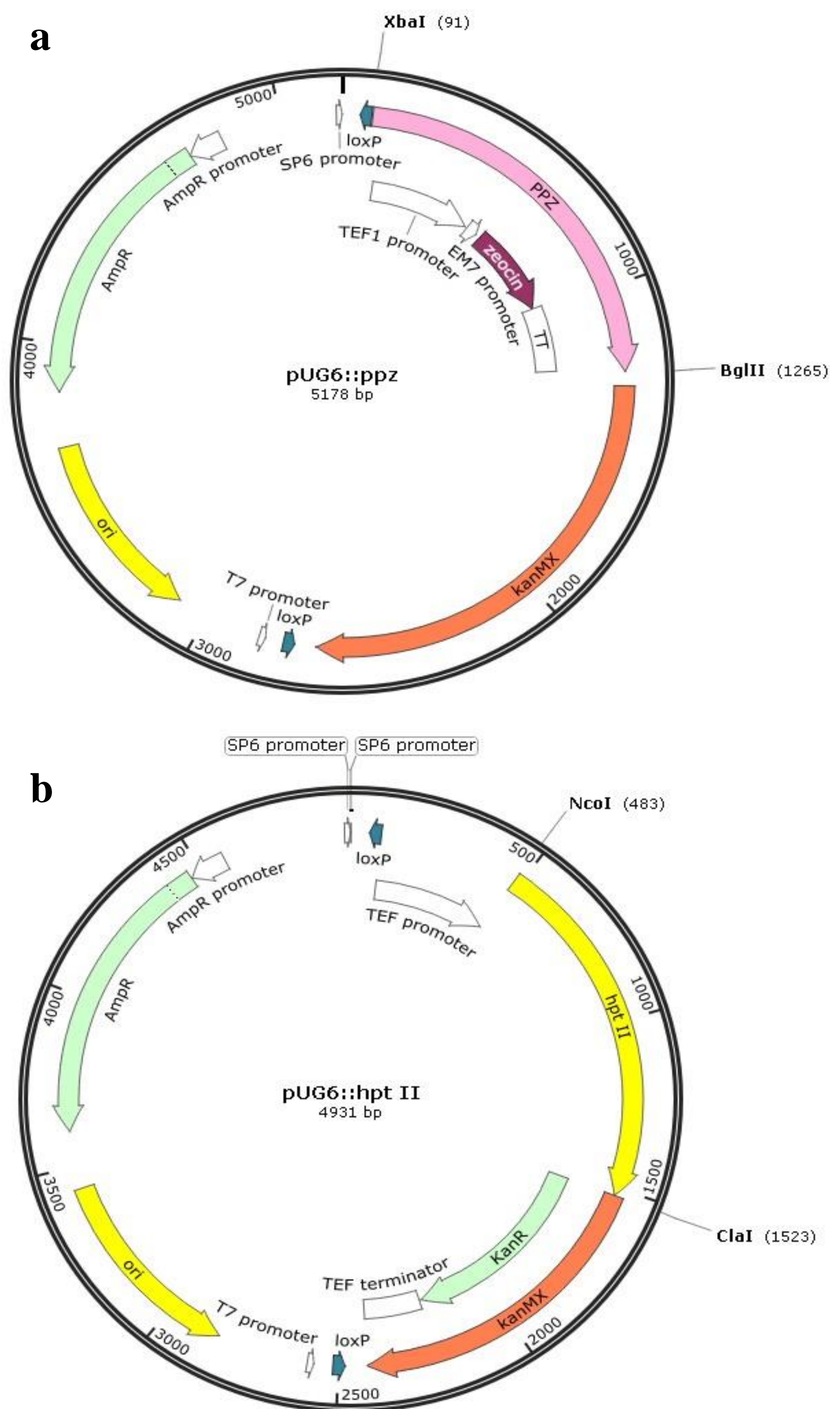

Fig. 4: Plasmid map of pUG::*ppz* and pUG::*hpt*II. a: Plasmid map of pUG::*ppz*; b : Plasmid map of pUG::*hpt*II

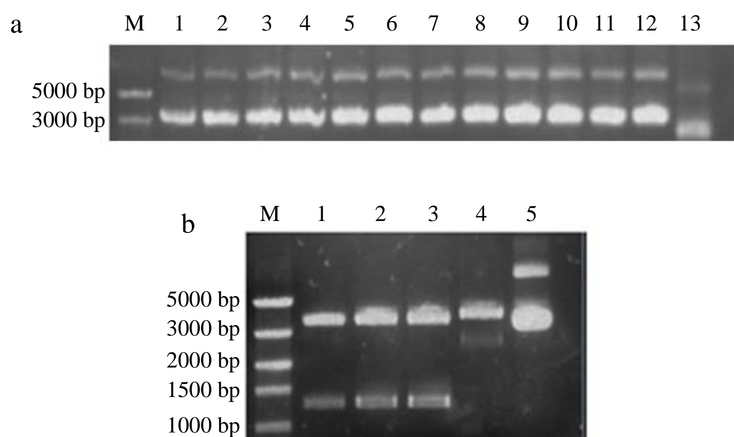

Fig. 5: Transformatnt verification of plasmid pUG::*ppz*. a. PCR identification of pUG::*ppz* (M: 5000bp DNA maker; 1-12: PCR result of the transformants; 13: PCR result of pUG6)*;* b. Double enzyme digestion identification of pUG::*ppz* (M: 5000bp DNA maker; 1-3: *Xba I/Bgl II* digestion of plasmid pUG*::ppz*; 4: Plasmid of pUG6; 5: Plasmid of pUG6::*ppz*)

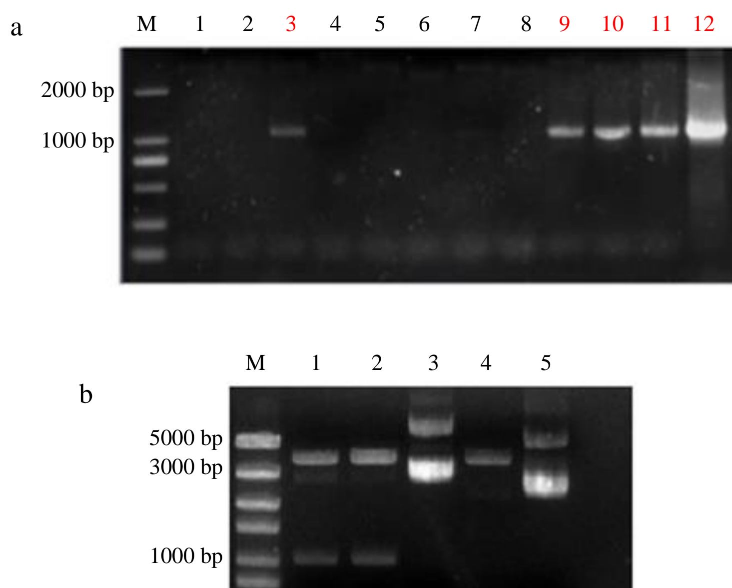

Fig. 6: Transformatnt verification of plasmid pUG:: *hptII*. a. PCR identification of pUG::*hptII* (M: 2000bp DNA maker; 1-11: PCR result of the transformants; 12: positive control); b. Double enzyme digestion identification of pUG::*hptII* (M: 5000bp DNA maker; 1-2: *Nco I*/*Cla I* digestion of plasmid pUG::*hptII*; 3: *Nco I*/*Cla I* digestion of plasmid pUG::*hyg*; 4: *Nco I*/*Cla I* digestion of pUG6; 5: Plasmid pUG6)

In order to further determine whether the *eyk1* gene was knocked out, the genomic DNA of the transformants was extracted for PCR verification. The genome DNA of each transformants as well as positive controls (Y-22, YE4-2) were extracted and diluted to the same concentration as template and eyk-F/R designed according to *eyk1* gene flanks were used as primers. As shown in Fig. 7b, the lanes 1-2 are *Y. lipolytica* Y-22 and YE4-2 respectively and the lanes 3-6 were transformants. The results showed that the *eyk1* gene fragment could be amplified from all of the strains. However, the brightness of stripe in lane 5 is 50% lighter compared with the strips of untransformed *Y. lipolytica* or other transformants. When the DNA of each sample was diluted again to the same concentration as templates, the same PCR result was obtained. Since the presence of single and diploid generation in *Y. lipolytica*, it is speculated that one allele in the diploid strain was knocked out, which led to the phenomenon. Therefore, the No. 5 transformant (YE4-2-5) was subjected to a subsequent shake flask fermentation test.

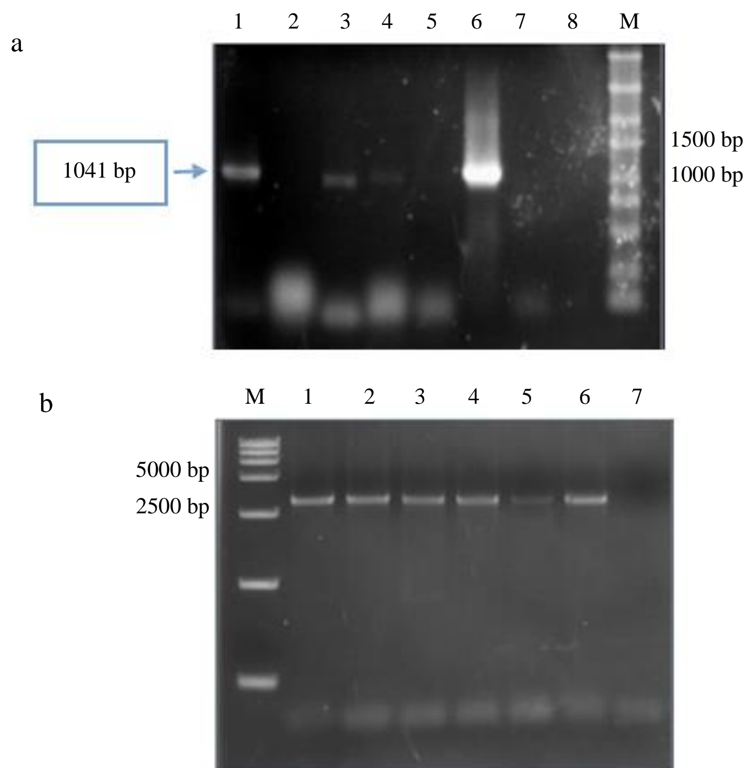

Fig. 7: Screening of *eyk1* deleted transformants. a. Sub-PCR identification of transformants transferred with the knockdown component eyk^{up} -loxp-*hpt* II-loxp-eyk ^{down} (M: 5000bp DNA maker; 1-5 PCR of the transformants; 6: Positive control (PCR of pUG6::*hptII*); 7: PCR of *Y. lipolytica* YE4-2; 8: Negtive control); b. Identification of inverter PCR (M: 5000bp marker; 1: PCR identification of Y-22 genome DNA; 2: PCR identification of YE4-2 genome DNA; 3-6: PCR identification of the transformants genome DNA; 7: Negative control)

Table 5: Fermentation of the recombinant strains

Strain	Glucose utilization (%)	Conversion rate (%)	Yeild (g/L)	Productivity (g/L/h)
YE4-2	54.20±2.18	32.50±2.62	58.3±1.09	0.61±0.08
YE4-2-5	65.02±4.36	48.51±1.25	94.6±3.78	0.99±0.26
Increased	19.96±1.33%	49.26±1.67%	62.26±1.72%	62.26±2.35%

The Productivity of Erythritol in the Recombinant Strain Y. lipolytica YE4-2-5 was Significantly Improved

The fermentation was carried out in the shake flask with 30% glucose as substrate and after 96 h of fermentation, cultures were collected for analysis. Results showed that the average conversion rate ($Y_{erythritol/glucose}$) of the starting strain YE4-2 was 0.325 and the average conversion rate of the recombinant strain No. 5 was 48.51%, which was 49.26% higher than that of YE4-2 (Table 5). The t-test results showed that the significance reached 5%.

In the past, erythritol productivity in *Y. lipolytica* has largely been improved by classical approaches that consisted of optimizing either the culture medium or culturing conditions (Mirończuk *et al*., 2014; Rymowicz *et al*., 2008; Yang *et al*., 2014). Recently, however, (Mirończuk *et al*., 2016) found that overexpression of glycerol kinase and glycerol dehydrogenase encoding genes (*gut1* and *gut2*) significantly increased the productivity of erythritol of *Y. lipolytica* A101. Compared with the wild-type yeast, overexpression of *gut1* alone increased the erythritol yield by 23%, while overexpression of *gut1* and *gut2* together increased the yield by 35%, however, overexpression of *gut2* alone led to a 28% decrease of the erythritol yield. Carly *et al*. (2017) overexpressed *gut1* (glycerol kinase), *gut2* (glycerol dehydrogenase), *yid A* (Erythrose-4PPhosphatatase), *tkl1* (transketolase), *alr* (erythrose reductase) in *Y. lipolytica*, which aimed to increase the flow of carbon from glycerol to erythritol. The results of fermentation with glycerol as substrate showed that the overexpression of *gut1* and *gut2* lead to similar results to that of (Mirończuk *et al*., 2016); while overexpressing *tkl1-gut1*, the yield of erythritol

was increased by 42%; however, overexpression of *alr, yid A* reduces the yield of erythritol. They also disrupted *eyk1* gene which involves in the erythritol catabolism based on the *tkl1-gut1* mutant strain and finally resulted in a 75% increase of the erythritol productivity. Our study also showed that one allae of *eyk1* knock out could significantly increase the erythritol productivity which further proved that *eyk1* gene is crucial on erythritol metabolism pathway; This laid a foundation for subsequent transformation and multiple allele knockout engineering.

Current biotechnological production of erythritol, take a highly concentrated glucose (typically 40% (w/v)) solution as substrate. These processes reach 40% (w/w) conversion rate of D-glucose to erythritol (Moon *et al.*, 2010; Jovanović *et al.*, 2014). In our study, the conversion rate of glucose to erythritol could reach 48.51±1.25%, which is greatly improved.

Conclusion

This study reported the establishment of genetic transformation system for the industrialized *Y. lipolytica* mutant YE4-2 and Cre/loxp homologous recombination knockout system containing the selective marker was constructed according to the strain's antibiotic sensitivity. After the recombination of the knock out component eyk^{up}-loxp-*hpt* II-loxp-eyk^{down} with *Y. lipolytica* YE4-2 genome DNA, the results show that the resistance gene has entered the yeast genome. While according to the PCR verification of *eyk1* gene, the brightness of the transformant *eyk1* band was about half of that in untransformed *Y. lipolytica* YE4-2 at the same DNA template concentration. Due to the presence of haploid and diploid in *Y. lipolytica*, it was preliminarily determined that one allele of *eyk1* in this recombinant strain was knocked out. The conversion rate of the recombinant strain from glucose to erythritol was significantly (5% significant by T test) improved from 32.50 to 48.51%, which was 49.26% higher than the average conversion rate of YE4-2 and the erythritol productivity is increased by 62.26%. Our study show that one allae of *eyk1* knock out could significantly increase the erythritol productivity which further proved that *eyk1* gene is crucial on erythritol metabolism pathway; This laid a foundation for subsequent transformation and multiple allele knockout engineering.

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Author's Contributions

Xinhe Zhao: Participated in the whole experiment process and also contributed to the interpretation of the results and manuscript preparation.

Tianlong Huang, Yaxin Zhang and Xiaojie Ren: Participated in part of the experimental design.

Yuanda Song: Ameliorated the manuscript.

Bei Guo: Contributed to the guidance of experimental design and ameliorated the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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Appendix

Primer list

Primers	Primer sequences（5'-3'）	Purpose and description
ppz-F2	GCTCTAGAGCGTGAGACCTTCGTTTGTGCG (*xba* I)	Amplify *ppz* fragment
ppz-R2	GAAGATCTTCTTTCGGTTAGAGCGGATGTG (*Bgl* II)	Amplify *ppz* fragment
eyk1-PF	GTTGTGTGATGAGACCTTGGTGC	Amplify *eyk1* fragment
eyk-TR	GTTTAGGTGCCTGAAGACGGTG	Amplify *eyk1* fragment
zeo-F	CAATCTAATCTAAGGGGCGGTGT	Zeocin resistance identification
zeo-R	GGGAGGGCGTGAATGTAAGC	Zeocin resistance identification
EL-F	CTTCTATAAGAAGCTCCTTTCCCCACAATTGGCCCACAC GACACTTCTACACACTTACACaacgcggccgccagctgaag	pUG6 disruption cassettes amplication primer
EL-R	GTTCCATCATAAATATCATTAAAAATTATATGCCATTTACA AGTGCTCGTACAAGTACTCctatagggagaccggcagat	pUG6 disruption cassettes amplication primer
hpt-F	CATGCCATGGCATGTATGAAAAAGCCTGAACT（*Nco* I）	Hygromycin B resistant gene primer
hpt-R	CCATCGATGGCGGTCGGCATCTACTCTATT（*Cla* I）	Hygromycin B resistant gene primer
eyk^{up}-F	TATCGGCAACCTTAGCGGCA	P fragment primer
eyk^{up}-R	AAAGGCCATTTAGGCCTGTAGAAGTGTCGTGTGGGC	P fragment primer (*sfi* I)
eyk^{down}-F	TATGGCCTTGATGGCCGAGAAGCGAAGGAATATGAG(*sfi* I)	T fragment (*sfi* I)
eyk^{down}-R	TTAGGTGCCTGAAGACGGTG	T fragment