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SHORT COMMUNICATION

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Enhanced acetate ester production of Chinese liquor yeast by overexpressing *ATF1* through precise and seamless insertion of *PGK1* promoter

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Abstract As the most important group in the flavor profiles of Chinese liquor, ester aroma chemicals are responsible for the highly desired fruity odors. Alcohol acetyltransferase (AATase), which is mainly encoded by *ATF1*, is one of the most important enzymes for acetate ester synthesis in *Saccharomyces cerevisiae*. In this study, we overexpressed *ATF1* in Chinese liquor yeast through precise and seamless insertion of *PGK1* promoter (*PGK1p*) via a novel fusion PCR-mediated strategy. After two-step integration, *PGK1p* was embedded in the 5′-terminal of *ATF1* exactly without introduction of any extraneous DNA sequence. In the liquid fermentation of corn hydrolysate, both mRNA level and AATase activity of *ATF1* in mutant were pronounced higher than the parental strain. Meanwhile, productivity of ethyl acetate increased from 25.04 to 78.76 mg/l. The self-cloning strain without any heterologous sequences residual in its genome would contribute to further commercialization of favorable organoleptic characteristics in Chinese liquor.

Keywords Chinese liquor yeast · Acetate esters · *ATF1* · Seamless *PGK1p* insertion · Self-cloning

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Introduction

Chinese liquor (or alcoholic spirit), one of the six most well-known distillates in the world, has a history dating back thousands of years. The current annual output of Chinese liquor is estimated to exceed five million metric tons in China. As the largest and most important group in the flavor profiles of Chinese liquor, ester aroma chemicals are responsible for the highly desired fruity odors. The major flavor-active esters in Chinese liquor are acetate esters such as ethyl acetate (solvent-like aroma), isoamyl acetate (banana-like aroma) and isobutyl acetate (fruity-like aroma) [15]. Several enzymes are involved in the formation of esters, of which the *ATF1*-encoded alcohol acetyltransferase (known as AATase I or Atf1p) is best studied and has the most activity in *Saccharomyces cerevisiae* [6, 8, 13]. It was also shown that deletion or overexpression of *ATF1* influenced the concentrations of ethyl acetate to a lesser degree than the concentrations of isoamyl acetate in beer, sake and wine [7, 9, 12]. For Chinese liquor yeast, little is known about the contribution of *ATF1* to a broad range of esters content.

Since genetic transformation of yeast was firstly established in 1978, tremendous progress has been achieved in yeast biology. However, the residual heterologous genes are problematical due to the fear of drug-resistance gene transfer to pathogenic organisms, the potential toxic and allergic effects of heterologous expression products [1]. Recently, self-cloning industrial yeast without any exogenous DNA residual after gene manipulation was constructed by a two-step replacement protocol [7, 11]. Multi-step of DNA ligation as well as the limited restriction sites resulted in difficulties in constructing chimeric plasmids, which were indispensable to achieve two-step integration. Consequently, we designed a novel PCR-mediated two-step

method combining advantages of fusion PCR and two-step integration procedure to achieve the precise overexpression modification.

In this study, we constructed a high acetate esters production industrial Chinese liquor strain, in which *ATF1* was overexpressed through two-step integration protocol using the selectable *URA3* marker, an ura3$^-$ yeast strain, and a drug, 5-fluoroorotic acid [4]. A plasmid carrying a fusion fragment consist of the upstream sequence, *PGK1* promoter (*PGK1p*) and downstream sequence of insertion loci was firstly constructed. The plasmid was linearized and subsequently subjected to the two-step integration protocol, obtaining a precise insertion of *PGK1p* into the upstream of *ATF1* without introduction of any extraneous DNA sequences. The mRNA level of *ATF1* and the acetyltransferase activity in transformant were investigated. The fermentation performance and acetate esters production of the engineered strain were also studied. This study lays a strong foundation for commercial application of Chinese liquor yeast with high ester property.

Materials and methods

Strains, medium and culture conditions

The haploid Chinese liquor yeast CLX14 provided by Angel yeast Co. Ltd, was used as the host strain. The laboratory strain W303-1a (*MATa ade2 ura3 leu2 trp1 his3 can1*) was used as a template to amplify the mutant *URA3* gene. *Escherichia coli* strain DH5α, which was used for plasmid construction and amplification, was incubated at 37 °C in Luria–Bertani medium (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl) supplemented with 100 mg/l ampicillin. Yeast strains were grown at 30 °C in YPD medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose). Cells transformed with linearized YIplac211-UPD were spread onto SD-URA3 medium (20 g/l glucose, 20 g/l agar powder and 6.7 g/l yeast nitrogen base without amino acids, supplemented with all the auxotrophic requirements except uracil) for selection of uracil prototrophic transformants. 5-Fluoroorotic acid (5-FOA) plate (6.7 g/l yeast nitrogen base without amino acids, supplemented with all the auxotrophic requirements, 20 g/l glucose, 100 mg/l 5-FOA) was used for selection of uracil auxotrophic transformants. All solid media used in this study contained 2 % agar.

Plasmid construction

The plasmid vector YIplac211 was used to construct the recombinant plasmid YIplac211-UPD. The oligonucleotide sequences of polymerase chain reaction (PCR) primers used in this study are listed in Supplementary Table 1. Plasmid DNA was prepared from DH5α and yeast chromosomal DNA was isolated by a yeast genomic DNA extraction kit (Beijing Solarbio Science & Technology). The procedure of integration plasmid YIplac211-UPD construction is summarized in Fig. 1a. Chimeric DNA fragment consisting of the 5′-upstream region (U) of the target loci, promoter of choice (P) and downstream sequence (D, N-terminal sequence of *ATF1*) was generated through two rounds of PCR. In the first round of PCR, U, P and D DNA segments were independently amplified from CLX14 chromosomal DNA template using primer pairs (UF and UR pair, PF and PR, DF and DR). In the second round of PCR, fusion PCR, the overlapping sequences served as primers to allow for extension to the subsequent chimeric products. PCR, in which purified U, P and D fragments were used as templates without primer addition, was firstly conducted. Cycling parameters: initial denaturation 50 °C (45 s), subsequent step 72 °C (2 min), 10 cycles total. Then, PCR production purified through gel extraction was used as template with primers UF, DR to generate complete gene sequence of UPD. The resulting fusion PCR product was double digested by *Bam*HI–*Kpn*I and inserted into the integrated vector YIplac211, creating the plasmid YIplac211-UPD.

Construction of the recombinant yeast strain

Yeast transformation was performed as lithium acetate procedure reported previously [10]. The host Chinese liquor yeast CLX14 was first subjected to *ura3* attenuation similarly as described in our previous work [4], obtaining CLX14-u3. The first step of integration was carried out through transformation of linearized recombinant plasmid YIplac211-UPD (digested by *Nru*I) into CLX14-u3, resulting in homologous recombination between the flanks of linearized plasmid and the chromosome sequence (Fig. 1b). Transformants were spread onto SD-URA3 medium and verified by colony PCR using primer pairs of YIP-UPD-F and YIP-UPD-R, YIP-UD-F and YIP-UD-R. The pop-out of the plasmid occurred through the integration recombination between DRs (direct repeats, U or D). Recombination of the U repeats led to recovery to a wild-type strain, but that of the D repeats resulted in a mutant possessing PGK1p-ATF1 without the extraneous DNA sequences (Fig. 1b). Transformants obtained from the first-step integration were cultured, spread onto 5-FOA plates and verified by colony PCR using primer pairs of UD-F and UD-R, UP-F and UP-R, PD-F and PD-R (Fig. 2b). The attenuated *ura3* in CLX14-u3-P was finally restored through transformation of wild-type *URA3* sequence, which was amplified via PCR using CLX14 as template.

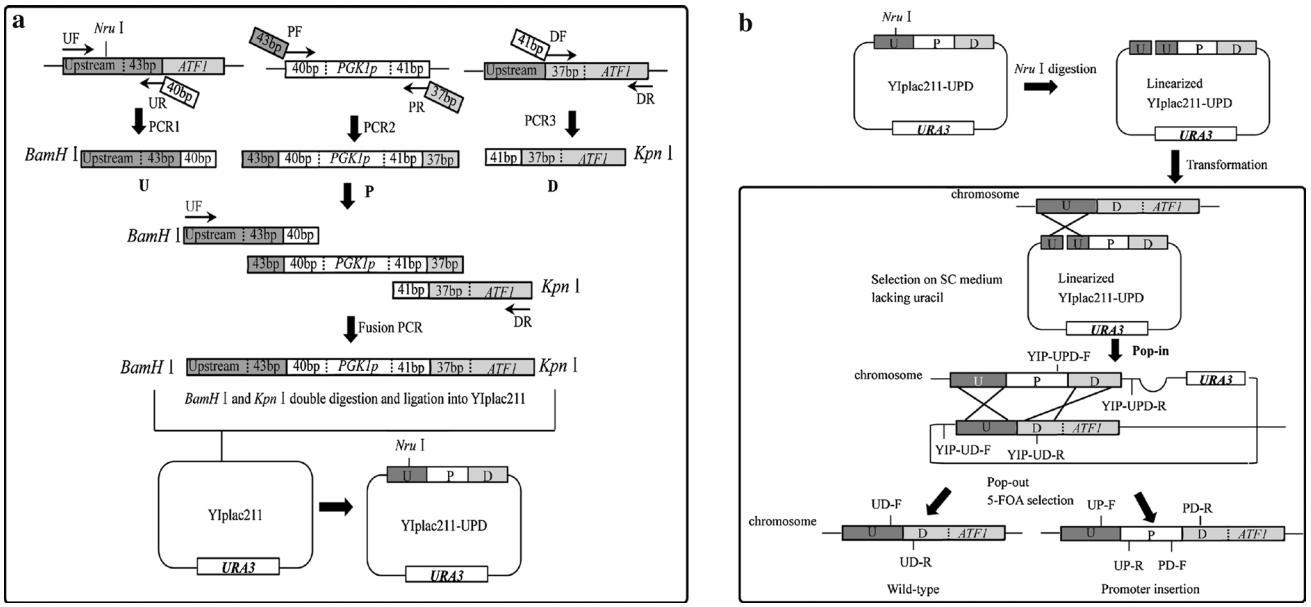

Fig. 1 Procedure of two-step integration method for promoter insertion. **a** Construction of the vector YIplac211-UPD. The *upstream* flank of *ATF1* (U), the *PGK1p* (P) and the *downstream* flank of *ATF1* (D) in the CLX14 genome was amplified in PCR1, PCR2 and PCR3, respectively. Fusion PCR was performed by mixing the PCR1, PCR2 and PCR3 products as templates in PCR4 to obtain UPD. After digestion of *Bam*HI and *Kpn*I, the fusion fragment UPD was inserted into YIplac211. The resulting plasmid was named YIplac211-UPD. The single *Nru*I site within *upstream* flank of *ATF1* was used for restriction enzyme digestion prior to transformation. **b** Schematic illustration of two-step integration strategy. YIplac211-UPD was digested with *Nru*I and introduced into the chromosome of CLX14-u3. The intermediate integrants, CLX14-u3-Y were isolated by selection on SC medium lacking uracil. Integration of linearized plasmid produced the tandem repeats of flanks. CLX14-u3-Y was spread on 5-FOA plates to obtain CLX14-u3-P. Integration homologous recombination between the *ATF1* upstream sequence resulted in a wild-type strain, but that between the *downstream* sequence resulted in promoter insertion without any extraneous DNA sequences

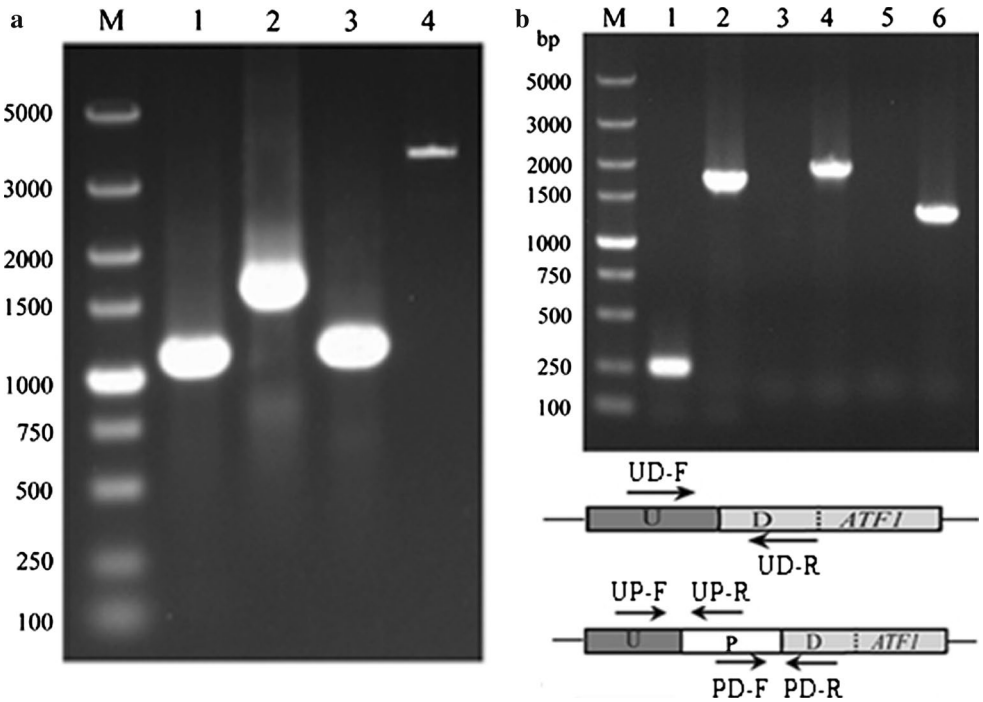

Fig. 2 Agarose gel analysis and sequence analysis. **a** Agarose gel showing production by two rounds of PCR. *Lane 1* The primers used were UF and UR, *lane 2* The primers used were PF and PR, *lane 3* the primers used were DF and DR (CLX14 genome as a template), *lane 4* the primers used were UF and DR (mixing U, P and D as templates). **b** Agarose gel showing production by colony PCR of the second-step integrants. *Lane 1* control CLX14-u3 as a template, *lane 2* CLX14-u3-P as a template (the primers used were UD-F and UD-R), *lane 3* control CLX14-u3 as a template, *lane 4* CLX14-u3-P as a template (the primers used were UP-F and UP-R), *lane 5* control CLX14-u3 as a template, *lane 6* CLX14-u3-P as a template (the primers used were PD-F and PD-R)

Real-time quantitative PCR and enzyme activity assays

Yeast mRNA was extracted and transcribed in reverse to produce cDNA using a cDNA synthesis kit (TIANScript RT Kit, TIANGEN, China). The *ATF1* gene expression was assessed via real-time quantitative PCR (RT-qPCR) using SYBR FAST qPCR Kit Master Mix (2×) Universal (KAPA Biosystems) with the primer pair ATF1-F and ATF1-R. The amplification protocol was a pre-incubation of 5 min at 94 °C, followed by 40 cycles of denaturation for 3 s (95 °C) and annealing and then polymerization for 30 s at 60 °C. In the final cycle, the temperature was held at 72 °C for an additional 10 min. The reaction was quantitatively analyzed using the $2^{-\Delta\Delta Ct}$ method. The *ATF1*-encoded AATase activity was measured using method as described previously [16]. One unit of AATase activity was defined as the amount of ethyl acetate (μmol) produced by certain weight of centrifuged yeast cell (1 g) per hour at 25 °C.

Fermentation experiments

The corn hydrolysate medium was prepared by gelatinizing the mixture of corn flour 60 g and 65 °C water 130 ml for 20 min in a 250-ml conical flask. Then the flasks were stored at 90 °C for 90 min with thermostable α-amylase (2×10^5 U/ml; Novozymes, Denmark), and subsequently saccharifying the mixture at 60 °C for 30 min with a saccharifying enzyme (200 U/ml; Novozymes, Denmark). The resulting mixture was cooled at room temperature to 30 °C for fermentation. Yeast cells were precultured in 4 ml of 8°Bx corn hydrolysate medium at 30 °C for 24 h, then transferred into 36 ml of 12°Bx corn hydrolysate in a 50-ml conical flask, and stored at 30 °C for 16 h. A total of 15 ml of the second-precultured yeast solution was transferred to the prepared corn semi-solid medium. The mixture was fermented at 30 °C till weight loss of CO_2 after interval 12 h was less than 1 g.

Gas chromatography (GC) analysis

GC has been widely used to analyze volatile compounds in Chinese liquor [5]. The corn broth after fermentation was distilled and used for GC analysis. The analysis was performed on an Agilent 7890C GC with AT.LZP-930 column (50 m × 320 μm internal diameter and 1 μm coating thickness). Nitrogen was used as the carrier gas and the GC conditions were used as follows: injector temperature was 200 °C, the split ratio was 5:1 and the injection volume was 1.0 μl with constant rate of 1 ml/min. The oven temperature program was as follows: 50 °C (8 min) followed by an increase to 120 °C at 5 °C/min, the final temperature was maintained for 8 min. Ethyl acetate, isoamyl acetate and isobutyl acetate were purchased from Merck.

Results

Construction of recombinant *Saccharomyces cerevisiae*

To achieve the precise and seamless insertion of *PGK1p* in the 5′-terminal of the target gene *ATF1*, combination of fusion PCR and two-step integration strategy was carried out in CLX14. U (1,048 bp), P (1,479 bp) and D (1,046 bp) were ligated via fusion PCR to obtain the resulting UPD fragment. Agarose gel electrophoresis of PCR production is shown in Fig. 2a. The resulting recombinants were verified via PCR using the primer pairs of YIP-UPD-F and YIP-UPD-R, YIP-UD-F and YIP-UD-R with CLX14-u3 (negative) and plasmid YIplac211-UPD (positive) as controls. Results (not shown) verified the integration of linearized plasmid YIplac211-UPD into the target loci.

When primer pairs of UP-F and UP-R, PD-F and PD-R, UD-F and UD-R were used to verify the recombinants after the second-step integration, a 1.9, 1.3 and 1.7 kb production was amplified with CLX14-u3 negative control. Results (Fig. 2b) showed the insertion of *PGK1p* before the *ATF1* ORF. Furthermore, sequencing results indicated the precise insertion of *PGK1p* in 5′-terminal of the target gene *ATF1* without any extraneous DNA residual.

Analysis of *ATF1* gene expression and measurement of AATase activity

To confirm the *ATF1* gene overexpression, we quantified the mRNA level of *ATF1* gene expression by RT-qPCR and measured the activity of *ATF1*-encoded acetyltransferase. The RT-qPCR results and AATase activity in the mutant were 40- and 5-fold higher than the parental strain, respectively (Fig. 3). These results confirmed the overexpression of the *ATF1* after *PGK1p* insertion led to a significant increase of gene expression and enzyme activity.

Fermentation characteristics of engineered strains

Stable performance of engineered strain is important for industrial fermentation. We further investigated the physiological characteristics (weight loss of CO_2, ethanol and residual sugar) of CLX14 and CLX14-P to assess their fermentation ability in corn semi-solid medium. The wild type and recombinant strain had similar fermentation characteristics regarding CO_2 loss (20 g), ethanol production (15 %, v/v, 20 °C) and residual sugar (29 g/l).

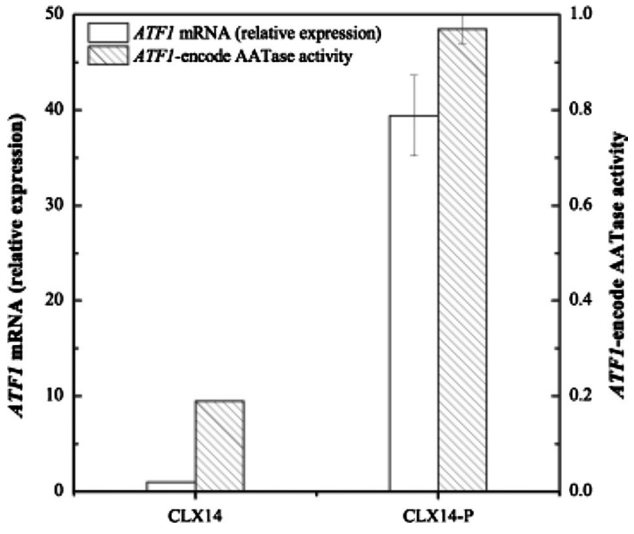

Fig. 3 *ATF1* gene expression levels and enzyme activity in the recombinant strain CLX14-P and CLX14. *ATF1* mRNA levels were analyzed by RT-qPCR and the experiments were repeated three times. Data are the average of three independent experiments. *Error bars* represent ± SD

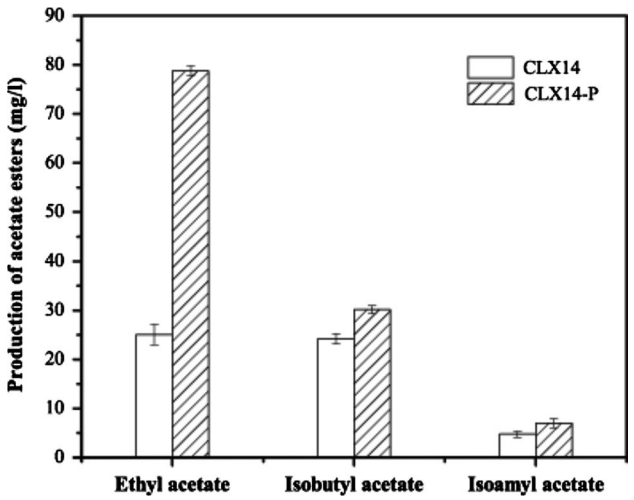

Fig. 4 Production of acetate esters in the recombinant strain CLX14-P and CLX14. Production of acetate esters in the semi-solid fermentation of corn. Data are the average of three independent experiments. *Error bars* represent ± SD

Effects of *ATF1* overexpression on the production of volatile flavor compounds

The ester content of CLX14-P and CLX14 was measured via GC analysis after corn fermentation. As shown in Fig. 4, ethyl acetate, isoamyl acetate and isobutyl acetate levels increased at different degree with ethyl acetate 3.1-fold higher (25.04–78.76 mg/l). These results confirmed that the overexpression of *ATF1* gene via *PGK1* promoter seamless insertion led to a significant increase of the ethyl acetate synthesis in CLX14 strain.

Discussion and conclusions

The harmonious complexity of perceived flavor in Chinese liquor is the result of specific ratios of many compounds, in which subtle combinations of trace ester aromatic compounds play a major role [17]. The distinctive flavor of Chinese liquor is affected by many variables including raw materials, yeast strain and fermentation conditions; amongst them yeast strain is the primary factor. The development of yeast strains capable of producing high amounts of esters during alcohol fermentation is an important biotechnological objective of the Chinese liquor industry [2, 3]. In the present study, we have overexpressed *ATF1* in industrial yeast through the precise and seamless insertion of *PGK1* promoter (*PGK1p*). After fermentation in corn semi-solid medium, the ester production of CLX14-P increased to 114.93 mg/l, which was twofold higher than 53.94 mg/l of the host strain, consequently enhanced the fruity odor of the distillates. The engineered strain with stable fermentation property provided a new dimension of optimized strains research in Chinese liquor industry.

Considerable progress has been made during the past two decades in the development of industrial strains possessing optimized quality. As a powerful tool to join fragments precisely without DNA digestion–ligation of restriction site, fusion PCR is widely applied for gene modification, including gene knockouts, construction of fusion genes, the synthesis of long DNA segments and other applications. The resulting mutant, CLX14-P, in which the *PGK1p* derived from the starting strain was seamlessly inserted into the upstream of *ATF1* without introduction of restriction sites, was obtained via two-step integration protocol. The strain construction process can be called "self-cloning", which means cloning of its own DNA [14]. Importantly, the selected promoter was inserted precisely in the 5′-terminal of the target gene. It is also noticeable that as the promoter of the target gene might be the terminator, or even ORF (open reading frame) of its upstream adjacent gene, our precise and seamless gene promoter insertion strategy, through which the intrinsic promoter sequence is preserved, can in the greatest extent eliminate abolishment of the upstream adjacent gene expressions. Therefore, as a promising approach for gene promoter modulation without leaving behind any heterologous DNA sequences, the PCR-mediated two-step procedure could be particularly used in multiple genomic manipulations and will facilitate the genomic manipulations of industrial yeast and related organisms.

In summary, we have constructed a moderately high ester productivity industrial strain CLX14-P. Consequently, the self-cloning strain returned to be prototrophic and did not contain any heterologous sequences in its genome. Therefore, engineered strain would be safe for public use and be easily accepted by consumers and thus pave the way to further commercialization of favorable organoleptic characteristics in Chinese liquor.

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