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Enhanced ethyl caproate production of Chinese liquor yeast by overexpressing *EHT1* with deleted *FAA1*

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Abstract The fruity odor of Chinese liquor is largely derived from ester formation. Ethyl caproate, an ethyl ester eliciting apple-like flavor, is one of the most important esters in the strong aromatic Chinese liquor (or Luzhou-flavor liquor), which is the most popular and best-selling liquor in China. In the traditional fermentation process, ethyl caproate in strong aromatic liquor is mainly produced by aroma-producing yeast, bacteria, and mold with high esterification abilities in a mud pit at later fermentation stages at the expense of both fermentation time and grains rather than by the ethanol-fermenting yeast *Saccharomyces cerevisiae*. To increase the production of ethyl caproate by Chinese liquor yeast (*S. cerevisiae* AY15) and shorten the fermentation period, we constructed a recombinant strain EY15 by overexpressing *EHT1* (encoding ethanol hexanoyl transferase), in which *FAA1* (encoding acyl-CoA synthetases) was deleted. In liquid fermentation of corn hydrolysate and solid fermentation of sorghum, ethyl caproate production by EY15 was remarkably increased to 2.23 and 2.83 mg/L, respectively, which were 2.97- and 2.80-fold higher than those of the parental strain AY15. Furthermore, an increase in ethyl octanoate (52 and 43 %) and ethyl decanoate (61 and 40 %) production was observed. The differences in fermentation performance between EY15 and AY15 were negligible. This study resulted in the creation of a promising recombinant yeast strain and introduced a method that can be used for the clean production of strong aromatic Chinese liquor by ester-producing *S. cerevisiae* without the need for a mud pit.

Keywords Strong aromatic Chinese liquor · *Saccharomyces cerevisiae* · Ethyl caproate · *EHT1* · *FAA1*

Introduction

As one of the six most famous distillates in the world, Chinese liquor (or alcoholic spirit) has a history dating back thousands of years. The invention and development of its manufacturing technique are considered as one of the major progresses in the technological history of ancient China [30]. The current annual output of Chinese liquor is estimated to exceed 5 million metric tons in China. According to the flavor characteristics, Chinese liquor can be classified into strong aroma, light aroma, and soy sauce aroma etc. Among others, strong aromatic liquor (or Luzhou-flavor liquor), which is the most common and best-selling liquor, accounts for 70 % of the total output of Chinese liquors [34]. As a characteristic flavor component of strong aromatic liquors, ethyl caproate is an important component that provides apple flavor to Chinese liquor, and determines the quality and aroma profiles of strong aromatic liquors.

Ester formation is highly dependent on the yeast strain used during production [19], and different *Saccharomyces cerevisiae* strains provide different aroma profiles to alcoholic products [12]. For instance, Chinese liquor yeast displays an undesirable capacity for ethyl caproate production [33]. In contrast to the pure yeast fermentation of beer, the traditional fermentation of strong aromatic liquors uses natural mixed culture starters (Daqu) in a mud pit, which is a special

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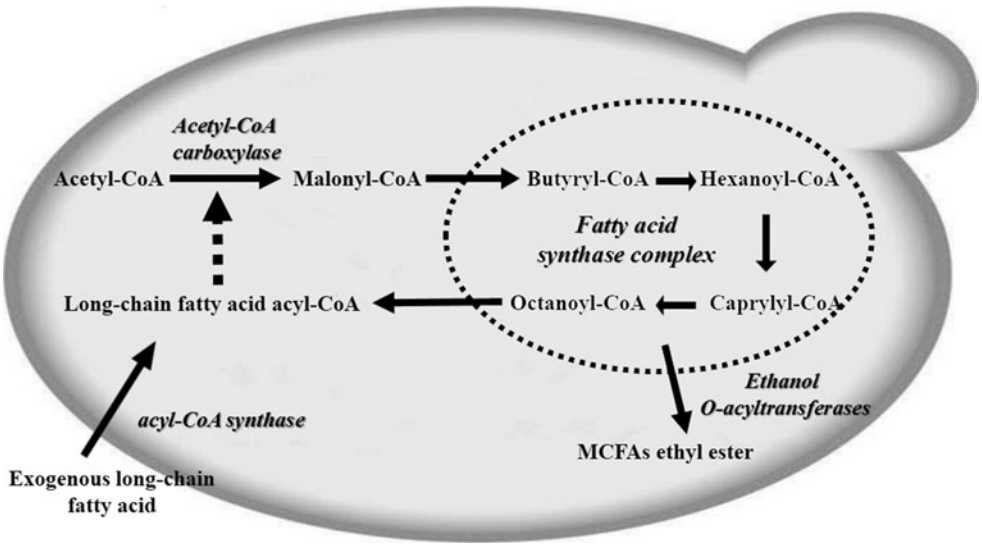

Fig. 1 Fatty acid biosynthesis and its relationship with medium-chain fatty acid ester formation in *Saccharomyces cerevisiae* [21]. *Dashed ellipse* represents fatty acid synthase complex; *dashed arrow* represents the inhibition of long-chain fatty acid acyl-CoA on acetyl-CoA carboxylase

"fermenter" composed of a muddy bottom and wall enriched with diverse microorganisms for strong aromatic liquor brewing. Ethanol is produced by *S. cerevisiae* in the pit during the main stage of fermentation; however, ethyl caproate is mainly produced during the later stages of fermentation by aroma-producing yeasts (*Hansenula* and *Candida*), bacteria, and molds (*Monascus* and *Rhizopus*) with high esterification abilities instead of *S. cerevisiae* [30, 32, 34]. To obtain a certain amount of ethyl caproate, researchers extend the fermentation period at the expense of large amounts of grain. However, with the extension of fermentation period, undesirable flavor compounds, such as geosmin, which are produced by the microbial community in the pit mud, have strong negative effects on the flavor and quality of liquor.

Studies have shown that in yeast ethyl caproate is synthesized from caproyl-coenzyme A and ethanol via a reaction catalyzed by ethanol hexanoyl transferase; ethyl caproate is also synthesized from caproic acid and ethanol catalyzed by esterase [8, 17, 18]. The rate of ethyl caproate formation is mainly dependent on the concentration of substrates (caproyl-CoA and ethanol) and the activity of enzymes [22]. Previous studies further revealed that ethyl ester synthesis in yeasts is catalyzed by ethanol *O*-acyltransferases, which are mainly encoded by *EHT1* and *EEB1* that belong to a three-member gene family (*EHT1*, *EEB1*, and *YMR210w*) [9, 13, 21–23]. Furthermore, Lilly et al. [13, 27] showed that the *EHT1* gene product (Eht1) is important in the production of ethyl esters (ethyl caproate, ethyl caprylate, and ethyl caprate) from medium-chain fatty acids (MCFAs) in wine yeast.

Precursor concentration is a limiting factor in ethyl ester synthesis [21]. MCFAs, which are indirectly regulated by the concentrations of long-chain fatty acid acyl-CoAs, are the main precursors in ethyl ester biosynthesis. In alcoholic fermentation, *S. cerevisiae* releases MCFAs, particularly octanoic acid and caproic acid [29], which are synthesized via a fatty acid synthase (FAS) complex during long-chain fatty acid synthesis [16]. Acetyl-CoA carboxylase functions as a key enzyme in the regulation of fatty acid biosynthesis. Two metabolic pathways are involved in long-chain acyl-CoA synthesis in *S. cerevisiae* [28]. One of these pathway is a de novo pathway involving acetyl-CoA carboxylase; the other pathway utilizes exogenous long-chain fatty acids catalyzed by acyl-CoA synthetases, which are encoded by at least six fatty acid activation genes including *FAA1* to *FAA4* as well as *Fat1* and *Fat2* [1, 26]. Changes in intracellular acyl-CoA pools lead to a higher level of long-chain fatty acyl-CoAs, which inhibit acetyl-CoA carboxylase, when exogenous long-chain fatty are abundant (Fig. 1). As a result, MCFAs synthesized and released from the FAS complex are reduced, thereby decreasing MCFA ethyl ester synthesis. Tadao et al. [28] demonstrated that *FAA1* deletion sake yeast can accumulate higher levels of ethyl caproate in sake mash.

The present study aimed to increase ethyl caproate production by using Chinese liquor yeast in the main stages of alcoholic fermentation instead of using other microorganisms (aroma-producing yeast, bacteria, and mold) in the later stages of alcoholic fermentation to shorten the fermentation period, reduce grain costs, and inhibit the negative effects of a pit mud on the flavor and quality of liquor. To achieve maximum production of ethyl caproate, overexpression of *EHT1* to enhance the enzymatic activities of ethanol hexanoyl transferase and deletion of *FAA1* to increase the levels of its precursors were performed. The results emphasized the importance of ethyl ester biosynthetic enzymatic activities and precursors in the production of ethyl esters, particularly ethyl caproate. This study introduced a method for the clean production of strong aromatic Chinese liquor by using ester-producing *S. cerevisiae* without the need for a mud pit and provides a strong foundation for pure yeast culture fermentation of Chinese strong aromatic liquor.

Table 1 Microbial strains and plasmids used in this study

Strains and plasmids	Relevant characteristic	Reference or source
Strain		
E. coli DH5α	supE44ΔlacU169φ80lacZΔM15) hsdR17 recAl endAl gyrA96 thi-1 relA	This lab
AY15	Commercial liquor yeast strain	Angel yeast
a8[a]	Haploid yeast strain from AY15, a mating type	This study
α5[a]	Haploid yeast strain from AY15, α mating type	This study
a8Δ*FAA1*	*FAA1* :: *loxP-KanMX-loxP*, a mating type	This study
α5Δ*FAA1*	*FAA1* :: *loxP-KanMX-loxP*, α mating type	This study
AY15Δ*FAA1*	*FAA1* :: *loxP-KanMX-loxP*, diploid yeast strain	This study
a8-1	*FAA1* :: *PGK1p-EHT1-PGK1t-loxP-KanMX-loxP*, a mating type	This study
α5-1	*FAA1* :: *PGK1p-EHT1-PGK1t-loxP-KanMX-loxP*, α mating type	This study
EY15	*FAA1* :: *PGK1p-EHT1-PGK1t-loxP-KanMX-loxP*, diploid yeast strain	This study
Plasmid		
pPGK1	Ap^r, containing *PGK1p-PGK1t* expression cassette	Lilly [13]
pUC19	Ap^r, cloning vector	This lab
pUG6	Kan^r, containing *loxP-KanMX-loxP* cassette	Gueldener [7]
pUC-FABK	Ap^r, Kan^r, containing FA-*loxP-KanMX-loxP*-FB	This lab
pFAPEKB	Ap^r, Kan^r, FA-*PGK1*$_p$-*EHT1-PGK1*$_T$-*loxP-KanMX-loxP*-FB	This lab

[a] a8 and α5 are high ethanol-producing haploids derived from AY15 strain (data not shown)

Materials and methods

Strains, plasmids, culture conditions, and fermentation

All of the strains and plasmids as well as their relevant genotypes used in this study are listed in Table 1. The diploid yeast strain AY15 and haploid (a8 and α5) were provided by the Microbiological Culture Collection Center of Tianjin Industrial Microbiology Key Laboratory, Tianjin University of Science and Technology, China.

Escherichia coli was grown at 37 °C in Luria–Bertani broth (composed of 1 % NaCl, 1 % tryptone, and 0.5 % yeast extract) supplemented with ampicillin (100 μg/ml) to select positive *E. coli* transformants. YEPD medium (composed of 1 % yeast extract, 2 % bacto-peptone, and 2 % glucose) was used to cultivate yeast. YEPD plates were supplemented with 1,000 μg/ml filter-sterilized G418 antibiotic (Promega, USA) to select yeast transformants harboring the *KanMX* gene. All of the solid media used in this study contained 2 % agar (Difco, USA).

Plasmid construction and yeast transformation

Polymerase chain reaction (PCR) primers used in this study are listed in Table 2. Plasmid DNA and genomic DNA of yeast were prepared as described by Sambrook and Russell [24]. pUC19 was used as backbone to construct recombinant plasmids pFAKPEB. The PCR-generated FA (411 bp) and FB (409 bp) fragments, which were homologous to the upstream and downstream regions of the *FAA1* gene, were inserted into the *Eco*RI/*Kpn*I and *Pst*I/*Hin*dIII sites of pUC19; pUC-FAB plasmid was then generated. The PCR-generated *loxP-kanMX-loxP* fragment (1,613 bp) was inserted into the *Kpn*I/*Bam*HI site to generate pUC-FABK plasmid. The PCR-generated *EHT1* fragment (1,356 bp) from a haploid yeast strain (a8 or α5) was inserted into the *Xho*I of pUC-PGK1 plasmid. PGK1p-EHT1-PGK1$_T$ fragment (3,145 bp) was amplified by PCR from pPGK1-E plasmid; *PGK1-EHT1*/*Bam*HI and pUC-FAB/*Bam*HI were linked to generate the pUC-FAPEB plasmid. The PCR-generated *loxP-kanMX-loxP* fragment (1,613 bp) was inserted into the unique *Kpn*I site of pUC-APEB plasmid to construct the recombinant pFAKPEB plasmid (Fig. 2).

The AKB and AKPEB fragments were amplified by PCR from the recombinant pUC-AKB and pFAKPEB plasmids, respectively. The amplified fragments were then transferred to the cell of a- and α-type haploids of AY15 according to the lithium acetate procedure [25]. The resulting a- and α-type recombinants haploids were verified by PCR analysis and 1,000 μg/ml G418 resistance assay (data not shown). For the engineered strains, the selected markers were removed using the *Cre/loxP* system.

Construction of the recombinant diploid yeast strain

Recombinant diploid yeast strain was obtained after the purified a- and α- type haploid recombinants were hybridized (data not shown). Haploid a- and α-type cells (0.5 ml each) were added to a tube containing 5 ml of YEPD. Yeast strains were cultivated, hybridized at 30 °C for 24 h, and

Table 2 PCR primers

Primers	Sequence (5′ → 3′)[a]	Restriction site
For plasmid construction		
EHT1-U	CCCCTCGAGATGTCAGAAGTTTCCAAATG	*Xho*I
EHT1-D	CCCCTCGAGTCATACGACTAATTCATCAA	*Xho*I
PGK1-U	CGCGGATCCTCTAACTGATCTATCCAAAA	*Bam*HI
PGK1-D	CGCGGATCCTAACGAACGCAGAATTTTCG	*Bam*HI
KAN-U	CGGGGTACCCAGCTGAAGCTTCGTACGCT	*Kpn*I
KAN-D	CGCGGATCCGCATAGGCCACTAGTGGATC	*Bam*HI
FA-U	CCGGAATTCACAAGGACAATAGTAAAGT	*Eco*RI
FA-D	GGGGTACCTTATTGTATCCTAACGAG	*Kpn*I
FB-U	AACTGCAGAGGAAAGCCTCATCATAC	*Pst*I
FB-D	CCCAAGCTT GGTAATCAATTTCCCTGA	*Hin*dIII
For PCR verification		
F-S	GGGACGGAGCGGGCGAAG	–
K-S	GGGACGGGCGACAGTCACATC	–
E-X	GGGTTTGGTTGATGCGAGTG	–
B-X	GGCTGTTGGCTGACCGAGAC	–
For real-time qPCR		
EHT1-F	AAGACGAGAAGGCGACAC	–
EHT1-R	CCACTGCGAGACAGGTTT	–
ACT1-F	CGTCTGGATTGGTGGTTCTA	–
ACT1-R	GTGGTGAACGATAGATGGAC	–

[a] Restriction enzyme sites are underlined

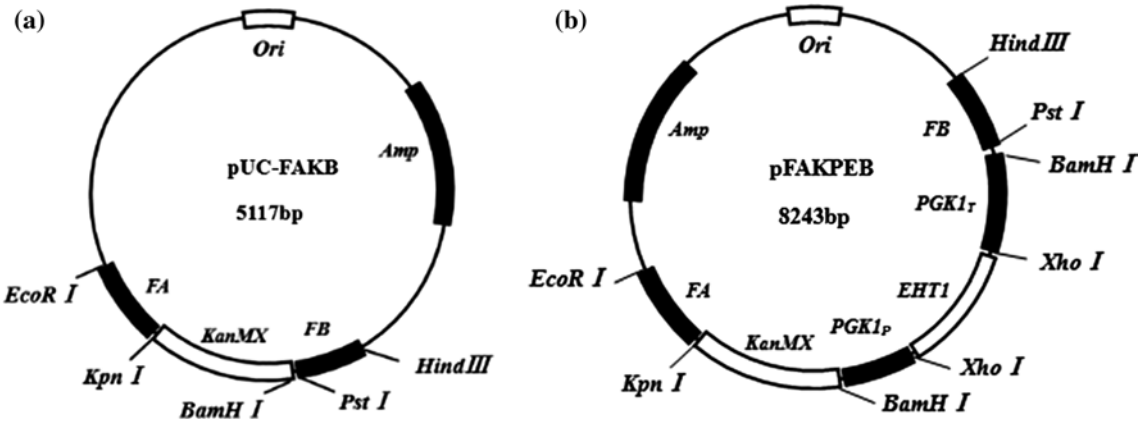

Fig. 2 Construction of pUC-FAKB and pFAKPEB plasmids

transferred to MacConkey medium (composed of 0.1 % glucose, 0.18 % NaCl, 0.82 % sodium acetate, 0.25 % yeast extract, and 2 % agar) at 25 °C for 6 days to produce spores. The resulting fusants exhibiting spore formation were verified under a microscope (Olympus, Japan).

Real-time quantitative PCR

Yeast cells were collected by centrifugation (4 °C, 5,000 rpm, 1 min) at the stationary phase (2×10^8 CFU/ml) from 2 ml fermentation medium and then washed thrice with distilled water for RNA extraction. The mRNA was extracted using a yeast RNA kit (Omega, USA); the changes in *EHT1* expression level were assessed by real-time quantitative PCR (RT-qPCR) using an Ultra SYBR Two-Step RT-qPCR kit with ROX (reference dye for real-time PCR; CWBIO, China). For each sample, 1 μg of total RNA was subjected to reverse transcription (RT) using an RT system (A3500; Promega). The concentrations of cDNA were determined and diluted to obtain a concentration of 100 ng μl^{-1}. The 25 μl PCR mixture consisted of 12.5 μl Platinum SYBR green quantitative PCR Super Mix-UDG with ROX (Invitrogen) and 1.25 μl of each primer (500 nM). 5 μl of cDNA was added to each reaction mixture. The PCR program

consisted of an initial denaturation for 2 min at 95 °C and amplification using 50 cycles of 15 s at 95 °C and 1 min at 60 °C. The expression level of the actin gene (*ACT1*) was relatively stable; thus, *ACT1* was used as the reference gene. The primers, which were used to amplify the small parts of the target gene *EHT1* and reference gene *ACT1*, are listed in Table 2. The expression level of the *EHT1* gene was normalized with respect to the expression level of *ACT1*.

Enzyme activity assays

A modified version of the method reported by Malcorps and Dufour [15] was used to assay the *EHT1*-encoded ethanol hexanoyl transferase activity. Ethanol hexanoyl transferase assays were conducted for 1 h at 30 °C in a reaction medium (200 μl) containing 200 mM KH_2PO_4, 100 mmol/l Tris–HCl (pH 7.5), 0.513 M ethanol, and 100 μM hexanoyl-CoA. Hexanoyl-CoA was purchased from Sigma. The reactions were terminated by adding 5.5 μM H_2SO_4 to lower the pH to 3.0; amyl acetate was added as an internal standard. The concentration of ethyl caproate produced was measured by gas chromatography–mass spectrometry (GC–MS). The specific activity is expressed as micromoles of ester formed per hour per milligram of protein. The total amount of protein in the samples was determined using a standard Bradford method [2].

Fermentation experiments

The corn hydrolysate medium was prepared by liquefying the mixture of 500 g of corn flour and 1,500 ml of water at 90 °C for 90 min with thermostable α-amylase (2×10^5 U/ml; Novozymes, Denmark), and by saccharifying the mixture at 60 °C for 30 min with a saccharifying enzyme (200 U/ml; Novozymes, Denmark). The resulting mixture was filtered using six layers of gauze; the sugar content of the liquid medium was then adjusted to 20 Brix degree (°Bx). Yeast cells were precultured in 4 ml of 8 °Bx corn hydrolysate at 30 °C for 24 h, subsequently transferred to 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 was transferred to a 500-ml conical flask containing 210 ml corn hydrolysate. The mixture was fermented at 30 °C for 15 days. For the solid fermentation of sorghum, 50 g of sorghum was immersed in water for 30 min at 95–98 °C to make the sorghum absorb water fully, and then cooked for 20–30 min till the crystalline structures of starch in sorghum were completely destroyed. The resulting mixture was then cooled to room temperature, stirred in 5 g Daqu for saccharification, and transferred to 500-ml conical flasks. After saccharification for 24 h, 10 ml of the second preculture in YEPD of yeast was added into the flasks. The mixture was also fermented at 30 °C for 15 days. The fermentation performance of biomass [optical density (OD) = 600 nm], residual sugar, and CO_2 weight loss were determined using a spectrophotometer, Brix hydrometer, and analytical balance, respectively. Ethanol production was monitored using an oenometer. The raw material liquor yield is expressed in kilograms of 65 % (vol) Chinese liquor produced by 1 kg of raw material. All of the fermentations were performed in triplicate.

$$\text{Raw material liquor yield} = \frac{65\%\ \text{(vol) Chinese liquor production (kg)}}{\text{raw material consumption (kg)}} \times 100\%$$

Headspace solid-phase microextraction (HS-SPME) and GC–MS analysis

HS-SPME is a simple, rapid, solvent-free, and low-cost sampling method to extract volatile and semivolatile compounds [14]. This technique has been applied to analyze volatile compounds in alcoholic beverages, such as Chinese liquor [5], wine [20], and cider [6, 31]. HS-SPME combined with GC–MS has been widely used to analyze volatile compounds in Chinese liquor [11]. The samples were distilled after fermentation and then used for GC–MS analysis. The sample (8 ml) was equilibrated at 50 °C for 10 min by using a magnetic stirrer, and then SPME fiber, which was coated with 50/30 μm DVB/CAR/PDMS, was exposed to the HS of the sample for 45 min at the same temperature to adsorb the analytes. The fiber was inserted into the injection port of the GC for 5 min to desorb analytes. Analysis was performed using an Agilent 7890C GC coupled with an Agilent 5975C MS system, which contained an HP-5 column (30 m × 0.20 mm internal diameter; film thickness, 0.32 mm; J&W Scientific, Folsom, CA, USA). The GC conditions were used as follows: injection temperature, 250 °C; carrier gas (helium), constant rate of 2 ml/min; and oven temperature program, 40 °C for 3 min then increased to 240 °C at a rate of 6 °C/min. The final temperature was maintained for 10 min; the electron impact energy was 70 eV; and the ion source temperature was set at 230 °C. Amyl acetate was used as the internal standard. Amyl acetate, ethyl acetate, ethyl caproate, ethyl lactate, ethyl butyrate, ethyl octanoate, ethyl decanoate, propanol, isobutanol, and isoamyl alcohol were purchased from Merck. All of the determinations were performed in triplicate.

Results

Improved ethyl caproate production by deleting *FAA1*

FAA1 gene (2,103 bp) was replaced with the constructed *FA-loxP-KanMX-loxP-FB* cassette to improve the

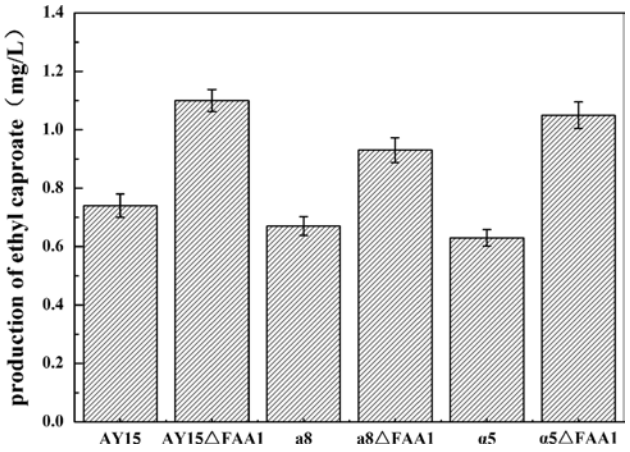

Fig. 3 Ethyl caproate production from the AY15, AY15Δ*FAA1*, a8Δ*FAA1*, a8, α5, and α5-Δ*FAA*1. Data are the average of three independent experiments. *Error bars* represent ± SD

production of ethyl caproate. The resulting engineered strain AY15Δ*FAA1* was constructed by hybridizing the engineered haploids a8Δ*FAA1* and α5Δ*FAA1*, which exhibited a higher ester-producing trait than other haploid transformants (data not shown). We investigated the effects of deleting *FAA1* gene on ethyl caproate production in the liquid fermentation of corn hydrolysate. The effects of deleting *FAA1* on ethyl corporate production are presented in Fig. 3. The production of ethyl caproate by the engineered strains AY15Δ*FAA1*, a8Δ*FAA1*, and α5Δ*FAA1* increased to 1.12, 0.93, and 1.01 mg/l, respectively, representing an increase of 57.1, 38.8, and 58.7 % compared with the parental strains AY15, a8, and α5, respectively. Furthermore, no significant difference was found between parental strains and engineered strains for ethyl octanoate and ethyl decanoate (data not shown). The results indicated that deleting the *FAA1* gene could improve ethyl caproate synthesis in yeast cells.

Further increased ethyl caproate production by overexpressing *EHT1* in a *FAA1*-deleted mutant

In order to further increase ethyl caproate production, *FAA1* gene (2,013 bp) was replaced with the constructed *FA-PGK1*$_P$*-EHT1-PGK1*$_T$*-loxP-KanMX-loxP-FB* cassette. The resulting engineered strain EY15, which combined the overexpression of the *EHT1* gene under the control of the *PGK1* promoter with the deletion the *FAA1* gene, was constructed by crossing a8-1 and α5-1 haploids that exhibited higher ester-producing traits than other transformants (data not shown). We tested the effects of combining *EHT1* overexpression with *FAA1* deletion on the production of ethyl caproate in the liquid fermentation of corn hydrolysate and solid fermentation of sorghum.

In the liquid fermentation of corn hydrolysate (Fig. 4a), the production of ethyl caproate by the engineered strains EY15, a8-1, and α5-1 were increased correspondingly by 48.6, 38.8, and 58.7 % compared with AY15Δ*FAA1*, a8Δ*FAA1*, and α5Δ*FAA1*. The production of ethyl caproate by EY15, a8-1, and α5-1 was increased to 2.23, 1.55, and 2.01 mg/l, which were approximately 2.97-, 2.1-, and 3.1-fold higher than those of the parental strains, respectively. Moreover, ethyl octanoate production was increased by 30, 50, and 52 %; ethyl decanoate production was increased by 70, 46, and 61 %. Negligible differences were observed between higher alcohol and ethyl acetate contents in the fermentation samples (data not shown).

To confirm the improved ester-producing capacity of EY15, a8-1, and α5-1, we further conducted solid fermentation of Chinese liquor in sorghum medium (Fig. 4b). Ethyl caproate production by the engineered strains EY15, a8-1, and α5-1 was increased to 2.83, 2.71, and 2.24 mg/l, which were 2.8-, 2.97-, and 2.1-fold higher than those of the parental strains, respectively. Ethyl octanoate production was increased by 43, 37, and 39 %; ethyl decanoate production was also increased by 40.1, 28.4, and 32.8 %.

The results of the two fermentation experiments indicated that the production of ethyl esters by the engineered strains EY15, a8-1, and α5-1 was significantly higher than those of the corresponding parental strains. To confirm the *EHT1* gene overexpression, we quantified the mRNA level of *EHT1* gene expression and measured the activity of *EHT1*-encoded acyltransferases. The RT-qPCR results showed the *EHT1* expression levels of a8-1, α5-1, and EY15 were 1.4-, 1.7-, and 1.8-fold higher than those of a8, α5, and AY15, respectively. Furthermore, the enzyme assay results indicated that the ethanol hexanoyl transferase activity of a8-1, α5-1, and EY15 was 2-fold higher than that of a8, α5, and AY15, respectively (Fig. 5). These results confirmed that the overexpression of *EHT1* leads to a significant increase in the level of gene expression and enzyme activity, thereby enhancing the ethyl caproate synthesis in EY15, a8-1, and α5-1.

Fermentation characteristics of engineered strains

Stable performance is important for industrial fermentation, in which diploid strains are frequently used because of their stable fermentation performance. We further investigated the physiological characteristics (biomass, residual sugar, and weight loss of CO_2) of diploid engineered strains EY15 to assess their fermentation performance in corn hydrolysate compared with that of AY15 and AY15Δ*FAA1*.

As shown in Fig. 6a, the *FAA1*-deleted mutants EY15 and AY15Δ*FAA1* exhibited a reduced growth rate, which reached the stationary phases of fermentation after 72 and 84 h, respectively. However, the effects of these mutants

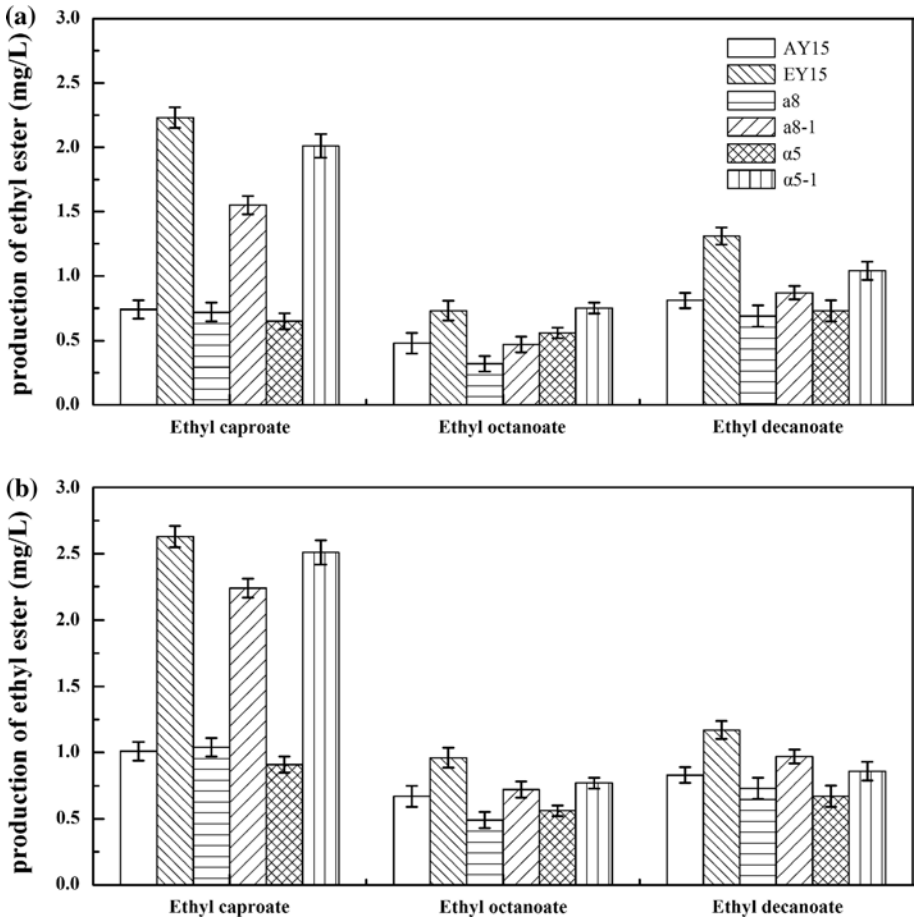

Fig. 4 Production of ethyl esters by AY15, EY15, a8, a8-1, α5, and α5-1. **a** Production of ethyl esters in the liquid fermentation of corn hydrolysate; **b** production of ethyl esters in the solid fermentation of sorghum. Data are the average of three independent experiments. *Error bars* represent ± SD

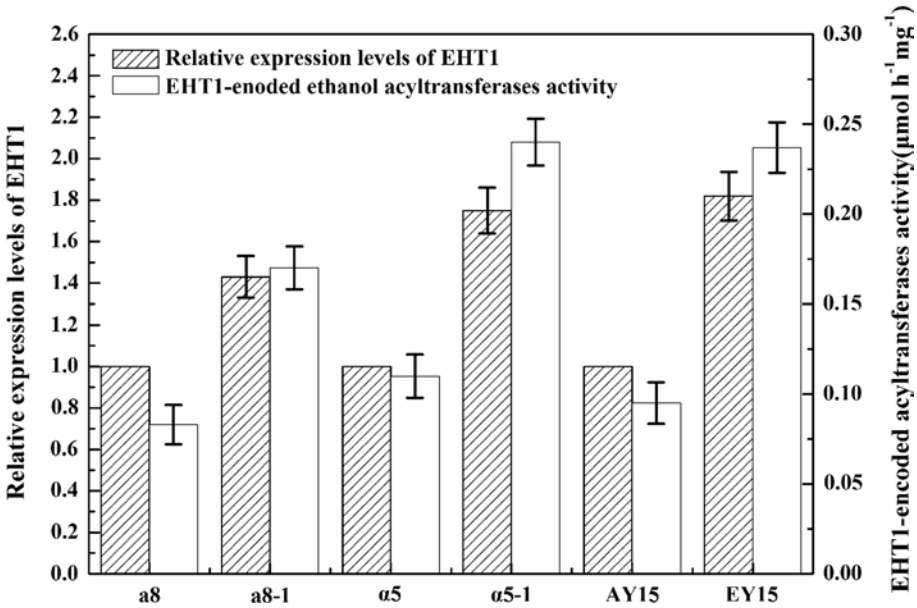

Fig. 5 Determination of *EHT1* gene expression levels and enzyme activity in the recombinant strain a8, a8-1, α5, α5-1, AY15, and EY15. *ACT*1 gene was used as the internal control in RT-qPCR, and the experiments were repeated three times. Data are the average of three independent experiments. *Error bars* represent ± SD

on the final biomass yield were not significant. Furthermore, the results of residual sugar and weight loss of CO_2 indicated a slight difference in alcohol fermentation performance (Fig. 6b). Although the *FAA1*-deleted mutant exhibited a reduced growth rate, the engineered yeast strains EY15 and AY15Δ*FAA1* showed negligible changes in the ethanol and liquor yield as assessed using two fermentation processes (Table 3).

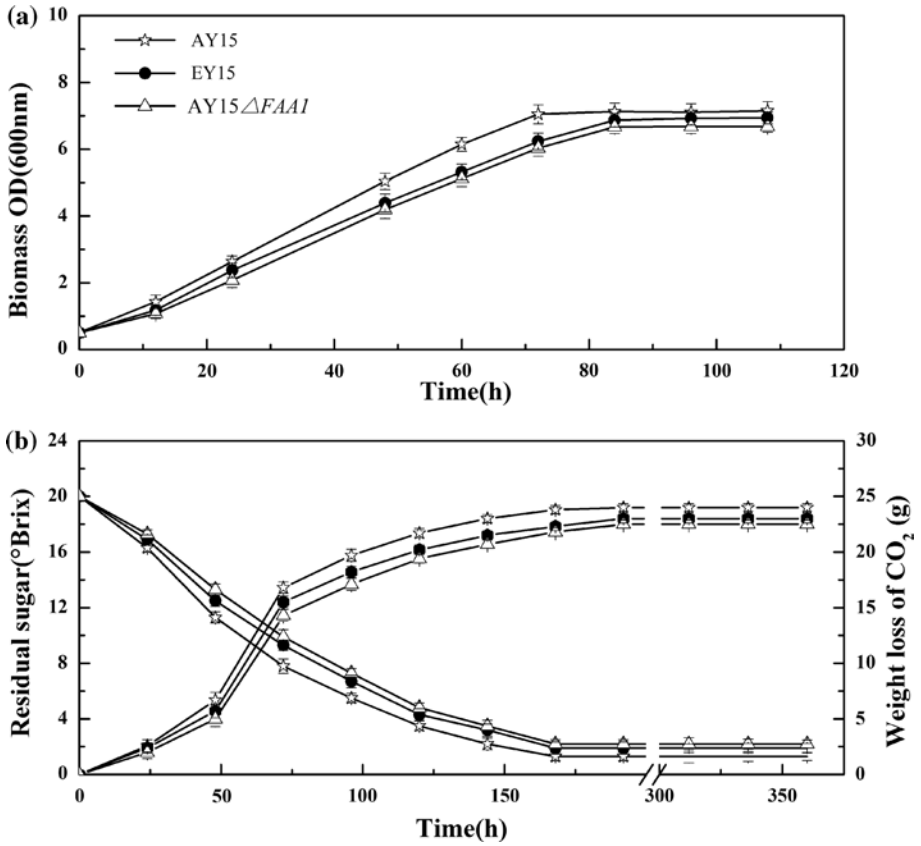

Fig. 6 Fermentation performance of the engineered strains EY15 and AY15Δ*FAA1* and the parental strain AY15. **a** OD = 600 nm; **b** residual sugar and weight loss of CO_2. Data are the average of three independent experiments. *Error bars* represent ± SD

Table 3 Ethanol and liquor yield in fermentation

Yeast strain	Liquid fermentation		Solid fermentation	
	Ethanol (v/v, 20 °C)	Raw material liquor yield[a] (%)	Ethanol (v/v, 20 °C)	Raw material liquor yield[a] (%)
AY15	11.7 ± 0.1	31.32 ± 0.26	8.8 ± 0.1	23.56 ± 0.26
AY15Δ*FAA1*	11.4 ± 0.2	30.50 ± 0.51	8.5 ± 0.1	22.75 ± 0.26
EY15	11.5 ± 0.1	30.78 ± 0.26	8.7 ± 0.2	23.28 ± 0.51

[a] According to the national standard the raw material liquor yield is expressed as 65° standard Chinese liquor yield. Data are the averages from three independent experiments

Discussion

The development of *S. cerevisiae* strains capable of producing high amounts of esters during alcohol fermentation is an important biotechnological objective of the Chinese liquor industry [3, 4, 10]. In the present study, AY15Δ*FAA1* strain was constructed, in which the *FAA1* gene was deleted, to improve the ethyl caproate production of commercial liquor yeast. After fermentation in the corn hydrolysate, the production of ethyl caproate by AY15Δ*FAA1* was increased to 1.12 mg/l, which was 1.48-fold higher than that of its parental strain AY15. The result supported the conclusion reported by Tadao et al. [28]. The increase in ethyl caproate production may be due to the deletion of the *FAA1* gene, which relieves the suppression of long-chain fatty acyl-CoAs on acetyl-CoA carboxylase. As a result, precursor caproic acids were released from the FAS complex, thereby resulting in an increase in ethyl caproate production.

The resulting engineered strain EY15 was investigated by combining the overexpression of *EHT1* gene under the control of the *PGK1* promoter with the deletion of *FAA1* gene in an industrial liquor strain to improve the yield of ethyl caproate further. In this study, the EY15 exhibited a significant improvement in the production of the MCFA esters (ethyl caproate, ethyl caprate, and caprylate), which consequently enhanced the fruity odor of the distillates. After 15 days of fermentation in different fermentation media (hydrolysate and sorghum) and different fermentation methods (liquid fermentation and solid fermentation),

the ethyl caproate concentrations of EY15 were 2.97- and 2.8-fold higher than those of the corresponding parental strain AY15. The levels of ethyl octanoate and ethyl decanoate were also increased. These results are consistent with those of Lilly et al. [13], who demonstrated that *EHT1* overexpression results in a significant increase in ethyl caproate, ethyl caprylate, and ethyl caprate. Furthermore, the significant increase in enzyme activities and mRNA levels of the *EHT1* gene confirmed the overexpression of *EHT1*. However, Saerens et al. [23] found that the overexpression of *EHT1* in an *S. cerevisiae* laboratory strain does not lead to an increase in ethyl ester. This apparent discrepancy can be ascribed to the use of different media and differences in genetic backgrounds of the host strains. Our results indicated that the overexpression of *EHT1* and the deletion of *FAA1* could significantly enhance the production of ethyl esters.

In liquor fermentation, the subtle differences in physiological characteristics among AY15Δ*FAA1*, EY15, and AY15 may be due to the deletion of the *FAA1* gene. This deletion decreases the content of long-chain fatty acid CoAs, which affect the synthesis of cell membranes, thereby resulting in a decrease in growth rate and biomass. In addition to the reduction of biomass yield, the negligible changes in ethanol production may be attributed to the fact that the ethanol has been used as a substrate in the synthesis of ethyl esters.

The EY15 strain can produce large amounts of ethyl caproate during the main stages of alcoholic fermentation, thereby shortening the fermentation period and reducing grain costs. In addition, this study provided a promising yeast strain that can be used to produce strong aromatic Chinese liquors without the use of mud pits. As a result, negative effects on the flavor caused by microorganisms in pit muds are reduced. Our engineered strain EY15 can significantly shorten the fermentation period of Chinese liquor, thereby increasing production efficiency, reducing costs, and improving the quality of Chinese liquor. The increase in ethyl caproate production achieved by using the engineered Chinese liquor yeast strains indicated that the new strains are useful for the development of novel industrial strains for the Chinese liquor industry. Our work provides a platform for the pure yeast fermentation of high-quality strong aromatic Chinese liquors.

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