Screening lager yeast with higher ethyl-acetate production by adaptive laboratory evolution in high concentration of acetic acid

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Research Article

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Abstract

Ethyl-acetate is important for the flavor and aroma of the alcoholic beverages, therefore, there have been extensive efforts toward increasing its production by engineering yeast strains. In this study, we reported a new approach to breed non-genetic modified producing yeast strain with higher ethyl-acetate production for beer brewing. First, we demonstrated the positive effect of higher acetic acid concentration on inducing the expression of *ACS*. Then, we applied adaptive laboratory evolution method to evolve strain with higher expression level of *ACS*. As a result, we obtained several strains with increased *ACS* expression level as well as ethyl-acetate production. In 3 L scale fermentation, the optimal strain EA60 synthesized more ethyl-acetate than M14 at the same time point. At the end of fermentation, the ethyl-acetate production in EA60 was 21.4% higher than M14, while the other flavor components except for acetic acid were changed in a moderate degree, indicating this strain had a bright prospect in industrial application. Moreover, this study also indicated that *ACS1* played a more important role in increasing the acetic acid tolerance of yeast, while *ACS2* contributed to the synthesis of cytosol acetyl-CoA, thereby facilitating the production of ethyl-acetate during fermentation.

Introduction

During alcoholic fermentation, yeast cells produced a wide range of aroma-active components which greatly affected the flavor of fermented alcoholic beverages (Bloem et al. 2016; Verstrepen et al. 2003). Of all these components, esters are considered as the most important aroma elements for their contributions to the fruity and flowery notes (Pires et al. 2014). According to the compositions, these esters could be categorized as the acetate esters and the medium-chain fatty acid ethyl esters. In the beer, acetate ester, especially for ethyl-acetate, serve as the main contributor to the flavor and fragrance since the small size and lipophilic nature allow it to easily diffuse from the cytoplasm into the extracellular medium (Dzialo et al. 2017; Nykänen et al. 1977; Pires et al. 2014). It is now generally accepted that high gravity or high concentration of free amino nitrogen of wort media greatly increased the final production of ethyl-acetate in the industrial fermentations (Lei et al. 2013a; Lei et al. 2013b; Peddie 1990). Furthermore, increasing the fermentation temperatures or reducing the dissolved oxygen were also reported contributing to the production of ethyl-acetate in beer (Fujii et al. 1997; Saerens et al. 2008). Nonetheless, these effects varied given the differences in genetic backgrounds of yeast.

In the recent decades, the mechanism for ethyl-acetate biosynthesis in yeast was elucidated. Alcohol acyltransferases (AATases) played a vital role in ethyl-acetate synthesis, and higher expression level of these AATases increased the production of ethyl-acetate. On the other hand, the substrate availability, especially for acetyl-CoA, is also important for the production of ethyl-acetate. Thus simultaneously engineering the AATase activity and acetyl-CoA availability could enhance the production of ethyl-acetate (Lilly et al. 2006; Mason and Dufour 2000; Verstrepen et al. 2003; Yoshimoto et al. 1998). However, due to the safety concerns, those genetic engineered strains were rarely applied in beer industries. Therefore, it is of great necessity to trigger the expression of these genes spontaneously to increase the production of ethyl-acetate in industrial producing strains.

The adaptive evolutionary engineering generally treats the cells under a certain pressure by repeated or prolonged cultivation until they have improved characteristics. However, the expression level of genes was tightly regulated for optimal growth in the organism's natural habitat. Therefore, it is important to design a specific pressure to trigger the expression of *ACS* or *ATF*. Since a recent study reported that overexpression of *ACS2* could increase acetic acid tolerance in *Saccharomyces cerevisiae* (Ding et al. 2015), we assumed that higher concentrations of acetic acid can trigger the expression of *ACS2* as well as *ACS1* in turn. Therefore, in this study, we will first investigate whether higher concentration of acetic acid could induce the expression of *ACS* genes. Then adaptive laboratory evolution (ALE) would be performed to evolve the strain with upregulated expression level of *ACS* genes. Next, the ethyl-acetate production and other fermentation performance of the evolved strain would be compared to the parental strain in a 3 L scale beer fermentation to evaluate the potential of the evolved strain in industrial application. Finally, the differential roles of *ACS1* and *ACS2* in ethyl-acetate synthesis during fermentation were elucidated. This work will provide a new approach to breeding non-genetic modified lager yeast with higher ethyl-acetate production, thereby improving the beer quality in flavor.

Materials And Methods

Strains and culture conditions

Strains used in this study were listed in Table 1. The lager yeast (*Saccharomyces pastorinous*) M14 (Liu et al. 2017) was used as the background strain, while M-ACS1 and M-ACS2 were constructed by overexpressing corresponding gene using an expression plasmid YEP352 under the control of *PGK1* promotor (Xu et al. 2018a). Primers used for amplification of *ACS1* and *ACS2* were listed in Table 2. EA60 was the evolved strain of M14 using the ALE method. The yeast strains were cultured in YPD medium or wort medium, supplemented with G418 when necessary, at 28°C.

Quantitative Real-Time-PCR analysis

Yeasts cells were collected at the mid-log phase and centrifuged at 3,000 × g for 5 minutes at 4 °C. Then the total RNA was extracted from cells using UNIQ-10 column Trizol total RNA isolation kit (Sangon Biotech), following the manufacturer's instruction. The three RNAs from 3 parallel samples were mixed and a total of 1 μg of RNA was used to synthesize the first-strand cDNA using PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time, Takara, Dalian, China). Then cDNA samples were diluted in water at a ratio of 1:5, and qRT-PCR was performed with the SYBR Premix Ex Taq^{TM} II kit (Takara, Dalian, China) in a LightCycler 480 PCR system (Roche Diagnostics, Shanghai, China) in triplicate (Xu et al. 2018b). *ACT1* was used as a standard control to normalize the gene expression. Primers used for RT-qPCR were listed in Table 2

Cellular acetyl-CoA level measurement

To measure the cellular acetyl-CoA level, 2 mL collected yeasts at the mid-log phase were quenched and then mixed with 2 mL boiling ethanol for 15 min to release the metabolite. Next, the mixture was

centrifuged at 12,000 × *g* for 10 minutes at 4 °C and the supernatant was dried by the vacuum freeze dryer. Finally, 200 μL ddH_2O was used to re-suspend the metabolite and the cetyl-CoA was detected by LC-MS/MS (Armando et al. 2012).

Beer fermentation

To prepare wort, pure Canadian malt (Supertime, China) was crashed and mixed with water in a ratio of 4.5:1 (kg/kg). The mash profile was processed as follows:48℃, 30 min; 63℃, 60 min; 72℃, 30 min and 78℃, 10 min. Then the wort was filtered and boiled for 60 min with hops (0.3‰). Finally, the boiled mixture was filtered again, adjusted to 11 °P and sterilized at 105℃ for 10 min.

The main fermentation process was detailed described in our previous works (Xu et al. 2018b). In brief, the yeast strains were initially pre-cultured in 100 mL wort at 28°C for 2 days and then collected after 12-h natural sedimentation at 4°C. After this, yeast was inoculated to 300 mL wort media with a cell density around 10^7 cells/mL for primary fermentation. Air locks were used to close the flasks, which ensured the exclusion of oxygen but allowed for the release of CO_2. The main fermentation process proceeded at 11°C for 7 days. The weight of the total fermentation equipment was recoded every day to calculate the weight loss. Three biological repeats of each experiment were performed. Strain M14 and EA60 were also used to perform a 3 L scale fermentation with 2 L wort media at 11°C for 10 days and two parallel experiments of each strain were carried out.

Adaptive laboratory evolution

The wort media supplied with 0.6% acetic acid was used throughout this section. Strain M14 was pre-cultured in the wort media at 28°C for 2 days, then cells were transferred into fresh media with 0.6% acetic acid every 24 h with an initial OD_{600} value at 0.1. During adaptive evolution, cells were collected at day 10, day 20, day 30, day 40, day 50 and day 60, and a detailed cell growth curve was monitored.

By-products analysis

Organic acids were analyzed using HPLC (Chromaster, Hitachi Ltd., Shanghai, China) with an Atlantis 100-C18 column (5 mm, 4.6´250 mm) (Waters, Shanghai, China) and an ultraviolet-visible detector. The mobile phase consisted of 20 mmol/L KH_2PO_4, with the flow rate of 0.5 mL/min, and the column temperature was maintained at 25°C. Alcohols and esters were measured using headspace gas chromatography (GC-2010 PerkinElmer TurboMatrix 16, Shimadzu, China) with a Rtx-Wax column (30 m, 0.32 mm, 0.25 mm), using 3-heptanone as an internal standard. Diacetyl content was measured using a diacetyl distiller based on the Chinese National standard for beer analysis, which had been described clearly (Shi et al. 2017). The ethanol content, apparent extract and attenuation were determined by Anton Paar Alcohol meter equipped with Alcolyzer Beer ME and DMA 4500M.

Results

Acetic acid triggers the expression of *ACS*

To evaluate the impact of acetic acid on the expression of *ACS* gene, we treated the yeast in wort media with different concentration of acetic acid. The cell growth of M14 was slightly affected when the acetic acid concentration was lower than 0.5%, while a dramatic growth defect was observed when the acetic acid concentration reached to 0.5% (Fig. 1a). When the acetic acid concentration reached 0.8%, the cell growth of M14 was completely inhibited (Fig. 1a). Meanwhile, we collected the cells and measured the mRNA level of *ACS1* and *ACS2* in M14 under these conditions by RT-qPCR. The results indicated that the expression levels of *ACS1* and *ACS2* in M14 were upregulated significantly when the acetic acid concentration was higher than 0.3% (Fig. 1b). Moreover, the relative expression level of *ACS1* was higher than that of *ACS2*. To explore whether *ACS1* played a more important role in acetic acid tolerance, we overexpressed *ACS1* and *ACS2* in M14 respectively (Fig. 1c) and evaluated the growth capacity of the generated strains (M-ACS1 and M-ACS2) under 0.6% acetic acid conditions. As a result, the cell growth of M-ACS1 was obviously better than that of M-ACS2 (Fig. 1d), indicating *ACS1* was more important than *ACS2* in relieving the pressure of acetic acid on cell growth. Nonetheless, the cell growth of M-ACS2 was still better than that of M14, which was in line with the previous work (Ding et al. 2015).

Breeding lager yeast with higher acetyl-acetate production using ALE

Then we used the ALE method to evolve the strain M14 under the pressure of 0.6% acetic acid. Within this procedure, the cell growth was gradually improved during evolution (Fig. 2a). After 60 runs transfer, we ended it and separated several single colonies on the plate. We randomly picked 10 colonies and performed beer fermentation experiments with these strains. The total weight loss of these strains at the end of main fermentation was similar to that of M14 (Fig. 2b), indicating the fermentation performance was not much affected. At the end of main fermentation, the ethyl-acetate productions of these strains were obvious increased (Fig. 2c). The optimal strain No.5 (called EA60 in the later text) produced 18.53 mg/L ethyl-acetate, which was improved by 40.3% when compared with parental strain M14. RT-qPCR results showed that the expression level of *ACS1* and *ACS2* in EA60 were upregulated by 3.7 and 2.1-fold respectively (Fig. 2d), which was in line with our expectation.

Next, we performed a 3 L scale fermentation with EA60 to evaluate its potential in industrial application. After inoculation into wort media, the cell growth of EA60 was superior to M14, resulting the cell amount of EA60 was higher than that of M14 (Fig. 3a). During the fermentation, EA60 consumed the fermented sugars quicker than M14, which reflecting on the apparent extract (Fig. 3b). Meanwhile, EA60 synthesized more ethyl-acetate than M14 at the same time course. At the end of fermentation, the ethyl-acetate production of EA60 was approximate at 18.60 mg/L, which was 21.4% higher than M14 (Table 3). The ethanol content of EA60 was decreased from 4.68% to 4.47% while the real attenuation was increased from 65.21% to 66.04%. In terms of the flavor components, we found the acetate was increased by 57.6% in EA60 while the other components such as higher alcohols and organic acid were changed in a moderate degree. Overall, these results suggested that the fermentation performance and the aroma profile of EA60 could meet the requirement for industrial production.

The role of *ACS1* and *ACS2* on ethyl-acetate production during fermentation

In EA60, the expression of *ACS1* and *ACS2* were both upregulated. Therefore, we would like to investigate which one contribute more to ethyl-acetate synthesis under beer fermentation conditions. We again used the engineered strains M-ACS1 and M-ACS2 to explore this question. We measured the cellular acetyl-CoA contents in the engineered strains at the mid-log phase and found that the cellular acetyl-CoA contents in these two strains were increased by 10.4% and 16.7%, respectively (Fig. 4a). However, during fermentation, we found that the production of ethyl-acetate of M-ACS1 was similar to that of M14 at the early fermentation but slightly higher than M14 at the later phase. By contrast, the ethyl-acetate synthesis of M-ACS2 was always higher than that of M14 at the same time course (Fig. 4b). At the end of fermentation, the ethyl-acetate production for M14, M-ACS1 and M-ACS2 was 13.27, 14.10 and 16.84 mg/L respectively ($p < 0.05$). These results suggested that *ACS2* was the main contributor to the synthesis of ethyl-acetate during fermentation, while *ACS1* contributed less despite the increased cellular acetyl-CoA level.

Discussions

Esters synthesized by lager yeast are important to the beer flavor, therefore breeding lager yeast with higher levels of these components is always a hot point in beer industry. Owing to the development of metabolic approaches and technologies, great efforts had been made to engineer the *Saccharomyces cerevisiae* or lager yeast with higher levels of ethyl-acetate production. However, in consideration of the consumers' acceptance and the policies regarding food safety, those genetic modified strains could not be applied in beer industry in China. Therefore building a bridge between biological knowledge and practical application is of great importance.

The availability of enzyme and substrate was the limiting factor for the synthesis of ethyl-acetate, thus increasing their abundances would be a promising strategy. In this study, we assumed that higher concentration of acetic acid could trigger the expression of *ACS*. Then through ALE, the expression of those genes would be upregulated spontaneously, resulting the evolved strain possessed higher acetyl-CoA level as well as higher ethyl-acetate production. As a result, we indeed found the expression of *ACS1* and *ACS2* in M14 were both increasingly upregulated along with the increased concentration of acetic acid. Meanwhile we found the upregulated level of *ACS1* was higher than that of *ACS2*. As Acs1p has a 30-fold higher affinity to acetate than Acs2p (van den Berg et al. 1996), higher expression level of *ACS1* may contribute more to relieve the pressure of acetic acid. This reason could be supported by the result that the cell growth of M-ACS1 in 0.6% acetic acid was better than M-ASC2 and M14. The previous work only reported that overexpression of *ACS2* could increase the yeast tolerance for exogenous acetic acid. Here, our results suggested that both *ACS1* and *ACS2* could confer yeast with such capacity and *ACS1* had a better effect.

Through ALE, we obtained several strains with higher production ethyl-acetate. Higher expression level of *ACS* in EA60 could explain the higher production of ethyl-acetate to a certain degree, but a further

comparative genomic analysis between strain M14 and EA60 was still needed to dissect the underlying regulation mechanism. As *ACS* enhanced the carbon flux toward acetyl-CoA (Lian et al. 2018; Lian et al. 2014), the intermediate product (acetaldehyde and acetate) was thereupon simultaneously increased in the final fermentation liquid. Whereas, the production of another acetate ester, isoamyl acetate, was not increased in EA60. This may due to the production of isoamyl acetate was subject to isoamyl alcohol content (Yoshioka and Hashimoto 1984).

Of note, the impact of *ACS1* and *ACS2* on ethyl-acetate synthesis was quite different under fermentation conditions. The cellular acetyl-CoA levels in M-ACS1 and M-ACS2 were both increased while the increased ethyl-acetate production of M-ACS1 was lower than expected. Such a phenomenon could be explained by the different distribution of Acs1p and Acs2p in yeast. A systematic study suggested Acs2p was exclusively located in the cytosol while Acs1p was subjected to dual distribution, e.g. to the peroxisomes under glucose repressing conditions but to the cytoplasm under de-repressing conditions (Fig. 4c) (Chen et al. 2012). Under the wort fermentation conditions where a plenty of fermented sugars exist, the Acs1p is of high likely to distributed to peroxisomes but not to cytosol. The lipid synthesis for cell growth preferred cytosolic acetyl-CoA, as a result, we found that the cell growth of M-ACS1 was similar to that of M14, while M-ACS2 showed a remarkable increase in cell growth (Fig. 4d). This result suggested that *ACS1* contribute to acetyl-CoA synthesis in the peroxisomes under fermentation conditions. Further, as AATase is located in the endoplasmic reticulum or lipid droplets (Fig. 4c) (Lin and Wheeldon 2014), those increased acetyl-CoA could not serve as effective substrate for the synthesis of ethyl-acetate.

Taken together, this study demonstrated that higher concentration of acetic acid could trigger the expression of *ACS*, and using ALE method, a non-genetic modified strain EA60 was obtained with higher level of ethyl-acetate for industrial application.

Declarations

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Table 1. Strains used in this study

Strains	Description	Resource
M14	Lager brewers' yeast	Our lab
M-ACS1	M14 derivate, overexpressing *ACS1* under the *PGK1* promotor	This study
M-ACS2	M14 derivate, overexpressing *ACS2* under the *PGK1* promotor	This study

Table 2. Primers used in this study

Primer	Sequence
Plasmid construction	
ACS1F	ctagctagcATGTCGCCCTCTGC
ACS1R	ccgctcgagTTACAACTTGACCG
ACS2F	ctagctagc ATGACAATCA AGG
ACS2R	ccgctcgag TTATTTCTTTTTTTGAGAG
RT-qPCR	
ACS1(qPCR)	GTGCTGACTTACTCTATG/ AGGTTATGATTGCTTCTG
ACS2(qPCR)	TACTGAAGGTATTCCAATG/ AGGTAGGTAAGTTCTCTG

Table 3. Parameters measured in the pilot scale fermentation. The pilot scale fermentation was performed in a 3 L Erlenmeyer flask with 2 L 11 °P wort at 11°C for 10 days with two repeats and the values were means ± SD of two independent experiments

	M14	EA60
Real attenuation (%)	65.21±0.42	66.04±0.51
Diacetyl (mg/L)	0.04±0.005	0.04±0.007
Ethanol (%)	4.68±0.1	4.47±0.1
Ethyl-acetate (mg/L)	15.32±0.11	18.60±0.17
Isoamyl acetate (mg/L)	0.53±0.03	0.50±0.05
n-Propanol (mg/L)	8.23±0.12	8.57±0.27
Isoamyl alcohol (mg/L)	40.62±1.12	38.91±0.98
Isobutanol (mg/L)	6.43±0.04	6.71±0.07
Acetate (mg/L)	99.01±2.13	156.0±5.11
Acetaldehyde (mg/L)	8.65±0.12	9.21±0.05
Malate (mg/L)	128.11±5.27	119.43±6.41
Citrate (mg/L)	97.26±1.28	100.33±0.85
Succinate (mg/L)	162.17±3.91	157.26±5.37

Figures

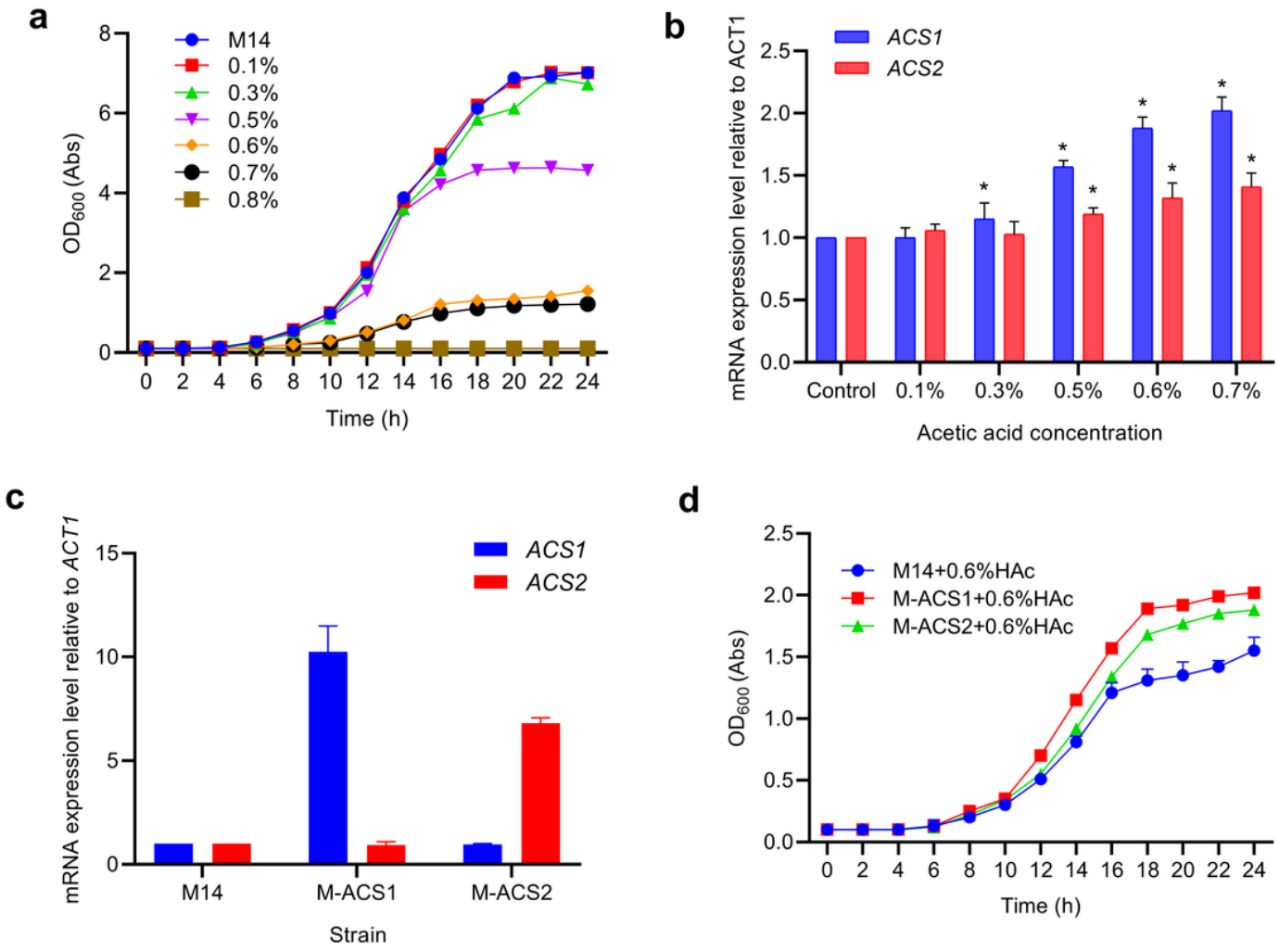

Figure 1

Acetic acid triggered the expression of ACS and higher expression levels of ACS confer the yeast with acetic acid tolerance. (a) The cell growth of parental strain M14 in the wort media with different concentration of acetic acid; (b) The relative expression level of ACS1 and ACS2 in M14 under different concentration of acetic acid; (c) The relative expression level of ACS1 and ACS2 in the engineered strains M-ACS1 and M-ACS2 which overexpression ACS1 and ACS2 respectively; (d) The cell growth of M-ACS1 and M-ACS2 in wort media supplied with 0.6% acetic acid.

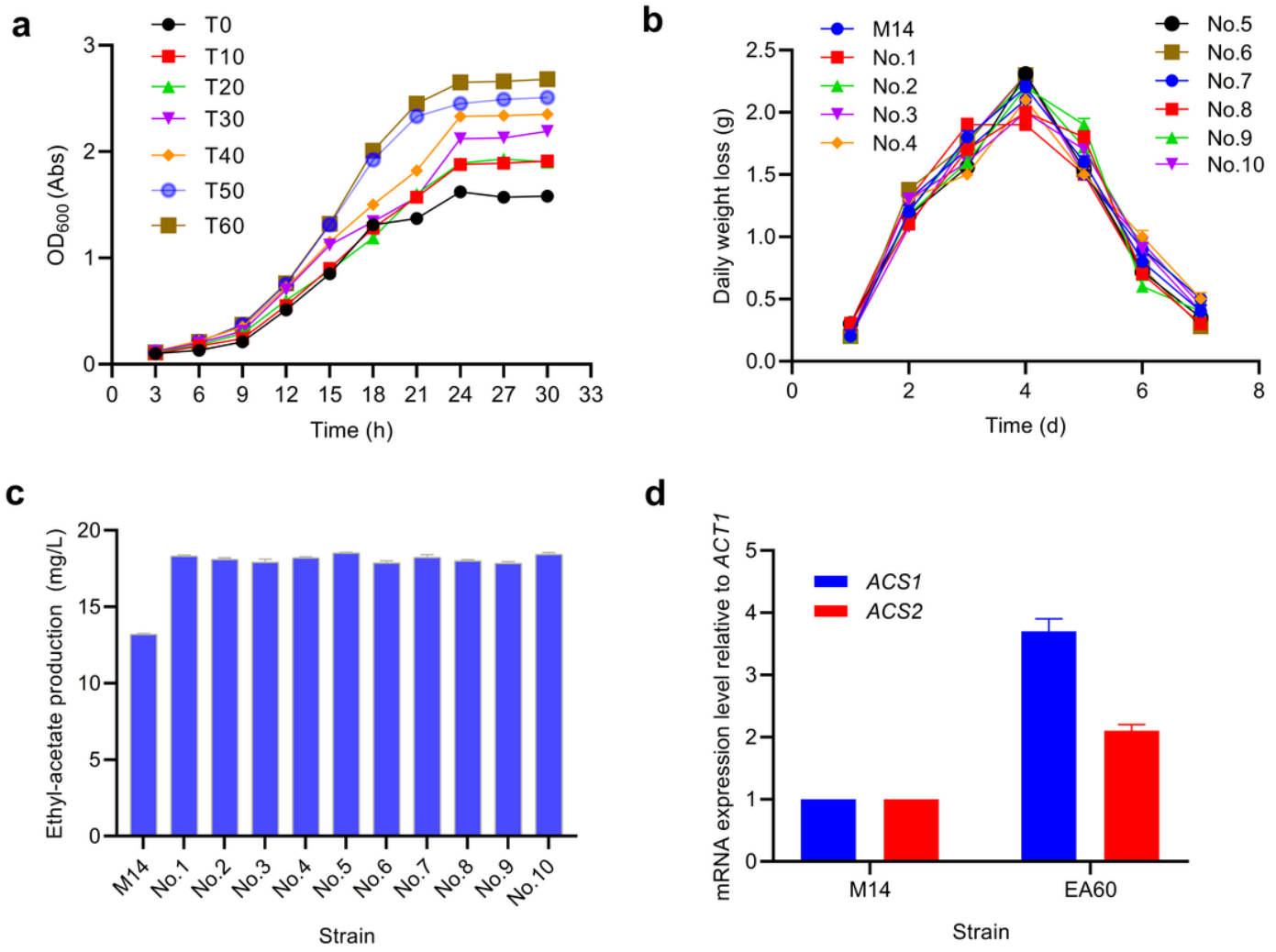

Figure 2

Breeding lager yeast with higher ethyl-acetate production. (a) The cell growth of the strains under 0.6% acetic acid during ALE process; (b) The daily weight loss (fermentation rate) of randomly picked strains seperated from the evolved strains which were transferred by 60 runs; (c) The ethyl-acetate production of the randomly picked strains; (d) The relatively expression level of ACS1 and ACS2 in the optimal strain EA60.

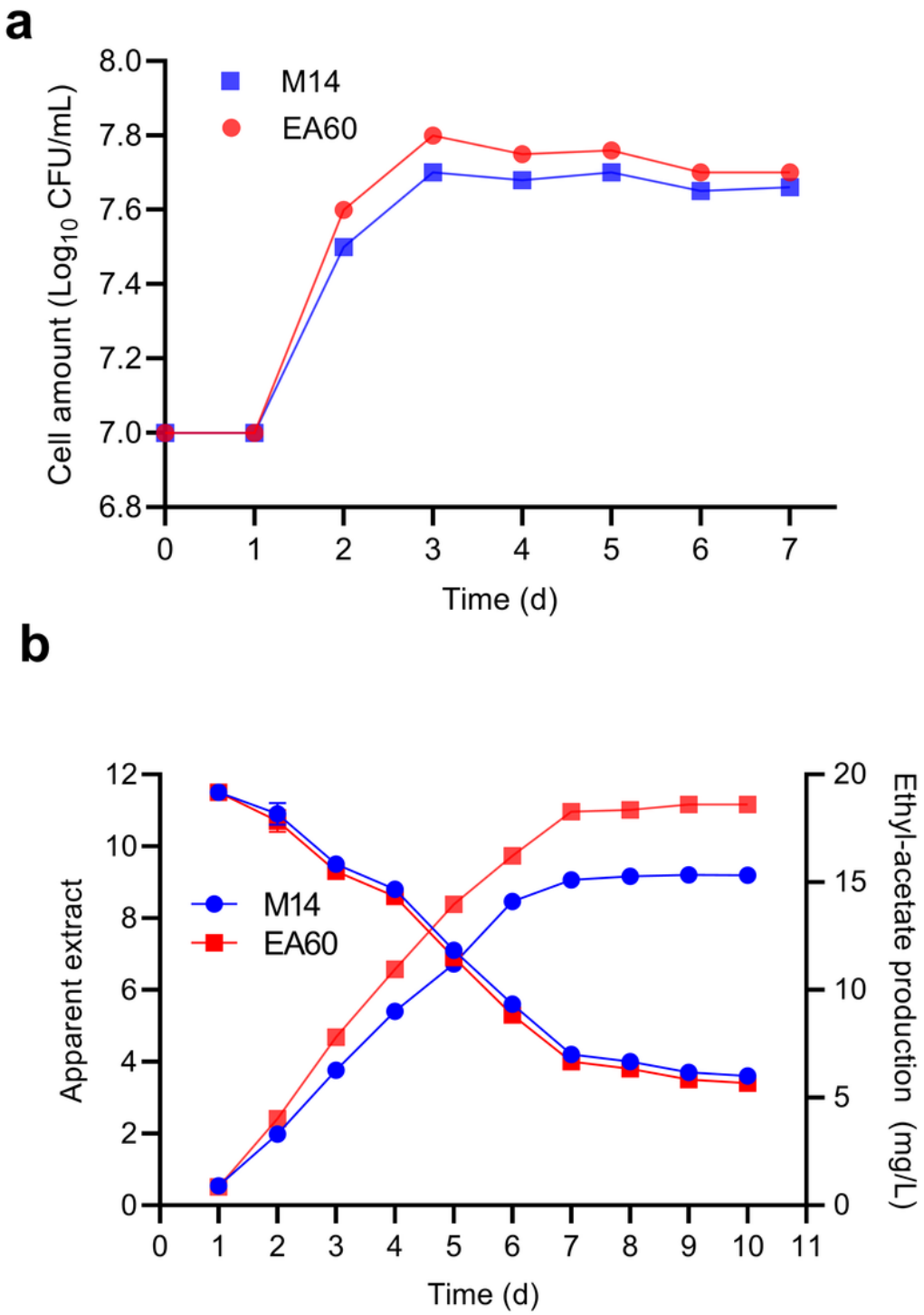

Figure 3

Time course of M14 and EA60 during alcoholic fermentation. (a) The cell amount during fermentation; (b) The dynamic changes of apparent extract and ethyl-acetate contents. The brewing experiment was carried in a 3 L Erlenmeyer flask with 2 L 11 °P wort at 11°C for 10 days with two repeats and the results were shown as means ± SD.

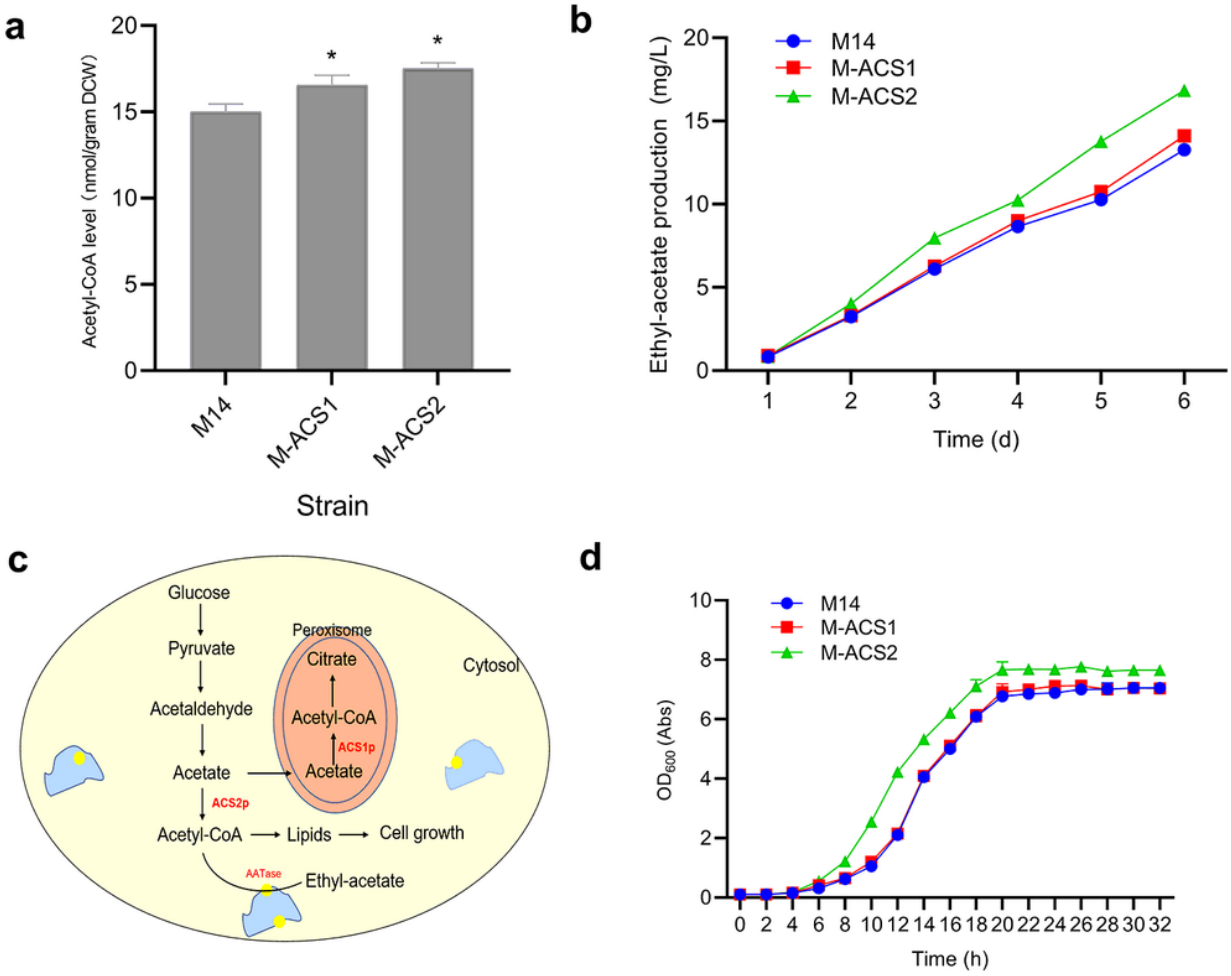

Figure 4

The different contribution of ACS1 and ACS2 to ethyl-acetate synthesis during alcoholic fermentation. (a) The cellular acetyl-CoA level of strain M14, M-ACS1 and M-ACS2; (b) The ethyl-acetate production; (c) The putative distribution of Acs1p and Acs2p in yeast cells; (d) The growth curve of strain M14, M-ACS1 and M-ACS2.