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RESEARCH ARTICLE

Optimization of fermentation conditions for production of ethyl caproate in *Baijiu* using a selected isolate of *Saccharomyces cerevisiae*

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ABSTRACT

Saccharomyces cerevisiae is indispensable in the production of *Baijiu*. This yeast not only produces ethanol, but also produces many flavor substances that impart unique quality characteristics to *Baijiu*. Ethyl caproate (EC) is the main flavor substance of strong-flavor *Baijiu* (SFB). *S. cerevisiae* that can produce a high yield of EC is critical for SFB production. In the present study, *S. cerevisiae* isolate YF1914 produced an EC content of 1.27 mg/L, which was greater than that produced by other test isolates obtained from several *Baijiu* brewing environments. Through single factor experiments, the influence of various factors on EC production by YF1914 was measured. Eight variables were tested using a Plackett-Burman design, and ethanol content, induction time, caproic acid content, initial pH, and temperature significantly affected EC production. Further studies were done using the steepest ascent path and response surface methodology (RSM) designs to identify optimized fermentation conditions for EC production. The optimum conditions were found to be: sorghum hydrolysate medium (SHM) with a sugar content of 12 Brix and an initial pH of 5.0, inoculum size 5% (v/v), incubation at 28 °C with shaking at 180 rpm for 32 h, addition of 10% (v/v) ethanol and 0.046% (v/v) caproic acid at 32 h, then static culture at 24 °C for 32 h. The optimized conditions produced an EC content of 21.98 mg/L or 17.3 times greater than the content obtained using initial conditions. Thus, a selected *S. cerevisiae* isolate with high EC yield was obtained, which can potentially improve SFB quality by increasing EC content.

Keywords: ethyl caproate; optimization; *Saccharomyces cerevisiae*; strong-flavor *Baijiu*

INTRODUCTION

Baijiu is a distilled liquor containing many flavoring substances such as esters, alcohols, and phenols (Hong et al., 2021). These compounds impart a distinctive flavor, making *Baijiu* unique among distilled liquors. Strong-flavor *Baijiu* (SFB) is the most important type, accounting for 70% of the *Baijiu* market. It is favored by consumers because of its strong aroma and rich flavor (Zhang et al., 2020; Zhao et al., 2017). Among the many flavor substances, ethyl caproate (EC) with a sweet aroma, cellar smell, and cucumber flavor, is the most important in determining the quality of SFB (Fan and Qian, 2006). However, the traditional fermentation process produces a low content of EC in raw, unblended *Baijiu* thus restricting the production of high-quality SFB (Li et al., 2019). Therefore, improving the content of EC in raw *Baijiu* is an important means to enhance the quality of SFB.

Previous research has shown that EC is mainly produced by microbial metabolism (Fan et al., 2020). Among the many functional microorganisms involved in *Baijiu* production, *Saccharomyces cerevisiae* is one of the most important (You et al., 2021). *S. cerevisiae* produces ethanol and flavor compounds such as esters (Wang et al., 2017; Zhang et al., 2020). Its biological characteristics are related to the yield and quality of *Baijiu* and these characteristics are influenced by the production environment. Generally, *S. cerevisiae* strains

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originating from different habitats often have different biological characteristics, due to genetic adaptation to specific environmental conditions. Currently *Baijiu* is produced in diverse settings in China. Breweries differ in their production environment and brewing methodology, resulting differences in microflora and biological characteristics of the same species (Wang et al., 2020). It is likely that *S. cerevisiae* strains from different *Baijiu* brewing enterprises differ in their EC yield. Identifying higher yielding strains could help brewers improve the quality of SFB (Li et al., 2020; Wang et al., 2020). This approach was successful with sake, where the EC content was greatly increased by using a *S. cerevisiae* isolate selected for high yield, thus improving the quality of the final product (Arikawa et al., 2000; Ichikawa et al., 1991; Kuribayashi et al., 2012; Takahashi et al., 2017). In contrast to the work with sake, there has been little research to measure EC yields with *S. cerevisiae* obtained from various *Baijiu* production environments, and the reported EC yields for the *S. cerevisiae* strains tested are low (< 0.5 mg/L) (Tan et al., 2016; Chen et al., 2016). Therefore, to identify a high EC yielding *S. cerevisiae* strain, the present study analyzed the ability of isolates obtained from different *Baijiu* production environments to produce EC. In addition, fermentation conditions for optimum EC production were identified. The present study identifies a *S. cerevisiae* isolate and specific production conditions that may be of great benefit in SFB brewing.

MATERIALS AND METHODS

Strains and reagents

Thirty *S. cerevisiae* strains were collected from different *Baijiu* brewing environments and maintained in our laboratory.

EC and heptane (chromatographic grade) were purchased from Sigma (St Louis, MO, USA). Sorghum was purchased in Xuchang, Henan province. All other chemicals were of analytical grade and commercially available unless otherwise stated.

Media preparation

Yeast extract peptone dextrose (YPD) medium and sorghum hydrolysate medium (SHM) were prepared as described previously (Fan et al., 2018).

Screening *S. cerevisiae* strains for high EC yield

Each *S. cerevisiae* strain was activated by inoculation into YPD medium at 28 °C and shaken at 180 rpm for 24 h. To induce the cell proliferation stage, the activated yeast cells were inoculated into 50 mL SHM and cultured for 24 h under the same conditions used for activation. Then, precursors (2% (v/v) ethanol and 0.02% (v/v) caproic acid) were added to allow synthesis of EC for 36 h during the induction stage. After culturing, the content of EC was determined by gas chromatography-mass spectrometry (GC-MS). The *S. cerevisiae* strain with highest yield of EC was used for optimization experiments (Supplementary Fig. S1).

Optimization of the production conditions with a single factor design

Various culture conditions (supplementary Table S1), including sugar content, initial pH, temperature (for the induction stage), shaking speed (for the induction stage), inoculum size, ethanol content, caproic acid content, time of precursors addition, and culture time (including time of both the cell proliferation stage and the induction stage), were optimized for EC production by using a single factor design under submerged fermentation in SHM as previously described.

Optimization of the production conditions using the Plackett-Burman (PB) experimental design

Based on the results from the single factor experiments, the PB design for eight variables, including sugar content (X_1), initial pH (X_2), temperature (X_3, for the induction stage), inoculum size (X_4), ethanol content (X_5), caproic acid content (X_6), time of precursors addition (X_7), and induction time (X_8), was used for screening (Table 1). Each variable was represented at low and high levels denoted by (− 1) and (+1), respectively (Table 1). EC production was the response value. A regression model, statistical significance (*F*-value analysis), and the proportion of variance explained were obtained using Design-Expert 11 (Stat-Ease Inc., USA).

Optimization of the production conditions by steepest ascent path design

Important variables screened with the PB design were used to construct the steepest ascent path. The direction of variables in the steepest ascent path design was determined according to the regression model from the PB design. Five experiments were conducted along the steepest ascent path and based on practical experience (Table 2).

Optimization of the production conditions using response surface methodology (RSM)

RSM was used to further optimize the screened variables for enhanced EC production, including induction time (A), ethanol content (B), and caproic acid content (C), using the Box–Behnken experimental design (BBD, Design-Expert Software 11.0) (Table 3). The center point of RSM was the point where the EC production was highest as identified with the steepest ascent path design.

Analysis of EC content

Samples were centrifuged at 13,000×g for 10 min at 4 °C. The supernatant was removed and mixed with an equal

Table 1: Test levels of variables and statistical analysis using the PB experimental design for EC production

Code	Variable	Low level (-1)	High level (+1)	Mean square	*F*-value	*P*-value	Rank	Significance
X_1	Sugar content (Brix)	10	14	0.0342	0.1456	0.7141	8	
X_2	Initial pH	4	8	6.30	26.82	0.0013	4	**
X_3	Temperature (℃)	16	20	1.91	8.11	0.0248	5	*
X_4	Inoculum size (%, v/v)	2.5	7.5	0.0385	0.1640	0.6976	7	
X_5	Ethanol content (%, v/v)	2	6	14.38	61.15	0.0001	1	**
X_6	Caproic acid content (%, v/v)	0.02	0.06	6.65	28.27	0.0011	3	**
X_7	Time of precursors addition (h)	24	40	0.2196	0.9342	0.3660	6	
X_8	Induction time (h)	8	40	11.60	49.35	0.0002	2	**

"*", significant at 5% level ($P < 0.05$); "**", significant at 1% level ($P < 0.01$).

Table 2: Experimental design and the results of steepest ascent for EC production

Groups	Ethanol content (%, v/v)	Induction time (h)	Caproic acid content (%, v/v)	Initial pH	Temperature (°C)	EC content (mg/L)
1	2	24	0.01	8	18	0.82
2	4	32	0.02	7	20	6.86
3	6	40	0.03	6	22	12.57
4	8	48	0.04	5	24	19.19
5	10	56	0.05	4	26	7.34

Table 3: The BBD design and the responses of the dependent variables

Test number	Ethanol content (%, v/v)		Induction time (h)		Caproic acid content (%, v/v)		EC content (mg/L)
	A	Code A	B	Code B	C	Code C	Y
1	8	0	24	0	0.04	0	19.03
2	8	0	24	0	0.04	0	19.23
3	10	+1	16	-1	0.04	0	11.32
4	8	0	16	-1	0.06	+1	16.55
5	6	-1	24	0	0.06	+1	17.01
6	6	-1	16	-1	0.04	0	17.99
7	10	+1	24	0	0.06	+1	17.06
8	10	+1	32	+1	0.04	0	21.7
9	8	0	24	0	0.04	0	19.13
10	8	0	16	-1	0.02	-1	6.42
11	8	0	24	0	0.04	0	19.25
12	6	-1	32	+1	0.04	0	17.11
13	10	+1	24	0	0.02	-1	8.69
14	8	0	32	+1	0.02	-1	13.26
15	6	-1	24	0	0.02	-1	10.15
16	8	0	24	0	0.04	0	18.95
17	8	0	32	+1	0.06	+1	18.06

volume of heptane. After incubation for 5 min, the organic phase was transferred to a new tube containing a small amount of anhydrous sodium sulphite for overnight at −20 ℃. Samples were filtered through a 0.22-μm nylon filter, then analyzed by GC-MS. The GC-MS conditions were as described by Fan et al (2018). The EC content in each sample expressed in mg/L was determined according with a calibration curve obtained by serial dilution of an EC standard solution. The EC calibration curve was generated using the external standard method. Specifically, 0.0869 g of EC was diluted by n-heptane into 100 mL, then serial diluted 50, 100, 200, 400, and 800 times. The EC content of the serial dilutions were measured by GC-MS to obtain a standard curve with the EC content as the X axis and the peak area as the Y axis as follows: $Y=4\times10^7X-514226$.

Statistical analysis

After verifying a normal distribution, a one-way ANOVA ($P < 0.05$) followed by Tukey's test was used to detect statistical differences among treatment groups. Each experiment was performed in triplicate and the mean value and standard deviation are reported. Data were processed and analysed using SPSS 24.0 (IBM Corp., New York, NY, USA), Design-expert 11 (Stat-Ease, Inc. USA), and Excel 2019 (Microsoft, USA).

RESULTS AND DISCUSSION

Screening for *S. cerevisiae* strains for high EC yield

In the present study, 30 *S. cerevisiae* isolates screened from samples related to *Baijiu* production were inoculated into SHM and their ability to produce EC was measured. All *S. cerevisiae* strains except two, F2405 and Y33175, could produce EC (Table 4). The EC yield for most of the *S. cerevisiae* strains was less than 1.0 mg/L, which is consistent with previous reports (Chen et al., 2016; Tan et al., 2016). These relatively low yielding strains were similar to the sake strain Kyokai no. 7 (Arikawa et al., 2000; Aritomi et al., 2004; Chen et al., 2016). Among the tested strains, only Y1360, YF1914, Y33197, and Y7#09 produced EC with a content greater than 1.0 mg/L with YF1914 producing the highest EC content of 1.27 mg/L. Thus, YF1914 was chosen for further experiments.

Optimization of production conditions with a single factor design

Effect of sugar content on EC Production

Because sorghum is the main raw material for *Baijiu* brewing, SHM was used as the initial fermentation medium to optimize the conditions for YF1914 to produce EC in submerged fermentation. The sugar content of SHM reflects the concentration of glucose, maltose, and other sugar sources, which can be used by yeast. The sugar content of SHM determines how much energy the SHM provides for yeast growth, reproduction, and metabolic activity (Fan et al., 2018). Therefore, an appropriate sugar content of SHM is conducive for yeast to produce EC. As sugar content increased, so did the concentration of EC produced by YF1914 (Fig. 1). In our previous studies, YF1914 showed a high glucose content tolerance (>80%, w/v) and it could produce highest ethyl alcohol when the glucose concentration was as high as 350 g/L (Fan et al., 2019; Fan et al., 2019). These results indicate that YF1914 has better growth, reproduction, and metabolic activity at higher sugar concentrations. However, the sugar content in SHM is often less than 150 g/L, which is insufficient for YF1914 to synthesize EC (Fan et al., 2019). Therefore, EC production by YF1914 increased with increasing sugar content (Fig. 1). In general, the sugar content of SHM is insufficient for YF1914 to grow, reproduce, and produce EC, which possibly indicates why a high EC content is difficult to achieve in *Baijiu* production. Even at the highest sugar content (14 Brix), EC production was relatively low. Compared with ethyl acetate produced by other yeasts, low sugar content is sufficient for ethyl acetate production (Fan et al., 2018; Fu et al., 2018). These effects are probably one reason why raw *Baijiu* often has poor quality from low EC content and high ethyl acetate content.

Effect of pH on EC production

pH is an important parameter regulating microbial metabolism. It not only affects the state of nutrients, but also affects microbial metabolism by altering microbial cell membrane potential and the activity of metabolic enzymes (Hashem et al., 2021). Specific metabolites produced by specific microorganisms have various optimal pH ranges. The maximal amount of EC produced by YF1914 occurred when the initial pH of SHM was either pH 5 or pH 6 (Fig. 2). The highest yield of EC was 2.1 mg/L at initial pH 6.0, which is consistent with the optimum growth pH for this strain (Fan et al., 2019). The yield of EC produced by YF1914 was related to its biomass, a result that is consistent with previous a report (Dufour et al., 2003). Thus, the synthesis of EC in the culture medium benefits from the metabolic activity of YF1914, rather than the chemical reaction of ethanol and caproic acid, as reported

Table 4: EC production by various *S. cerevisiae* strains

No.	EC content (mg/L)	No.	EC content (mg/L)	No.	EC content (mg/L)
Y115	0.25±0.02	P5503	0.13±0.05	Y4#16	0.53±0.10
Y1360	1.06±0.07	M3	0.73±0.23	Y4408	0.44±0.04
Y4#14	0.64±0.12	YF1914	1.27±0.12	Y3401	0.61±0.11
Y8#01	0.51±0.10	Y8#01	0.83±0.04	F13004	0.47±0.08
Y33199	0.21±0.05	Y1217	0.39±0.08	Y32702	0.91±0.13
Y32704	0.32±0.00	Y33197	1.03±0.11	Y33175	n.d.
Q5503	0.89±0.06	Y32508	0.13±0.02	Y32488	0.74±0.16
F1504	0.69±0.01	PC1	0.45±0.17	F10404	0.36±0.09
F2405	n.d.	Y7#09	1.13±0.08	F13011	0.92±0.11
Y4#04	0.39±0.11	Y8#15	0.33±0.14	AQ	0.19±0.07

"n.d.": not detected.

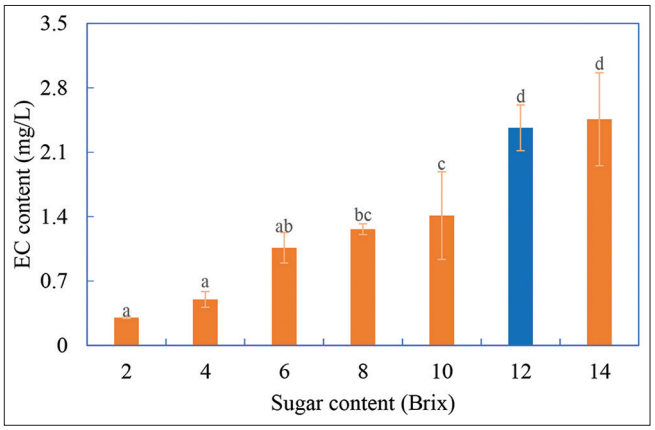

Fig 1. Effect of sugar content on EC production by YF1914. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

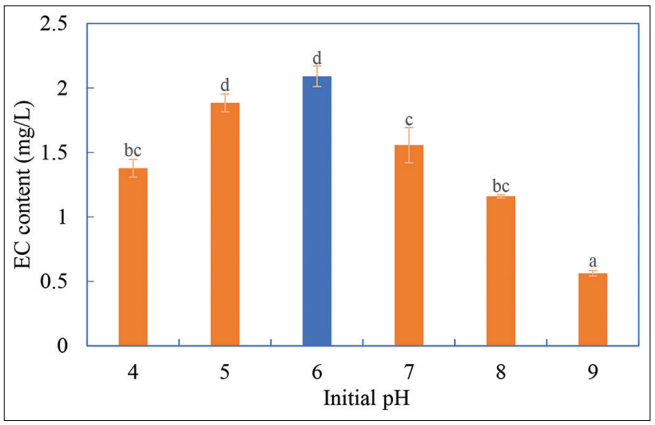

Fig 2. Effect of initial pH on EC production by YF1914. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

previously (Fan et al., 2021; Zhang et al., 2017). Fortunately, EC production under acidic conditions was slightly higher than under alkaline conditions (Fig. 2), corresponding to the optimum pH value for the *Baijiu* brewing process (Fan et al., 2019; Gao et al., 2021). This characteristic of YF1914

should enable it to be more easily applied to commercial *Baijiu* brewing.

Effect of temperature on EC production

Our previous study showed that YF1914 has a wide temperature adaptability range from 20-50 °C, which is important for its practical application in *Baijiu* brewing (Fan et al., 2019). The yield of EC first increased and then decreased with increasing temperature (for the induction stage), and the highest yield of EC was 3.9 mg/L at 18 °C (Fig. 3), a temperature outside the optimum range for growth of YF1914 (25-35 °C) (Fan et al., 2019). Compared with 18 °C, the optimum temperature range for growth would increase cytotoxicity to yeast cells from ethanol and caproic acid. Also, the optimum growth temperature range prevents acyl CoA accumulation and does not result in increased medium-chain fatty acid esters content (Dufour et al., 2003). Unfortunately, the temperature of the *Baijiu* brewing process is generally high, between 25-37 °C, and only in the later stage of fermentation does the temperature decline to about 25 °C (Zhang et al., 2021). These temperature effects may be another reason why EC content is not high in raw *Baijiu*, and a prolonged fermentation period is required to increase EC content.

Effect of shaking speed on EC Production

Many reports have shown that the amount of dissolved oxygen affects microbial metabolic pathways, thus affecting the content of their products (Barberel and Walker, 2000). The effect of dissolved oxygen on the EC yield of YF1914 was investigated by varying shaking speed (for the induction stage). The yield of EC decreased with increasing shaking speed, and yield was the highest in a static state (Fig. 4), which is consistent with our previous reports (Fan et al., 2021; Qin et al., 2021). Long-chain saturated fatty acids can accumulate and the activity of acetyl CoA carboxylase can be inhibited at low dissolved oxygen levels followed by an accumulation of medium-chain fatty acyl CoAs with acyl CoAs released from fatty acid synthase promoting the synthesis of EC (Dufour et al., 2003). The low oxygen demand for EC production by yeast benefits the current *Baijiu* production process. Because the highest EC yield occurred under static conditions, shaking speed was not investigated in the PB experiment.

Effect of Inoculum Size on EC Production

Results from the experiments on the effect of pH on YF1914 EC yield showed that the yield of EC was positively correlated with yeast biomass. Therefore, the effect of inoculum size on EC production was analyzed. Lower inoculum size had lower EC production, and the EC yield increased with the increasing inoculum size (Fig. 5). The highest EC yield was obtained at the inoculum size of 5% (v/v). Increasing the inoculum size beyond 5% inhibited

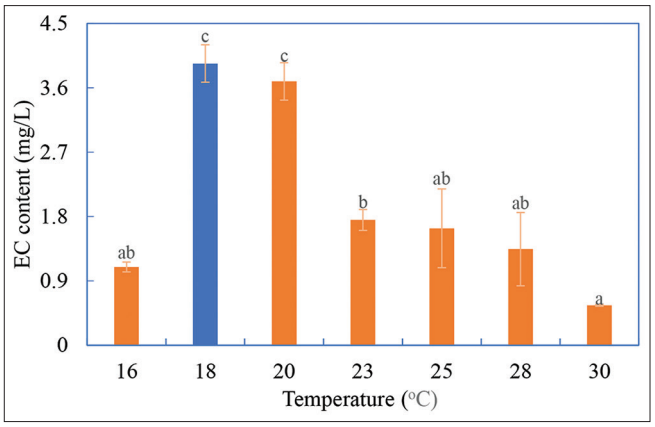

Fig 3. Effect of temperature on EC production by YF1914. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

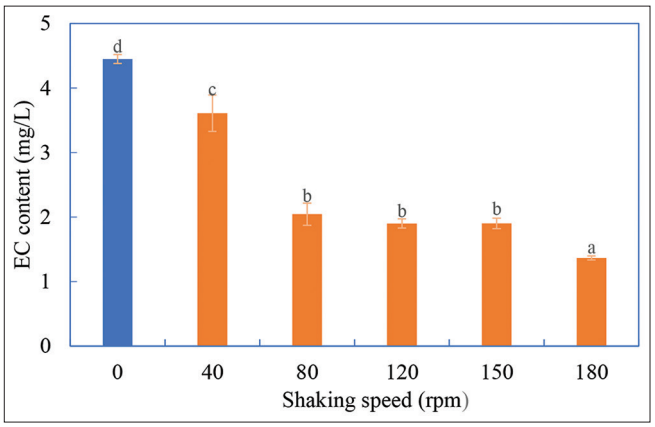

Fig 4. Effect of shaking speed on EC production by YF1914. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

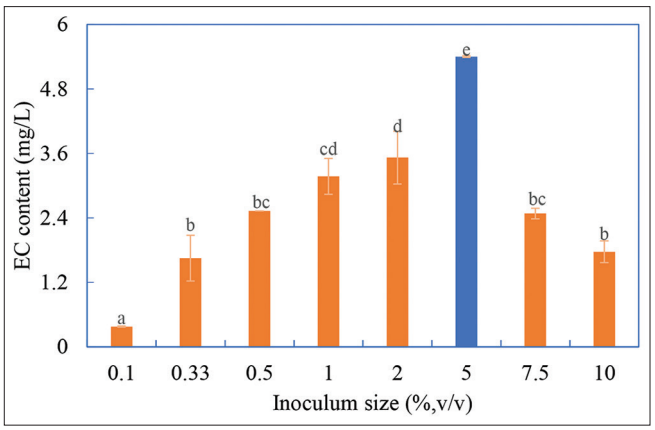

Fig 5. Effect of inoculum size on EC production by YF1914. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

EC yield, possibly due to the consumption of a large amount of nutrients for rapid cell growth and reproduction. In fact, such high biomass production is uncommon in the *Baijiu* brewing process. This result also explains why the

accumulation of EC in raw *Baijiu* requires a long brewing period. In addition, this result provides a theoretical basis for increasing the content of EC in raw *Baijiu* by enhancing the content of functional microorganisms with greater EC yielding capacity. Indeed, many researchers are seeking to enhance functional microorganisms to improve EC yield (Li et al., 2020).

Effect of Ethanol Content on EC Production

EC is formed by ethanol and caproic acid under the action of microbial esterases (Fan et al., 2021). Thus, ethanol is an important precursor for microbial synthesis of EC, but the compound has some toxic effect on microbial cells. This view is supported by the results of the present study (Fan et al., 2021). A high concentration of ethanol affects the growth and metabolism of the microorganism. Therefore, ethanol affects the microbial synthesis of EC based on these two effects. For YF1914, the yield of EC increased continuously with increasing ethanol content until the ethanol content reached 4% (v/v) (Fig. 6). Perhaps the toxic effect of ethanol on cells was less than its effect as a precursor in this range. However, when ethanol content was higher than 4%, the toxic effect of ethanol on YF1914 cells overcame its role as a precursor and the yield of EC synthesized by YF1914 decreased. This result also shows that EC in *Baijiu* is mainly produced by microbial enzymes, rather than chemical reactions. A small amount of EC was produced by YF1914 without additional ethanol, likely because *S. cerevisiae* can produce some ethanol in SHM (Fan et al., 2019). In the *Baijiu* brewing process, *S. cerevisiae* can produce sufficient ethanol slowly, providing ethanol for the synthesis of EC. The main purpose of the present study was to obtain a *S. cerevisiae* strain with a high EC yield, thus providing a valuable functional strain for improving EC content in raw *Baijiu*.

Effect of Caproic Acid Content on EC Production

Caproic acid is similar to ethanol in that it is a precursor of microbial synthesis of EC and it has toxic effects on microbial cells. A caproic acid content that is either too low or too high will result in lower EC generation by YF1914, due to either the lack of precursor or cell toxicity (Fig. 7). When the concentration of caproic acid was 0.04% (v/v), the EC yield of YF1914 was highest at 3.64 g/L. No EC was produced by YF1914 when the concentration of caproic acid was higher than 0.08% (v/v), which was likely due to the toxic effect of caproic acid on the yeast cells. However, the concentration of caproic acid is often insufficient in the *Baijiu* brewing process, resulting in low EC content (Chen et al., 2014; Li et al., 2020). No EC was produced when caproic acid was absent from the medium (Fig. 7), which confirms the requirement of caproic acid for microbial synthesis of EC. Consequently, many researchers strengthen pit mud by screening for higher caproic acid

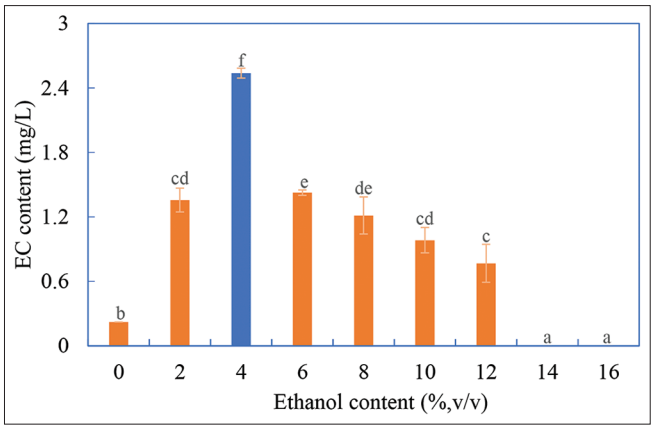

Fig 6. Effect of ethanol content on EC production by YF1914. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

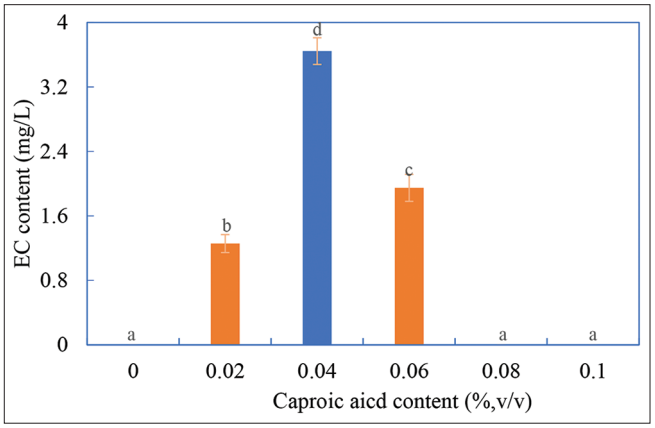

Fig 7. Effect of caproic acid content on EC production by YF1914. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

producing strains to improve caproic acid content (Li et al., 2020).

Effect of Time of Ethanol and Caproic Acid Addition on EC Production

Both ethanol and caproic acid are required for microbial synthesis of EC but both can have toxic effects at higher concentrations. Aside from the concentration of these two precursors, the condition of microorganisms when exposed to precursors can also influence the degree of toxicity. Therefore, the effect of the time of addition of the precursors on EC production was analyzed. Ethanol and caproic acid were added together at different time points (Supplementary Table S1). After both the precursors were added, the fermentation was allowed to continue for 36 h. No EC was produced when precursors were added before 16 h (Fig. 8). This result may be related to the yeast biomass and physiological state. During this period, yeast cells were mainly in the lag phase and the logarithmic prophase. The ability of cells to resist precursors was weak, and the biomass was low. Therefore, cell growth

was inhibited greatly after precursor addition and no EC production could be detected. Thereafter, the yeast cells were in metaphase and late logarithmic growth, and the stable growth phase. Cells in this stage had good tolerance to ethanol and caproic acid and sufficient biomass to quickly convert the precursors into EC. The highest yield of EC occurred when precursors were added at 32 h. Cells were sensitive to ethanol and caproic acid in the decline phase, causing biomass and EC yield to decrease with the addition of precursors. This pattern also was consistent with *Clavispora lusitaniae* YX3307 (Fan et al., 2021). These results show the difficulty in regulating EC content in *Baijiu* production. The concentration of precursors, microbial biomass, and the growth status of cells all influence the final content of EC.

Effect of Culture Time on EC Production

Consistent with the results from the experiments with precursor timing, no EC was detected in the first stage in the absence of precursors, especially caproic acid (Fig. 9). After adding precursors, EC production increased rapidly,

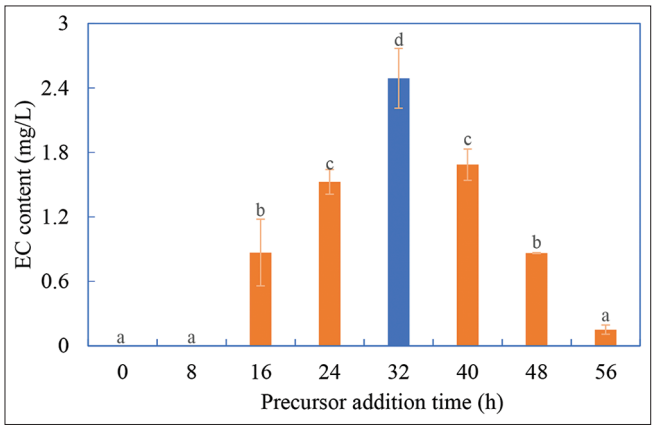

Fig 8. Effect of time of precursors addition on EC production by YF1914. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

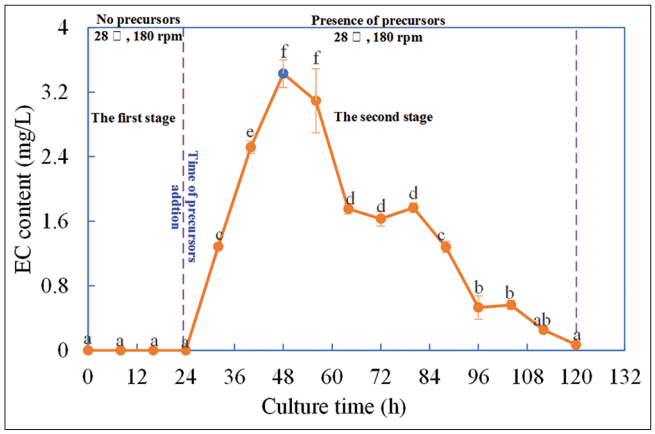

Fig 9. Effect of culture time on EC production by YF1914. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

especially within 24 hours likely due to high biomass accumulated in the first stage. These cells quickly convert highly toxic caproic acid into weakly toxic EC, which may be a stress response (Qin et al., 2021). In the *Baijiu* production it is difficult to ensure that the concentration of ethanol and caproic acid quickly reach the appropriate concentration simultaneously. In addition, the yeast biomass and growth state must be correct for optimal EC production. These factors explain why *Baijiu* brewing requires time to accumulate EC. The yield of EC was highest between 48 and 56 h. As fermentation time is extended beyond this time, nutrients in the medium become limiting and EC is metabolized to maintain cell growth and reproduction, resulting in a continuous decline in EC content.

Using a PB design to Optimize production conditions

The single factor experiments demonstrated that EC yield is influenced by multiple factors. Therefore, all factors except shaking speed were evaluated for optimization using the PB design. A design matrix of seventeen runs with eight variables along with the corresponding responses for EC production was used (Table 5). EC yield varied with the test conditions, ranging between 0.22 and 5.96 mg/L, indicating these factors strongly affected EC production (Table 5). A first order polynomial equation was fitted to the results:

$$Y=3.20-0.0543X_1-0.7377X_2+0.4057X_3+0.0577X_4+1.24X_5+0.7573X_6+0.1377X_7+1.00X_8 \quad (1)$$

Where Y is EC content, and X_1, X_2, X_3, X_4, X_5, X_6, X_7 and X_8 are sugar content, initial pH, temperature, inoculum size, ethanol content, caproic acid content, time of precursors addition, and induction time, respectively.

The coefficient of each variable represents their effect on EC production. The effect was identified by the Fisher's test and *P* values. The *F*-test value and *P* value of the model were 53.79 and 0.0001 ($P<0.01$), respectively, indicating the model was highly significant. The coefficient of determination (R^2) was 0.9703, suggesting the model could be used to guide further research. Based on the coefficient of regression, five out of the eight variables, X_5, X_8, X_6, X_2 and X_3, significantly influenced EC production (Table 1). These five factors merit further study. X_1, X_4, and X_7 had no insignificant effect on EC production and can be omitted from further study. In the following experiments, X_1, X_4, and X_7 were set to 12 Brix, 5% (v/v), and 32 h, respectively, according to the single factor results described above.

Optimization of the production conditions by steepest ascent path design

Results from the PB design showed that increased ethanol content, induction time, caproic acid content, and temperature have positive effects on EC production,

whereas higher initial pH had a negative effect. Therefore, ethanol content, induction time, caproic acid content, and temperature should be increased, and initial pH should be reduced in the steepest ascent path design. The step lengths of factors were according to equation (1) of the step length of the steepest ascent method and experimental experience. EC production increased along the path and reached the peak of 19.19 mg/L in test 4 under induction for 48 h at 24° C, initial pH 5.0, 8% ethanol, and 0.04% caproic acid, then decreased (Table 2). Thus, the fourth set of tests was used as the central point of the RSM.

Optimization of the production conditions using a RSM design

Based on results from the PB and steepest ascent path designs, three-factors including ethanol content, induction time, and caproic acid content at three level were considered for the RSM design. A total of 17 experiments for BBD were carried out to analyze the interactions between the factors and optimize the best fermentation conditions for EC production. Various maximum and minimum levels of three factors were used for the 17 experimental runs (Table 3). The regression model for EC production was performed with a 2nd-order polynomial equation:

$$Y=19.12-0.4362A+2.23B+3.77C+2.81AB+0.3775AC-1.33BC-1.22A^2-0.8715B^2-4.67C^2 \quad (2)$$

Where Y is EC production, and A, B, and C represent ethanol content, induction time, and caproic acid content, respectively.

The *F*-value for the regression model (*F*-value was < 0.0001) indicated the model was statistically significant (Table 6). The determination coefficient (R^2) was 0.9990, indicating that the sample variation of 99.90% for EC

Table 5: PB design matrix for evaluating factors influencing EC production

Run order	Std order	X_1 (Brix)	X_2	X_3 (°C)	X_4 (%, v/v)	X_5 (%, v/v)	X_6 (%, v/v)	X_7 (h)	X_8 (h)	EC content (mg/L)
1	17	12	6	18	5	4	0.04	32	24	4.15
2	10	10	4	16	2.5	2	0.02	24	8	0.38
3	2	10	8	20	2.5	6	0.02	24	40	3.67
4	1	14	4	20	2.5	6	0.06	24	8	5.31
5	6	12	6	18	5	4	0.04	32	24	4.67
6	5	12	6	18	5	4	0.04	32	24	4.59
7	9	10	8	16	2.5	6	0.06	40	40	5.73
8	16	12	6	18	5	4	0.04	32	24	4.77
9	12	14	8	20	2.5	2	0.02	40	8	0.22
10	11	14	8	16	7.5	6	0.06	24	8	2.70
11	3	10	8	20	7.5	2	0.06	40	8	1.30
12	15	12	6	18	5	4	0.04	32	24	4.85
13	14	10	4	20	7.5	2	0.06	24	40	5.16
14	7	14	4	20	7.5	6	0.02	40	40	5.96
15	13	14	8	16	7.5	2	0.02	24	40	1.14
16	8	14	4	16	2.5	2	0.06	40	40	3.53
17	4	10	4	16	7.5	2	0.02	40	8	0.79

Table 6: Regression coefficients and their significances for EC production from the results of the BBD

Source	Sum of squares	DF	Mean square	*F*-value	*P*-value	Significant
Model	301.52	9	33.50	784.99	< 0.0001	**
A-Ethanol content	1.52	1	1.52	35.67	0.0006	**
B-Induction time	39.83	1	39.83	933.19	< 0.0001	**
C-Caproic acid content	113.70	1	113.70	2664.13	< 0.0001	**
AB	31.70	1	31.70	742.68	< 0.0001	**
AC	0.5700	1	0.5700	13.36	0.0081	**
BC	7.10	1	7.10	166.41	< 0.0001	**
A^2	6.23	1	6.23	146.00	< 0.0001	**
B^2	3.20	1	3.20	74.93	< 0.0001	**
C^2	91.98	1	91.98	2155.25	< 0.0001	**
Residual	0.2988	7	0.0427			
Lack of Fit	0.2327	3	0.0776	4.69	0.0847	not significant
Pure Error	0.0661	4	0.0165			
Cor Total	301.82	16				
	R^2=0.9990	R^2_{Adj}=0.9977	CV=1.30%			

"**", significant at 1% level ($P < 0.01$).

production could be attributed to the independent variables and only about 0.10% of the total variation was unexplained by the model. The model terms of A, B, C, AB, AC, BC, A^2, B^2 and C^2 were significant, and the lack of fit was not significant with a *P*-value of 0.0847. Thus, the model is adequate for prediction within the range of variables used.

All variables had a strong linear effect on the response ($P < 0.01$). Ethanol content had a negative effect, while induction time and caproic acid content had positive effects. The significant positive effect of induction time and caproic acid content indicates that these factors caused EC production to increase. The interaction between any two of the factors was significant and all had significant positive quadric effects on EC production.

Three-dimensional (3D) response surface plots for EC production illustrate the effects of the independent variables and the interactions between two variables by keeping the third variable at zero (Fig. 10). All response surface graphs displayed an obvious convex shape with an open downward direction, indicating the optimum levels for EC production were well-defined. The elliptic order of contour indicates that the interaction between any two of three factors was significant.

EC production increased slightly as ethanol content and induction time together increased (Fig. 10a). EC production increased first then decreased gradually as the caproic acid content increased, whereas there was no significant change of EC production with the increase of ethanol content (Fig. 10b). In addition, EC production increased then decreased as caproic acid content increased and slightly increased with increasing induction time (Fig. 10c).

Optimal values of the variables were calculated from the data obtained with RSM using the following critical values: A (ethanol content) = 10%, B (induction time) = 32 h, and C (caproic acid content) = 0.046%. The model predicted that the EC production could reach 22.06 mg/L using these optimized values. A validation experiment was performed, and the yield of EC achieved 21.98 mg/L, which conformed well to the value predicted by the model. Optimization resulted in a 16.3-fold increase in EC production compared to 1.27 mg/L.

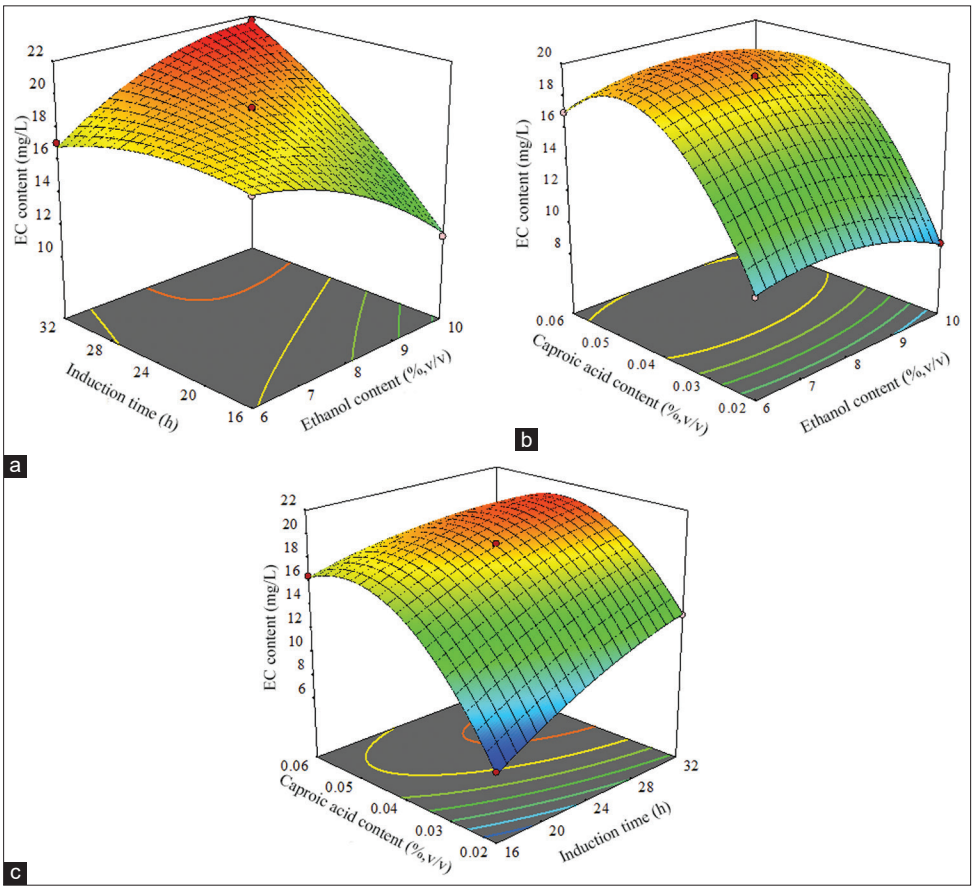

Fig 10. The 3D surface interaction plots of the effects of ethanol content, induction time and caproic acid content on the EC production response using the BBD. Interaction of (a) ethanol content and induction time, (b) ethanol content and caproic acid content, and (c) induction time and caproic acid content.

CONCLUSION

In the present, *S. cerevisiae* strains isolated from *Baijiu* brewing environments were screened for EC production and strain YF1914 was selected. Experiments were performed to optimize EC production by YF1914 using single factor, PB, steepest ascent path, and RSM designs. After optimization, an EC yield of 21.98 mg/L was achieved under the following conditions: YF1914 was cultured in SHM, 12 Brix, initial pH 5.0, inoculum size 5%, 28 °C, shaking at 180 rpm for 32 h, followed by addition of 10% ethanol and 0.046% caproic acid, and final static-state fermentation at 24 °C until 64 h. *S. cerevisiae* YF1914 applied in practical production and using the optimized fermentation conditions described here would likely increase the EC content in raw *Baijiu*, and thus enhance the production of high-quality *Baijiu*.

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Author contribution

The concept and design of the study were done by Fan Guangsen and Li Xiuting; experiments were done by Liu Pengxiao, Yin Huan, and Gong Yi; analysis and interpretation of data were done by Xu Jiangqi and Teng Chao; manuscript writing was done by Yang Ran; review was done by Chang Xu.

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