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Research Article

Optimization of Caproic Acid Production from *Clostridium kluyveri* H588 and its Application in Chinese Luzhou-flavor Liquor Brewing

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Abstract: This study focused on the optimization of caproic acid production from *Clostridium kluyveri* H588 and its application in Luzhou-flavor liquor brewing. The critical variables that affected caproic acid production were identified by Plackette-Burman design (ethanol, sodium acetate, yeast extract and initial pH) and further optimized by using a four factor central composite design of response surface methodology. The results demonstrated that the optimum conditions for caproic acid production were determined to be ethanol concentration of 4.046% (v/v), sodium acetate concentration of 0.982% (w/v), yeast extract concentration of 0.145% (w/v) and initial pH of 6.41. Caproic acid production (214.23 mg/100 mL) in the optimized condition was in good agreement with the value predicted by the model equation (219.11 mg/100 mL), thereby assuring its effectiveness. Furthermore, the optimized condition of the lab-scale bioreactor was scaled-up to 5000 L fermentor and the obtained caproic acid broth was subjected to pit-filling fermentation. The result demonstrated this method of pit-filling fermentation was efficient in improving the quality of Luzhou-flavor liquor.

Keywords: Daqu, fermented *Zaopei*, luzhou-flavor liquor, microbe

INTRODUCTION

Chinese liquor is a traditional alcoholic beverage, which is consumed widely in China and plays an important role in Chinese life and culture (Zhang *et al*., 2012). In general, it is typically classified into five categories based on aroma characteristics: Luzhou-flavor style, light aroma style, soy sauce aroma style, sweet honey style, and miscellaneous style (Li *et al*., 2013, 2012). Among them, Luzhou-flavor liquor is the most popular distilled liquor in China.

In the traditional solid fermentation of Luzhou-flavor liquor, pit mud is the basis of liquor flavor, as it habitated a large number of microbes such as caproic acid bacteria, lactic acid bacteria, butyric acid bacteria, acetic acid bacteria and methane bacteria etc., which were believed to play key roles in the formation of aroma substance, and consequently gave Luzhou-flavor liquor special feature described as highly flavored, sweet and refreshing (Chen *et al*., 2010). Among them, caproic acid bacteria is one of the most important acid producing microbes, as it was intimately associated with the production of ethyl caproate and its precursor caproic acid. In Luzhou-flavor liquor, ethyl caproate is the dominant aromatic ingredient and its content affects the taste of spirits. Therefore, in Chinese brewing liquour industry, in order to improve the flavor of Luzhou-flavor liquor, it should be necessary to increase the production of ethyl caproate. Normally, high concentration of caproic acid could promote high production of ethyl caproate, conversely, low concentration of ethyl caproate was produced. In addition, the caproic acid bacteria could help to generate butyric acid, valeric acid and other trace elements, which also contributed to the special flavor of Luzhou-flavor liquor (Fan and Qian, 2006). Therefore, the growth metabolism of the caproic acid bacteria could greatly influence the quality of the finally obtained liquor. It was reported that the caproic acid fermentation broth had being successively used in pit mud maintenance, acceleration the aging of pit mud and preparation of esterifying liquid (Song, 2001; Xu, 2012; Li and Li, 2012). But its use in pit-filling fermentation was rarely investigated.

In present study, a Plackette-Burman design followed by a central composite design of response surface methodology has been used to optimize

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Table 1: Level of the variables and statistical analysis of Plackette-Burman design

Factors	Code	Low level (-1)	High level (+1)	Effect	Coef.	t-value	p-value
$(NH_4)_2SO_4$ (w/v, %)	A	0.10	0.2	-2.527	-1.264	-0.62	0.580
K_2HPO_4 (w/v, %)	B	0.05	0.1	11.548	5.774	2.83	0.066
$CaCl_2$ (w/v, %)	C	0.05	0.1	-7.668	-3.834	-1.88	0.157
Sodium acetate (w/v, %)	D	0.50	1.0	33.077	16.539	8.10	0.004
$MgSO_4.7H_2O$ (w/v, %)	E	0.05	0.1	-5.880	-2.940	-1.44	0.245
Yeast extract (w/v, %)	F	0.10	0.2	19.101	9.550	4.68	0.018
Ethanol (v/v, %)	G	2.00	4.0	-17.823	-8.912	-4.37	0.022
Initial pH	H	6.00	7.0	15.245	7.623	3.73	0.033

R^2 = 97.82%, R^2 (adj) = 92.00%; Coef.: Coefficient

conditions of caproic acid production, and the optimized condition of the lab-scale shake flask was scaled-up to 5000 L pilot-scale fermentor. Additionally, the obtained caproic acid broth was subjected to the pit-filling fermentation for efficient improving the quality of Luzhou-flavor liquor.

MATERIALS AND METHODS

Microorganism and culture maintenance: *Clostridium kluyveri* H588 used in this study was obtained from Key Laboratory of Bio-engineering of Huainan Normal University, People's Republic of China. It was maintained on the modified Barker medium (w/v): 0.5%, K_2HPO_4 0.04%, $MgSO_4.7H_2O$ 0.02%, $(NH_4)_2SO_4$ 0.1%, yeast extract 1%, $CaCO_3$ 0.05% and ethanol 2% and sealed with paraffin oil and then kept at 4°C. Inoculum of *Clostridium kluyveri* H588 was prepared in the seed medium (w/v) containing sodium acetate 0.5%, K_2HPO_4 0.04%, $MgSO_4.7H_2O$ 0.02%, $(NH_4)_2SO_4$ 0.1%, yeast extract 1%, $CaCO_3$ 0.05% and ethanol 2% in 500 mL flasks with 450 mL of culture medium. Pre-cultivation was performed anaerobically at 35°C for 7 days, then the obtained culture broth was used as inoculum for caproic acid fermentation experiments.

Shake flask batch fermentation: The caproic acid fermentation experiments were performed in 500 mL Erlenmeyer flasks each containing 450 mL liquid volume. For optimization studies, the composition of the fermentation medium was varied according to the experimental design. The initial pH of each medium was also adjusted to the required value either with 1 M HCl or 1 M NaOH. The medium in the flasks was then sterilized at 121°C for 15 min. After inoculation with 10% (v/v) of inoculum, the flasks were incubated statically at 35°C in an incubator chamber. Sampling was taken after 10 days for caproic acid concentration analysis. The optimization was performed according to either Plackette-Burman or Central Composite Design. Table 1 to 3 show the experimental variables studied.

Plackett-Burman experimental design: The Plackette-Burman design, a useful method for process conditions optimization, was utilized in present study as a first optimization step to investigate which factors have a significant effect on caproic acid production. For the selection of these factors, the Minitab (16.0) statistical software package was introduced to generate and analyze the design. The Plackette-Burman experimental design is a two factorial design based on Eq. (1):

$$Y = A_0 + \sum A_i X_i \quad (1)$$

where, Y is the response (caproic acid production), A_0 is the model intercept, A_i is the linear coefficient and X_i is the level of the independent variables. This model does not consider the interaction among variables and it is used to screen and evaluate the important variables that influence the response. According to Plackette-Burman design, each variable was examined in two levels: -1 for low level and +1 for high level (Pan *et al*., 2008; Bie *et al*., 2005). In this study, eight selected factors were examined in 12 experimental designs. All experiments were repeated three times and the averages of caproic acid concentration (Y) were taken as the response. The factors that were included in the screening test and their settings are given in Table 2.

Table 2: The Plackette-Burman design variables (in coded levels) with caproic acid concentration as response

Run	A	B	C	D	E	F	G	H	Y (mg/100 mL)
1	1	-1	1	-1	-1	-1	1	1	102.556
2	1	1	-1	1	-1	-1	-1	1	169.172
3	-1	1	1	-1	1	-1	-1	-1	108.010
4	1	-1	1	1	-1	1	-1	-1	156.282
5	1	1	-1	1	1	-1	1	-1	138.013
6	1	1	1	-1	1	1	-1	1	151.255
7	-1	1	1	1	-1	1	1	-1	155.382
8	-1	-1	1	1	1	-1	1	1	133.068
9	-1	-1	-1	1	1	1	-1	1	176.873
10	1	-1	-1	-1	1	1	1	-1	104.699
11	-1	1	-1	-1	-1	1	1	1	142.370
12	-1	-1	-1	-1	-1	-1	-1	-1	121.436

Table 3: Coded and real values for each variable of the central composite design

		Coded levels				
Variables	Symbol	-2	-1	0	+1	+2
Ethanol (v/v, %)	X_1	1.00	2.0	3.00	4.0	5.00
Sodium acetate (w/v, %)	X_2	0.25	0.5	0.75	1.0	1.25
Yeast extract (w/v, %)	X_3	0.05	0.1	0.15	0.2	0.25
Initial pH	X_4	5.50	6.0	6.50	7.0	7.50

Central composite experimental design and optimization by response surface methodology: The levels of significant parameters and the interaction between various process conditions, which significantly influenced the caproic acid production, were analyzed and optimized using the central composite design (Yan *et al*., 2011). The central composite design is one of the most widely used response surface designs for fitting second-order models. In this study, the four different parameters were chosen as main variables and designated as x_1, x_2, x_3, and x_4, respectively, in Table 3. The low, middle, and high levels of each variable were designated as -2, -1, 0, +1 and +2, respectively, and were assigned in the variable levels x_i coded as X_i according to Eq. (2):

$$X_i = \frac{x_i - x_0}{\Delta x} \quad (2)$$

where, X_i is the coded value of the i^{th} test variable, x_i is the reel value of the i^{th} test variable, x_0 is the real value of the x_i at the centre point of the investigated area, and Δx is the step change of variable. The second-order model used to fit the response to the independent variables is shown in Eq. (3):

$$Y = \beta_0 + \sum_{i-1}^{k} \beta_i X_i + \sum_{i-1}^{k} \beta_{ii} {X_i}^2 + \sum_{i}^{k}\sum_{j}^{k} \beta_{ij} X_i X_j \quad (3)$$

where,

Y : The predicted response value

β_0, β_i, β_{ii} and β_{ij} : Constant, linear, quadratic and interaction coefficients, respectively

k : The number of factors studied in the experiment

X_i and X_j : The coded independent factors under study

The experiment design and the results of the central composite design are presented in Table 4. The fit of the regression model obtained was checked using the adjusted coefficient of determination R-squared. Statistical and numerical analyses involving multiple regressions and the Analysis of Variance (ANOVA) were carried out with the aid of the Design Expert 7.1.6 statistical software. Moreover, the statistical significance of the model was determined by the application of Fischer's F-test. Contour and surface plot of response values were obtained using the same software. Finally, a validation experiment was carried out under theoretical optimum conditions as predicted by the model. All experiments were repeated three times and the results represented the mean values of three independent experiments.

Seed development for the pilot-scale fermentation: The inoculum for the 5000 L fermentation was prepared as follows. A 500 mL Erlenmeyer flask containing 405 mL of the seed medium (second-stage seed) was inoculated with 45 mL inoculum suspension of *Clostridium kluyveri* H588 (first-stage seed) and incubated anaerobically for 7 days at 35°C in an

Table 4: Central composite design for the experimental design and predicted results

	Code values				Real values				Caproic acid concentration (mg/100 mL)	
Run	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4	Actual	Predicted
1	-1	1	1	-1	2.0	1.00	0.20	6.0	149.401	151.58
2	0	0	0	0	3.0	0.75	0.15	6.5	204.123	203.63
3	-1	-1	-1	-1	2.0	0.50	0.10	6.0	105.670	105.10
4	1	1	1	-1	4.0	1.00	0.20	6.0	194.878	196.44
5	-1	1	-1	1	2.0	1.00	0.10	7.0	169.330	167.54
6	0	0	-2	0	3.0	0.75	0.05	6.5	131.768	134.58
7	-1	-1	1	-1	2.0	0.50	0.20	6.0	137.016	126.18
8	0	0	0	0	3.0	0.75	0.15	6.5	203.281	203.63
9	-1	1	-1	-1	2.0	1.00	0.10	6.0	153.012	151.42
10	-2	0	0	0	1.0	0.75	0.15	6.5	127.855	135.26
11	-1	-1	-1	1	2.0	0.50	0.10	7.0	139.724	133.46
12	0	0	0	-2	3.0	0.75	0.15	5.5	156.613	160.04
13	-1	1	1	1	2.0	1.00	0.20	7.0	169.992	167.69
14	1	1	-1	1	4.0	1.00	0.10	7.0	184.080	190.22
15	0	0	0	0	3.0	0.75	0.15	6.5	203.235	203.63
16	1	-1	1	1	4.0	0.50	0.20	7.0	171.567	168.45
17	1	1	-1	-1	4.0	1.00	0.10	6.0	205.371	203.70
18	1	1	1	1	4.0	1.00	0.20	7.0	181.427	182.95
19	0	0	0	0	3.0	0.75	0.15	6.5	202.996	203.63
20	2	0	0	0	5.0	0.75	0.15	6.5	205.109	201.45
21	0	-2	0	0	3.0	0.25	0.15	6.5	129.570	137.21
22	0	2	0	0	3.0	1.25	0.15	6.5	201.924	198.03
23	1	-1	-1	1	4.0	0.50	0.10	7.0	156.019	154.80
24	0	0	2	0	3.0	0.75	0.25	6.5	147.462	148.40
25	-1	-1	1	1	2.0	0.50	0.20	7.0	151.907	154.54
26	0	0	0	2	3.0	0.75	0.15	7.5	174.585	174.91
27	0	0	0	0	3.0	0.75	0.15	6.5	203.864	203.63
28	1	-1	1	-1	4.0	0.50	0.20	6.0	166.942	169.69
29	0	0	0	0	3.0	0.75	0.15	6.5	204.291	203.63
30	1	-1	-1	-1	4.0	0.50	0.10	6.0	158.435	156.03

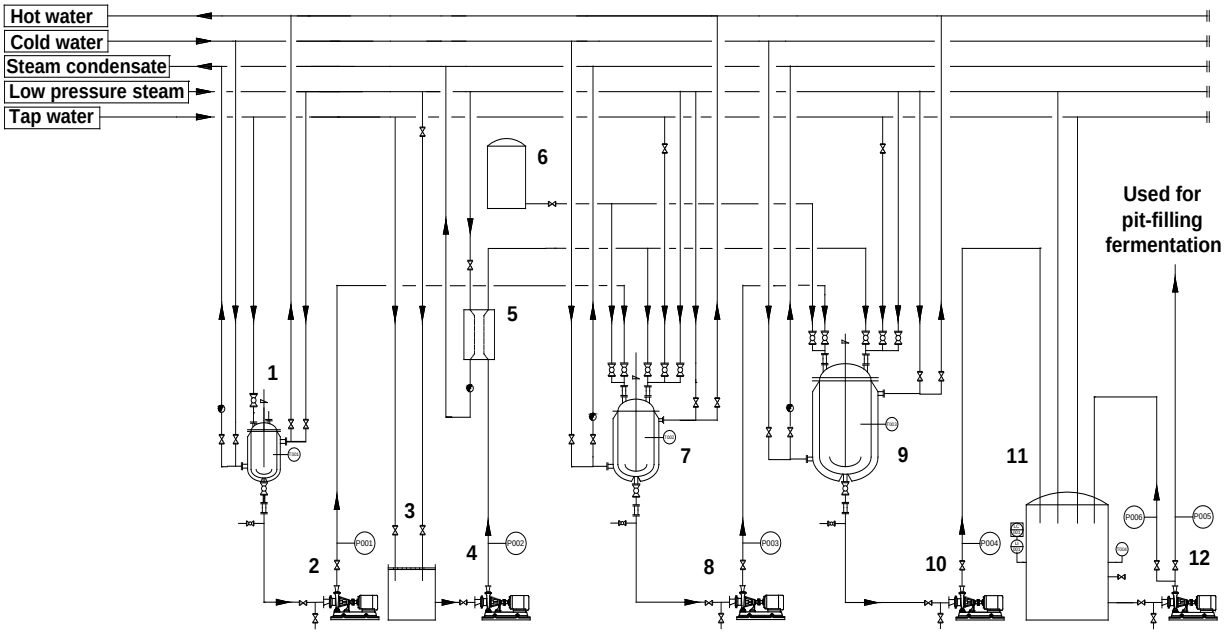


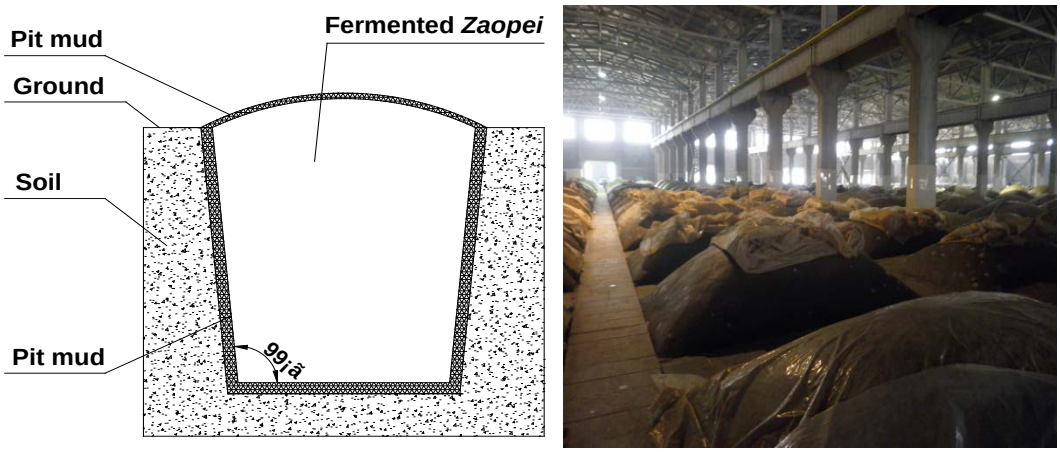
Fig. 1: The layout of the 5000 L caproic acid production pilot plant
1: 50 L seed preparation reactor; 2: Pump; 3: Mixing tank; 4: Pump; 5: Plate heat exchanger; 6: Ethanol tank; 7: 500 L seed preparation reactor; 8: Pump; 9: 5000 L reactor; 10: Pump; 11: Product storage tank; 12: Pump

incubator chamber. The third-stage seed was prepared by inoculating 450 mL second-stage seed into the 50 L seed preparation reactor containing 4050 mL seed medium, and the culture condition was the same to those mentioned above. Then the entire volume was inoculated into the 500 L seed fermentor (v/v, 10%). Also, after incubating under the same conditions and reaching middle exponential phase the fermentation broth (fourth-stage seed) was used as the seed of the 5000 L scale fermentation.

Pilot-scale batch fermentation: In this pilot-scale batch fermentation study, a fermentor of 5000 L with 4500 L working volume was used. The pilot-scale fermentation was carried out under the optimized caproic acid production conditions. The experiment was conducted in a pilot-scale plant built at Anhui Yingjia Group Co., Ltd., Luan city, People's Republic of China. Figure 1 illustrates the schematic diagram of the experimental apparatus used in this study. The apparatus was composed of four parts including a mixing tank, an inoculum preparation system, a caproic acid fermentor and a storage tank. All facilities were made of stainless steel.

Prior to the start of the pilot-scale fermentation, the fermentor operation requires a thorough manual cleaning followed by sterilization-in-place. Sterilizations were performed using low pressure steam to heat vessels and transfer lines, while steam condensate was drained from low points through steam traps. Transfer lines were held at 121°C or a higher temperature for at least 30 min and adequacy of pipe surface temperature was verified using a temperature indicator. All empty vessels except the mixing tank with their associated housings were sterilized prior to use by heating to 121°C and holding for 30 min. The fermentation medium was prepared by mixing the media components except ethanol in the mixing tank. Then the medium was sterilized through a plate heat exchanger and subsequently pumped into the caproic acid production reactor. Afterward, the temperature of the medium was adjusted to 35°C by wetting the bioreactor externally with cold tap water, and the initial pH was adjusted at desired level by the addition of 5 M KOH or 1 M HCl, then the mixture was added with ethanol and inoculated with 10% (v/v) of the seed culture. The whole was allowed to ferment at constant temperature of 35°C, adjusted continuously by cooling or heating with water. The caproic acid content was determined every day until the end of fermentation. After caproic acid fermentation, the broth was pumped into the storage tank and used for pit-filling fermentation mentioned in next paragraph.

Pit-filling fermentation: The pit-filling fermentation experiments were performed using three industrial traditional fermenting pits and their profile is shown in Fig. 2. For each pit, the top length and width is 3600 and 2300 mm, respectively, the bottom length and width is 2800 and 1540 mm respectively, and the depth is 2400 mm. Another three pits owned the same geometric volume, were used as the control. All the pits were built in 2012.



(A) (B)

Fig. 2: The profiles of the Luzhou-flavor soil pit (A) and the pit filled with *Zaopei* (B)

In the brewing process, the raw materials, namely multiple-grains (sorghum, rice, glutinous rice, maize and wheat), were milled into grain power and cooked with crude distillers' grains prepared from previous processes. Subsequently, the starter Chinese Daqu was put into the cooked mixture, which was indispensable to fermentation. After these materials were mixed as the ratio defined by the process, they were placed into the soil cellar and covered with pit mud, then subjected to liquor fermentation. As for the control pit, it was fermented at the natural conditions for 70 days. While for the experimental pit, part of the upper pit mud was clawed and 500 L of the caproic acid fermentation broth was filled in the fermented *Zaopei* from the top to the bottom as soon as the ethanol fermentation basically finished (Occurred after 20 days in this study). Subsequently, the test pit was sealed by the pit mud and subjected to fermentation again for about 50 days. After 70 days of fermentation, the *Zaopei* of the test and the control pits was took out and distilled with steam to extract ethanol and other flavor compounds, respectively and then the quality of each raw liquor was employed as the criterions for pit-filling fermentation assessment.

Analytical methods: The cell concentration was monitored spectrophotometrically at absorbance of 600 nm. The uninoculated sterile medium was used as a control. The Optical Density (OD) value was then converted to Dry Cell mass (DCW) using a calibration curve. The relationship between DCW and OD was found to be DCW (mg/L) = $162.4 \times OD_{600} - 2.03$; $R^2 = 0.9981$. The caproic acid in the fermented broth and main aromatic compounds in the distilled liquor were measured by using Agilent 7890 gas chromatography with a CP-Wax 57CB Acidic capillary column (50 m×0.25 mm i.d. and 0.20 μm film thickness) and a Flame Ionization Detector (FID). The chromatogram was run at 270°C injection temperature and 300°C detector temperature using N_2 as a carrier gas and H_2 as a flaming gas (Zhang *et al*., 2012, 2013). For the fermented broth, it should be filtered through a filter with a diameter of 0.22 μm, then the obtained solution was subjected to a Gas Chromatograph equipped for determination of caproic acid concentration. The sensory quality of raw liquor was evaluated in triplicate by three well trained experts. They had more than 15 years experience in the sensory analysis for Chinese liquor. Sensory attributes, including taste, smell and aftertaste, were evaluated using a 10 point hedonic scale, where 1 means dislike extremely and 10 means like extremely (Li *et al.*, 2011).

RESULTS AND DISCUSSION

Screening the significant variables on caproic acid production: In this section, 12 experiments were used to screen the relatively important factors of 8 variables affecting caproic acid production. The factors include $(NH_4)_2SO_4$, K_2HPO_4, $CaCl_2$, sodium acetate, $MgSO_4.7H_2O$, yeast extract, ethanol and initial pH of the medium according to the pre-experiments. The upper and lower levels of each variable were chosen according to the preliminary investigation of the variables. Table 1 showed the effects of these factors on the response and significant levels. The experimental data analysis indicated that there was a wide variation of caproic acid contents from 102.556 to 176.873 mg/100 mL in the 12 experiments (Table 2). This variation demonstrated that caproic acid production optimization was important for enhancing productivity.

The analysis of the regression coefficients and the t values of 8 factors (Table 1) showed that D (ethanol), F

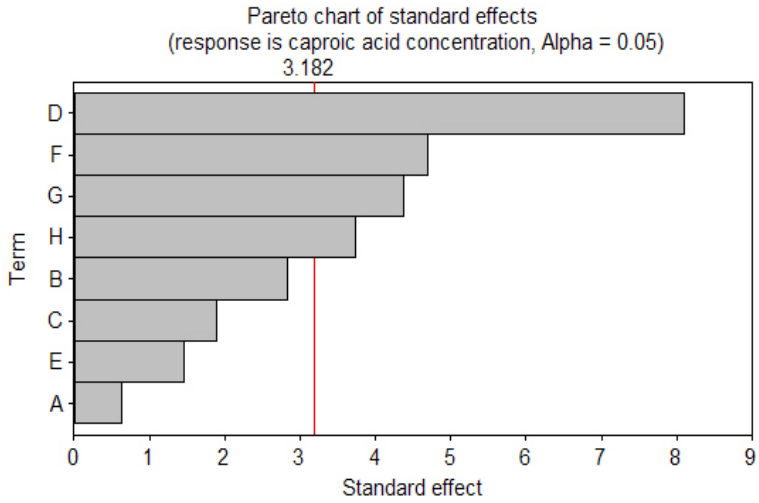


Fig. 3: Pareto chart of eight-factor standard effects on caproic acid concentration

(sodium acetate), G (yeast extract) and H (initial pH) with confidence levels above 95% were considered as the significant parameters, whereas A ($(NH_4)_2SO_4$), B (K_2HPO_4), C ($CaCl_2$) and E ($MgSO_4.7H_2O$) having confidence levels below 95% were considered insignificant and were excluded in the next optimization experiment, but instead were used in all trials at their low levels in consideration of lowering cost. In the results, R^2 was found to be 0.9782, which means that the model could explain 97.82% of the total variation in the system.

In the Pareto chart (Fig. 3), the maximal effect was presented in the upper portion and then progress down to the minimal effect. Figure 3 showed that the most important factors determining caproic acid production were D (ethanol), F (sodium acetate), G (yeast extract) and H (initial pH). Ethanol and sodium acetate are the major carbon sources for the growth of strain *C. kluyveri*, so both are important factors for caproic acid production. Yeast extract contains the substance of biotin, which is indispensiable to the growth of *C. kluyveri* (Bornstein and Barker, 1991). While initial medium pH affects many activities of microbe such as enzymatic processes, signaling pathways and transportations of various components across the cytoplasmic membrane and cell wall (Moon and Parulekar, 1991). Therefore, it is also important for enhancing the productivity of caproic acid. Although ethanol, sodium acetate, yeast extract and initial pH were identified as four significant factors, the optimal levels of the individual factors were still unknown at this stage. They could be determined by the following optimization experiments.

Response surface analysis for the optimization of three factors:

Response surface analysis regression and model analytics: Base on the Plackett-Burman experimental results, to build a second-order (quadratic) model for the response variable (Caproic acid concentration), a more detailed study using a response surface method was conducted with ethanol, sodium acetate, yeast extract and initial pH. The central composite design was used to study the interactions among the significant factors and also determine their optimal levels. The levels of the factors chosen were set based on the Plackette-Burman analysis. Each variable was redefined and studied at five coded levels (-2, -1, 0, +1 and +2), and the average value of the high and low level for every chosen variables by Plackette-Burman design were taken as a central real value of zero.

Table 3 shows the coded and real values of the variables at various levels. For each run, the experimental responses, as well as the predicted response obtained from the regression equation, are shown in Table 4. By using multiple regression analysis on the experimental data, the following second-order polynomial Eq. (3) was established to explain the degree of caproic acid production:

$$Y = 203.63 + 16.55X_1 + 15.20X_2 + 3.45X_3 + 3.72X_4 + 0.34X_1*X_2 - 1.85X_1*X_3 - 7.40X_1*X_4 - 5.23X_2*X_3 - 3.06X_2*X_4 - 5.625E - 004X_3*X_4 - 8.82X_1^2 - 9.00X_2^2 - 15.54X_3^2 - 9.04X_4^2$$

where, Y is the predicted degree of caproic acid concentration, X_1, X_2, X_3 and X_4 are the coded values of ethanol, sodium acetate, yeast extract and initial pH, respectively.

The predicted values were compared with the measured values from the experiment, it indicated that these data were in reasonably close agreement. The suitability of the model was checked by the coefficient of determination R^2, which was calculated to be 0.9830, showing that 98.30% of the variability in the response could be well described by the model. In addition, the value of predicted R^2 (0.9023) is in reasonable agreement with the adjusted R^2 (96.72%). This ensured

Table 5: Regression coefficients and their significance for response surface quadratic model

Term	Coefficient	D.F.	S.D.	95% CI low	95% CI high	F-value	p-value
Model	203.63	1	2.14	199.06	208.20	62.040	<0.0001
X_1	16.55	1	1.07	14.27	18.83	238.590	<0.0001
X_2	15.20	1	1.07	12.92	17.49	201.410	<0.0001
X_3	3.45	1	1.07	1.17	5.74	10.390	0.0057
X_4	3.72	1	1.07	1.44	6.00	12.050	0.0034
$X_1{}^*X_2$	0.34	1	1.31	-2.46	3.13	0.066	0.8014
$X_1{}^*X_3$	-1.85	1	1.31	-4.65	0.94	2.000	0.1780
$X_1{}^*X_4$	-7.40	1	1.31	-10.20	-4.60	31.800	<0.0001
$X_2{}^*X_3$	-5.23	1	1.31	-8.03	-2.43	15.890	0.0012
$X_2{}^*X_4$	-3.06	1	1.31	-5.86	-0.26	5.440	0.0340
$X_3{}^*X_4$	-5.625E-004	1	1.31	-2.80	2.80	1.838E-007	0.9997
$X_1{}^2$	-8.82	1	1.00	-10.95	-6.68	77.430	<0.0001
$X_2{}^2$	-9.00	1	1.00	-11.14	-6.87	80.690	<0.0001
$X_3{}^2$	-15.54	1	1.00	-17.67	-13.40	240.300	<0.0001
$X_4{}^2$	-9.04	1	1.00	-11.18	-6.90	81.350	<0.0001

R^2 = 98.30%; R^2 (pred) = 90.23%; R^2 (adj) = 96.72%; C.V. = 3.33%; S.D.: Standard deviation; D.F.: Degree of freedom

Table 6: Analysis of Variance (ANOVA) for the response surface quadratic model

Source	S.S.	D.F.	M.S.	F-value	p-value (prob.>F)
Model	23928.24	14	1709.16	62.04	<0.0001
Lack of fit	411.82	10	41.18	145.57	<0.0001
Pure error	1.41	5	0.28		
Residual	413.23	15	27.55		
Cor. total	24341.48	29			

S.S.: Sum of square; D.F.: Degree of freedom; M.S.: Mean square

a satisfactory reflection of the quadratic model to the experimental data. The Coefficient of Variation (*CV*) indicates the degree of precision with which the treatments are compared. A lower *CV* means a higher credibility of the model. The lower value of *CV* (3.09%) demonstrated the performed experiments were highly reliable. The p-values are also used to judge the significance of each of the coefficients (Table 5), which, in turn, may demonstrate the patterns of the interaction among the variables. Smaller value of p indicates that the corresponding coefficient is more significant. Values of the probability less than 0.05 indicate that the model terms are significant. In this case X_1, X_2, X_3, X_4, X_1*X_4, X_2*X_3, X_2*X_4, X_1^2, X_2^2, X_3^2, and X_4^2, respectively were the significant model terms (Table 5). Values greater than 0.1000 indicate the model terms are not significant.

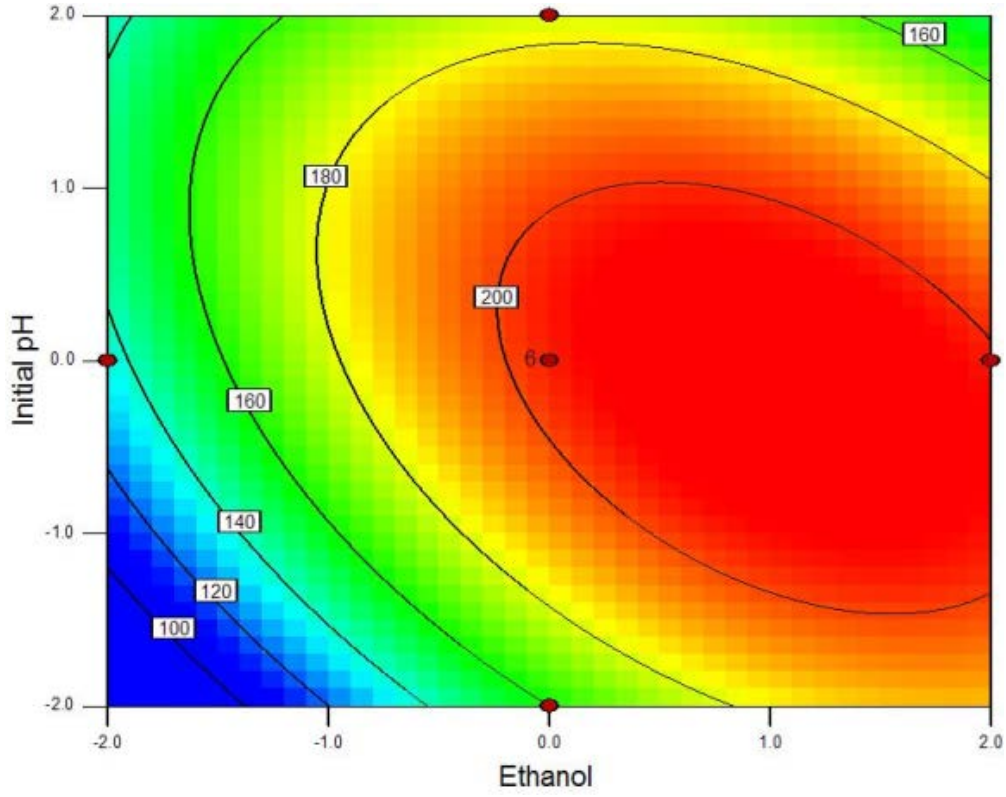
Furthermore, the Analysis of Variance (ANOVA) for quadratic regression model is given in Table 6. The Model F-value of 62.04 implies the model is significant. There is only a 0.01% chance that it could occur due to noise. The "Lack of Fit F-value" of 145.57 implies the lack of fit is significant. There is only less than 0.01% of error probability. The model was found to be effective in prediction within the range of variables employed.

Interactions among the factors: Response surface plot provides a way to predict the production of caproic acid for various values of the experimental variables and the contours of the plot are helpful in determination of the type of interactions between experimental variables. In present study, the three-dimensional response surfaces and two-dimensional contour plots shown in Fig. 4 and 5 were based on the regression model keeping two variables constant at their zero level, while changing the other two within their test range. The contour curves directly illustrate whether the interactions exist between the variables, if the contour lines are parallel with either of axes, no interaction exists between these two variables, or else the interaction does exist (Dong *et al*., 2009).

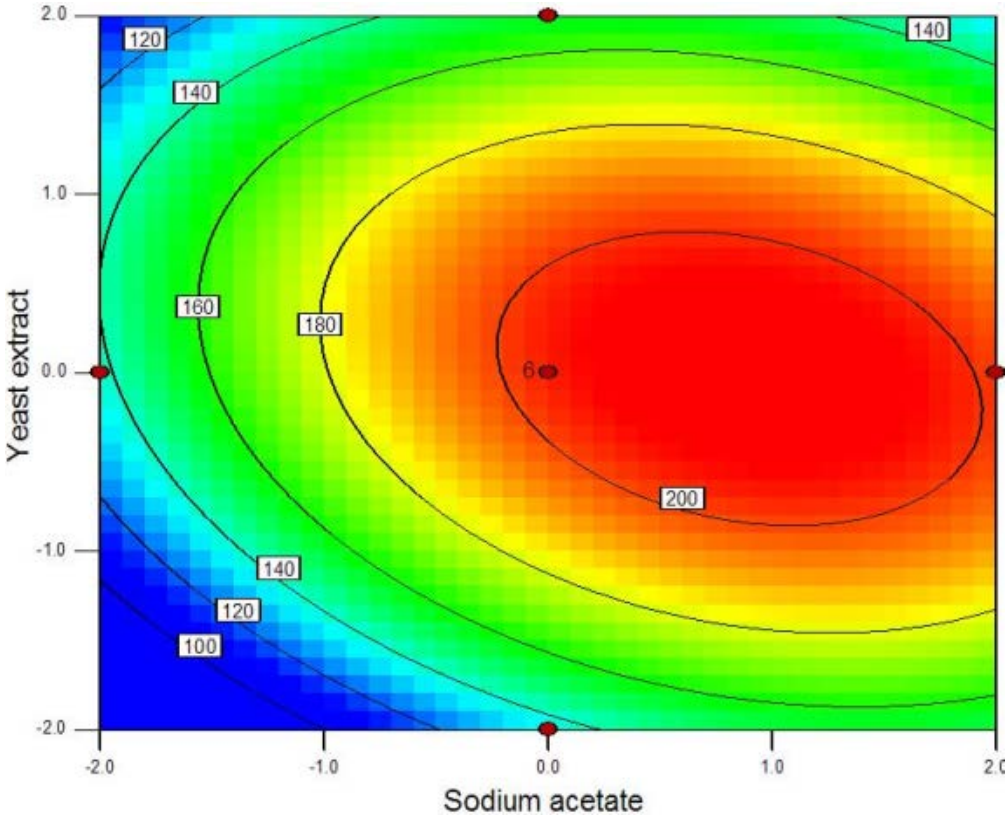
Elliptic contours in Fig. 4 illustrated that there were striking interactions between the variables. And the summits of the response surface of caproic acid concentration suggested that the optimal conditions lie inside the tested range of the variables (Fig. 4). Figure 4A shows the effect of ethanol and initial medium pH on caproic acid concentration. At the relative high ethanol and medium initial pH, the maximum caproic acid concentration could be achieved. Plots showing interaction between sodium acetate and yeast extract, sodium acetate and initial medium pH for caproic acid production are depicted in Fig. 4B and C. As shown in Fig. 4B, the maximum caproic acid production was observed with relative high sodium acetate and yeast extract. At the medium initial pH and relative high sodium acetate, the caproic acid concentration reached maximum (Fig. 4C).

The optimum values of the selected variables were calculated from the data obtained using the response surface plot system, which gave the following results in terms of coded values: X_1 = 1.046, X_2 = 0.928, X_3 = -0.109 and X_4 = -0.185. Correspondingly, the maximum point of the model was attained, which was a ethanol concentration of 4.046% (v/v), sodium acetate concentration of 0.982% (w/v), yeast extract concentration of 0.145% (w/v) and initial medium pH of 6.41, respectively. The maximum predicted value of caproic acid concentration was 219.11 mg/100 mL.

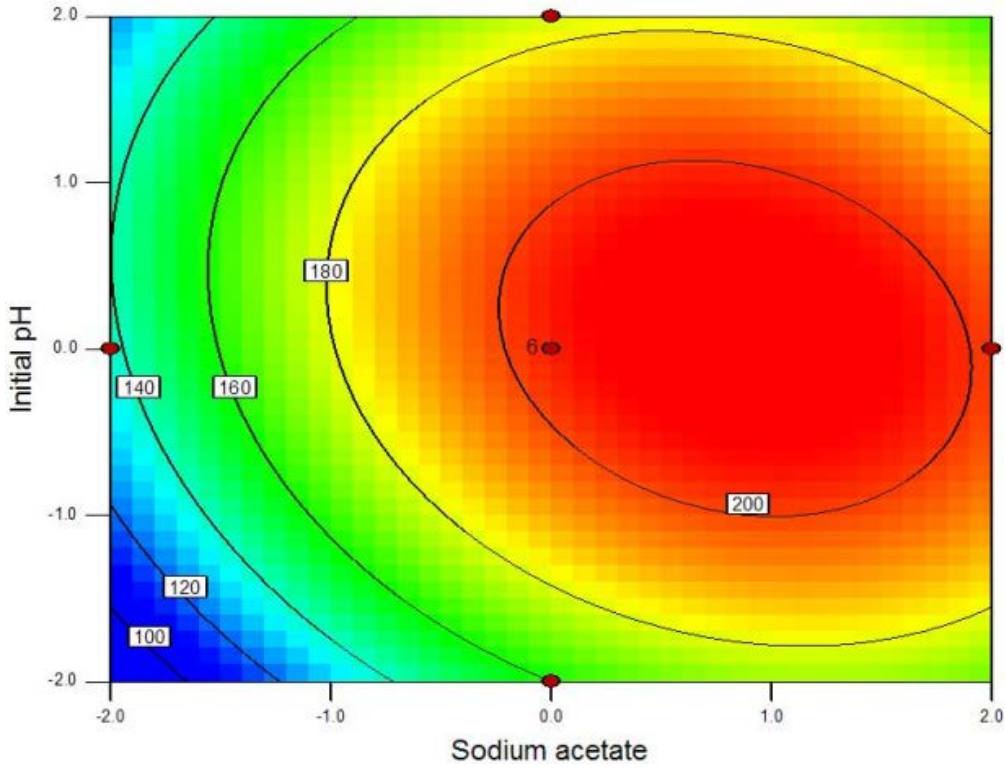
In order to confirm the predicted results of the model, caproic acid production experiments were carried out triplicately under the theoretical optimum condition and a mean value of 214.23 mg/100 mL was


Initial pH
Ethanol
2.0
1.0
0.0
-1.0
-2.0
160
180
200
160
140
120
100
6

(A)

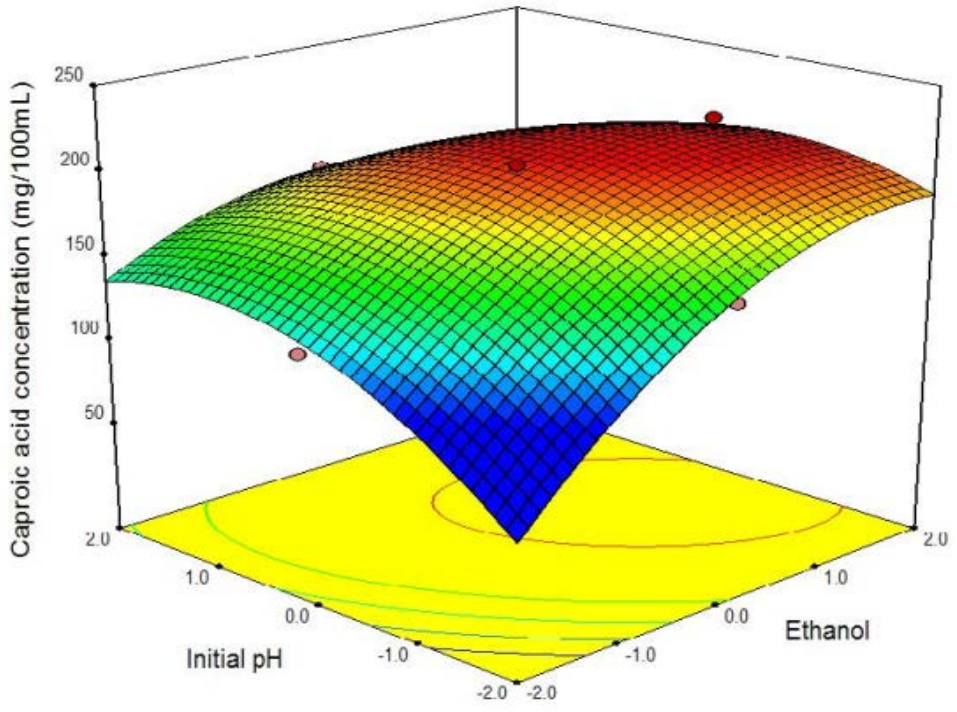

Yeast extract
Sodium acetate
2.0
1.0
0.0
-1.0
-2.0
120
140
140
160
180
200
140
120
100
6

(B)

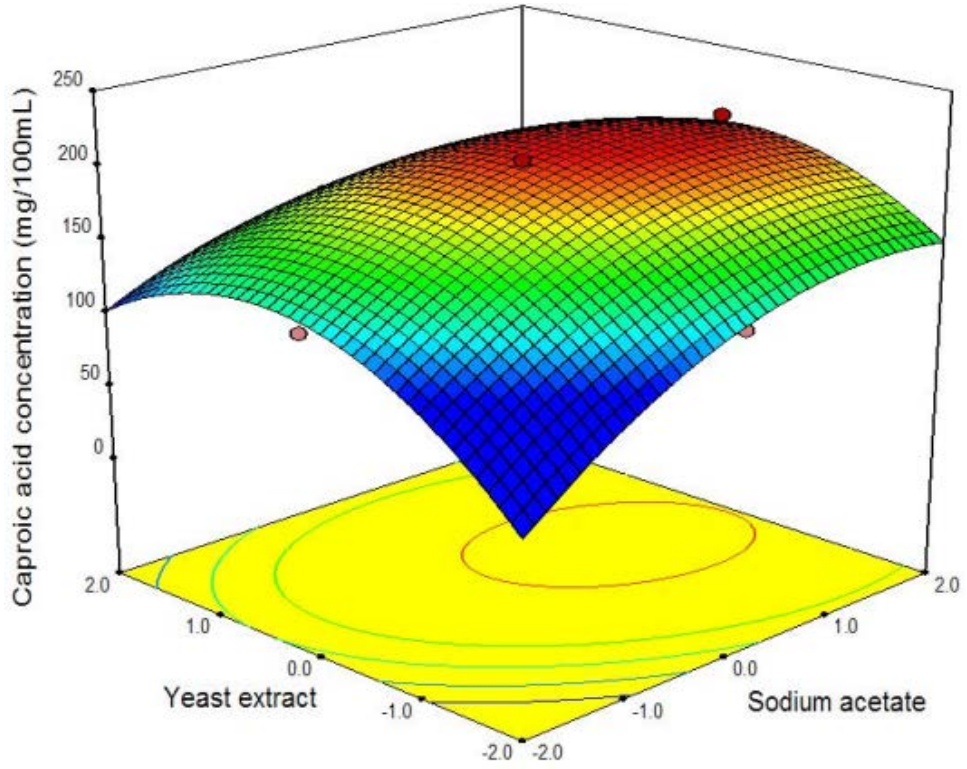


(C)

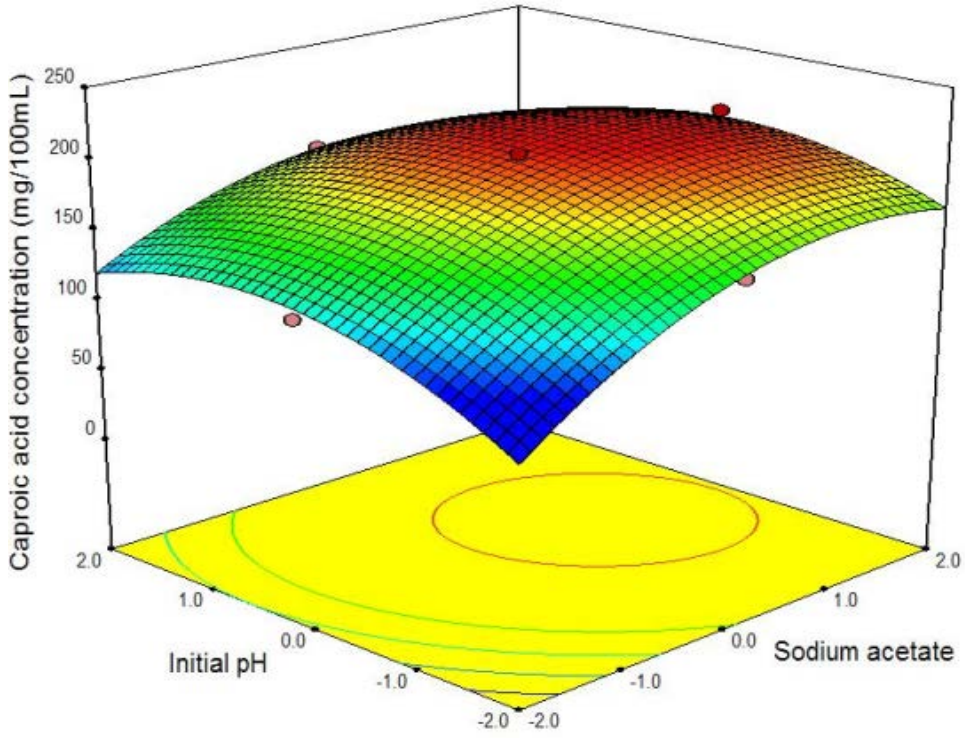
Fig. 4: Contour plots of the central composite design for the optimization of the caproic acid production. Effect of (A) ethanol and initial medium pH, (B) sodium acetate and yeast extract, (C) sodium acetate and initial medium pH. Other factors were constant at zero levels



(A)



(B)



(C)

Fig. 5: Response surface plots of the central composite design for the optimization of the caproic acid production. Effect of (A) ethanol and initial medium pH, (B) sodium acetate and yeast extract, (C) sodium acetate and initial medium pH. Other factors were constant at zero levels

obtained. The excellent correlation between estimated and test values verifies the model validation and existence of an optimal point.

Scaling-up and application in Luzhou-flavor liquor brewing: To study the possibility of industrializing caproic acid production, the optimized parameters have been tested during caproic acid fermentations carried out on pilot-scale of 5000 L reactor and three repeated batch fermentations were performed. The variation of caproic acid concentration and cell biomass with time are shown in Fig. 6. For cell biomass, after 2 days of lag-phase, the cells growth of *Clostridium kluyveri* H588 grew well in the medium and achieved a maximum biomass of 331 mg/L after 7 days. The growth curve of the strain displayed an S-shape with a lag phase (0-2 days), an exponential phase (2-7 days), and a stationary phase after 7 days. After the stationary phase, a large decrease in the number of viable cells was observed, indicating that a product inhibition and/or nutrient depletion affect biomass yield. Fig. 6 illustrated that caproic acid content increased slightly at the first 3 days of the fermentation process. It also showed a sharp increase in caproic formation during the period from 4 to 7 days, reaching the maximum value of 210.01 mg/100 L and then slightly levels off. Compared with the lab-scale shake flask fermentation, the pilot-scale fermentation was faster and a higher cell concentration being achieved, this might due to the difference in the geometry of each system.

Once the caproic acid fermentation was finished, the obtained culture broth was subjected to pit-filling fermentation for 50 days, then the fermented *Zaopei* of the experimental and control pits was took out and distilled with steam to extract ethanol and other flavor compounds, respectively. In this study, the raw liquor distilled from each pit of *Zaopei* was placed separately, and the quality of the raw liquor was employed as the

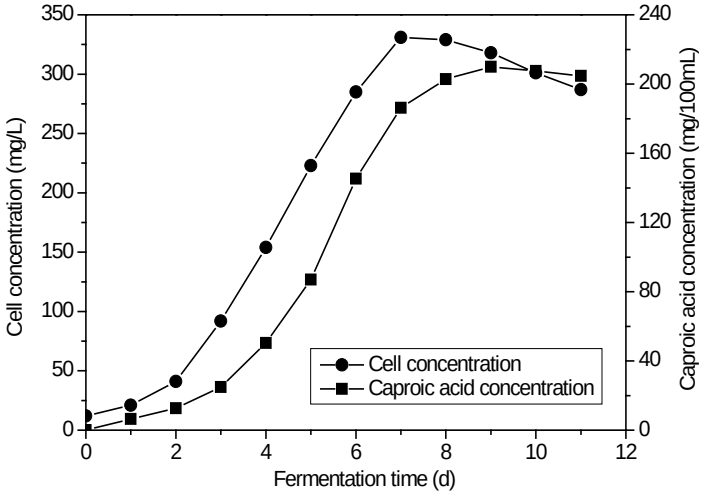


Fig. 6: The variation of caproic acid production and cell biomass concentration as a function of time

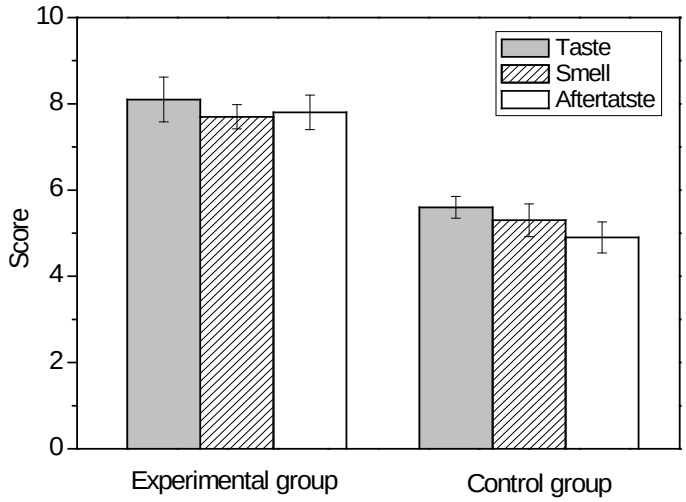


Fig. 7: The quality evaluation of the raw liquors obtained from the fermented *Zaopei* of the experimental and control pits

Table 7: The concentrations of flavor compounds in the raw liquor obtained from the fermented *Zaopei* of experimental and control pits, respectively (mg/100 mL)

Compounds	Control	Pit-filling fermentation
Ethyl acetate	89.2±2.31	110.5±3.75
Ethyl caproate	110.8±3.22	275.2±2.15
Ethyl butyrate	52.1±1.24	82.9±1.02
Ethyl lactate	354.6±3.68	126.8±2.41
Acetic acid	46.3±1.87	68.3±1.37
Caproic acid	18.9±0.65	32.1±1.14
Butyric acid	17.5±0.74	28.3±1.38
1-propanol	15.6±0.89	29.4±1.07
Isobutanol	10.2±0.58	18.3±0.96

criterions for different pit of fermented *Zaopei* assessment. Each result represented the mean value of the correspondingly data obtained from the experimental and control pits, respectively. Table 7 illustrated the concentrations of flavor compounds in the raw liquor obtained from the fermented *Zaopei* of the experimental and control pits, respectively. It was reported that ethyl caproate, ethyl acetate, ethyl butyrate and ethyl lactate are the most important flavor ingredients in Luzhou-flavor liquor, and their proportions determine the aroma quality and the style of the spirits. Among these four major esters, ethyl caproate is the dominant aromatic ingredient in Luzhou-flavor liquor and its content affects the taste of spirits, usually high concentration of ethyl caproate could cause high quality of Luzhou-flavor liquor, while high ethyl lactate concentration would make the wine bitter, stuffy and lacking of fragrance and finally destruct the typical character of Luzhou-flavor liquor (Zhang *et al*., 2012, 2013). From Table 7, it could be easily found that the maximum ethyl caproate concentration (275.2±2.15 mg/100 mL) was obtained from the *Zaopei* of the experimental pits, which was 148.4% higher than that from the control pits. Also, higher concentrations of ethyl acetate and ethyl butyrate were occurred in the raw liquor distilled from the experimental pits. While the ethyl lactate distilled from the control pits possessed higher concentration.

Figure 7 is the comparative experiments results, which was used to explore the effect of pit-filling fermentation on the quality of the correspondingly obtained raw liquor. It could be observed from Fig. 7 that the liquor distilled from the *Zaopei* of the experimental pits (pit-filling fermentation) possessed the best quality, including taste, smell and aftertaste. This phenomenon may come down to the lowest concentration of ethyl lactate and the highest concentrations of other three esters, in particular the highest ethyl caproate existed in the raw liquor. Besides, higher concentration of organic acids, such as caproic acid, acetic acid and butyric acid, obtained from the *Zaopei* of the experimental pit may also contribute to the liquor flavor and taste. As it was reported that organic acids could help to make the flavor and taste of liquor harmonious and increases the soft of the liquor.

As a conclusion, the pit-filling fermentation of caproic acid culture broth was successfully utilized in improving the quality of Luzhou-flavor liquor, especially the newly built pits. But it should be noted that the moment the caproic acid culture broth filled into the fermented *Zaopei* should be as soon as the ethanol fermentation finished. Otherwise, low yield of liquor was obtained. The reason might due to high content of caproic acid inhibit the starch saccharification and ethanol fermentation.

CONCLUSION

Plackette-Burman design and central composite design have proved to be effective for optimizing the conditions of the caproic acid production. The optimal conditions for caproic acid production were as follows: ethanol concentration of 4.046% (v/v), sodium acetate concentration of 0.982% (w/v), yeast extract concentration of 0.145% (w/v) and initial pH of 6.41, under these conditions, experimental results of caproic acid production was obtained as 214.23 mg/100 mL, which was almost equal to the value predicted by the model equation (219.11 mg/100 mL), thereby assuring its validity. In addition, a simple strategy for scale-up was to transfer the optimized conditions of a lab scale flask shake to 5000 L pilot-scale fermentation and the resulted fermentation broth was used for the pit-filling fermentation with promising results.

ACKNOWLEDGMENT

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