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Optimization of alcohol-free beer prepared from fermented wort by *Pichia kluyveri var kluyveri*

An increasing number of studies have examined the preparation of alcohol-free beer beverages by special yeast fermentation methods. Here, *Pichia kluyveri var kluyveri* was used to prepare alcohol-free beer beverages by the fermentation of wort. The single-factor test method and response surface method were used to determine the optimal preparation parameters for alcohol-free beverages. The optimal fermentation process was as follows: fermentation without shaking; original wort concentration, 12 °P; fermentation temperature, 20.41 °C; inoculation quantity, 2.69 % (v/v); and fermentation time, 180.44 h. The feasibility of the process was verified by the triangle bottle test and fermentor test. Lastly, the fermented beverage was modified with a sour taste agent, and samples with 0.4 ‰ citric acid added had better taste. This method has the advantages of full body, rich fruit aromas, short fermentation period, and no additional equipment input. Thus, this method is suitable for wide use.

Descriptors: *Pichia kluyveri var kluyveri*; alcohol-free beer; single-factor; response surface; optimization

1 Introduction

Since the end of the 20th century, the variety of non-alcoholic beers has increased for both health and social reasons [1]. In most countries of the European Union, excluding Spain, the United Kingdom, the Netherlands, and Denmark, beer with an alcohol content less than 0.5 % (ABV) is considered alcohol-free beer [2]. In this study, alcohol-free beverages refer to products with alcohol contents less than or equal to 0.5 % (ABV).

Methods of obtaining alcohol-free beer can be divided into two categories: the physical method of removing ethanol and the biological method of limiting the production of ethanol [3]. There are two types of physical methods: thermal methods and membrane-based methods [4]. Thermal methods cause significant losses of beer flavour and wine body because of thermal damage, while the main disadvantage of the membrane system is its high installation and operating costs [2]. Biological methods are receiving increased attention by technologists, especially the limited fermentation process, mash alteration, and the use of special yeasts. The most major advantage of biological methods is that no special equipment is required; indeed, traditional brewery equipment can be used for production [1, 2].

Advances have been made in the use of specific yeasts in biological methods. For example, *Saccharomycodes ludwigii* has been used in the commercial production of alcohol-free beer, and several studies have been conducted on this yeast [5, 6]. In addition, the process of wort fermentation during the preparation of low alcohol or alcohol-free beverages has been studied in many species, including *Candida* spp., *Cyberlindnera* spp., *Torulaspora delbrueckii*, *Zygosaccharomyces* spp., *Pichia* spp., and other non-*Saccharomyces* species [7]. Although most non-alcoholic beers currently made with special yeasts require no additional equipment investment, they are generally sweet and lacking in flavour and body [2].

Pichia kluyveri can be isolated from some fermentation product preparation processes, fruit prize, and fruit epidermis. *P. kluyveri* has great potential for use – and has occasionally been used – in the preparation of fermentation products. For example, the quality and flavour of cocoa were improved by *P. kluyveri* fermentation [8, 9]. The quality and flavour of coffee were also improved using *P. kluyveri* starter to ferment coffee beans [10]. Orange juice fermentation beverages can be prepared by *P. kluyveri*. Such orange juice is rich in bioavailable carotenoids and is highly beneficial to human health when consumed regularly [11]. Another study found that the consumption of fermented orange juice reduced cardiovascular risk factors in healthy mice [12]. *Alicia Gutierrez* et al. found that *P. kluyveri* was one of the most promising strains for the development of beer, wine, and cider beverages [13]. *Liuping*

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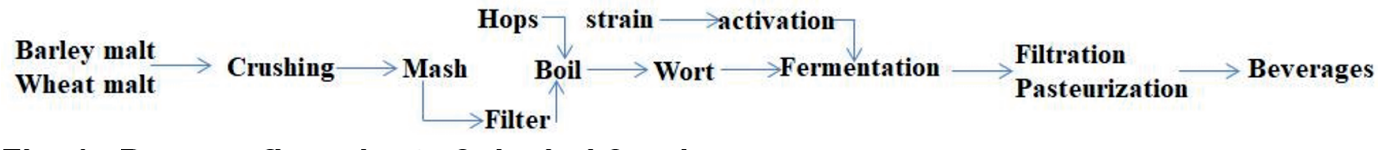

Fig. 1 Process flow chart of alcohol-free beverages

Fan et al. used a new *P. kluyveri* yeast strain to ferment bayberry juice into a non-alcoholic fermented bayberry juice that has a low alcohol content (< 0.5 %), unique fruit flavour, mellow taste, and fermented aroma [14].

Optimization of important process conditions is important for enhancing the economic and logistical efficiency of the biological process [15]. Various experimental design methods can be used to optimize the fermentation process [16]. Traditional process optimization usually involves altering factors one by one. This method cannot guarantee optimal values of variable parameters nor can it describe the interaction between various variables [16, 17]. The response surface method can overcome the disadvantage of using a single-factor optimization method and is a widely used statistical and mathematical tool. The response surface method is based on the fitting of a polynomial equation and experimental data to optimize the response affected by multiple independent variables to better describe the interaction between each variable [18].

Here, alcohol-free beverages were brewed with wort, hops, *P. kluyveri var kluyveri*, and water. A single-factor experiment was conducted with a Box-Benhnken design (BBD). The optimal fermentation process was obtained by further optimization of the BBD experiment. We then validated the triangle bottle and the fermentor. Finally, the best combination of factors for improving the taste of the fermented product was determined. These studies provided insight into how technology could be used to scale-up the production of alcohol-free beverages.

2 Materials and methods

2.1 Microorganisms, malt, and hops

The test strain *P. kluyveri var kluyveri* SS1-5 was screened from grape juice of cabernet sauvignon in Yantai. Strains were preserved in the General Microbiology Center of China Microbial Species Conservation Administration, NO. CGMCC NO. 4494.

Australia barley malt and wheat malt were used to prepare wort. Two types of hops were used: hetzbrook fragrant granular hops and hetzbrook bitter granular hops.

2.2 Culture medium for preservation and activation of strains

YEPD solid medium contained glucose 2 %, yeast powder 1 %, peptone 1 %, and agar 2 %, volume with distilled water, and 115 °C for 30 min of sterilization. YEPD liquid medium contained glucose 2 %, yeast powder 1 %, peptone 1 %, volume with distilled water, and 115 °C for 30 min sterilization. Wort medium was wort at 12 °P, sterilized at 115 °C for 30 min, and natural pH.

2.3 The process flow

The process flow chart is shown in figure 1.

2.4 Preparation of wort

Wort was prepared by heating the single mash and extracting saccharification. The ratio of barley malt to wheat malt was 5:1. After crushing the raw material, it was soaked for 10 min at a feeding temperature of 37 °C. The temperature was then kept at 52 °C for 40 min, 65 °C for 60 min, and then 72 °C for 15 min. The wort was detected with iodine solution. After resting at 78 °C for 10 min, reflux was performed, and the wort was filtered after clearing. The grains were washed at 78 °C twice. Hops were added twice at 2.75 g/kg wort. Bitter hops were added after initial boiling for 5 min, and fragrant hops were added for 10 min before the end of boiling. The boiling time was 65 min. The final wort concentration was then maintained at its target value; after boiling, it was left to stand for 20 min. Finally, hops grains and hot solidified matter were separated and cooled.

2.5 Single-factor experimental design

The concentration of the original wort, inoculation quantity, fermentation temperature, fermentation time, and rotating speed of the shaker are the most important factors affecting the preparation of fermented beverages [19, 20]. Sensory scores were used to evaluate the effects of the original wort concentration (6 °P, 8 °P, 10 °P, 12 °P, and 14 °P), inoculation quantity (0.5 %, 1 %, 3 %, 5 %, and 10 %), fermentation temperature (8 °C, 16 °C, 20 °C, 24 °C, and 28 °C), fermentation time (3 d, 5 d, 7 d, 9 d, 11 d, and 13 d), and rotational speed of the shaker (0 rpm, 40 rpm, 100 rpm, 160 rpm, and 200 rpm) on the quality of alcohol-free beverages.

2.6 Response surface methodology design

According to the results of the single-factor experiment, three factors – fermentation temperature, inoculation quantity, and fermentation time – were selected for study. The BBD was used for the experiment according to three levels of these factors (Table 1).

The experiment was designed using Design-Expert software. The BBD experiment with three factors and three levels led to a total

Table 1 The Box-Benhnken design factors and levels

Symbol	Independent variable (units)	Coded level		
		−1	0	+1
X_1	Fermentation temperature (°C)	12	20	28
X_2	Inoculation quantity (%)	1	3	5
X_3	Fermentation time (d)	3	7	11

Table 2 Experimental design and results of the Box-Behnken experiments

Run	X_1	X_2	X_3	Sensory score (point)
1	0	0	0	92.2
2	0	−1	1	86.0
3	0	1	1	78.0
4	−1	−1	0	76.0
5	−1	0	1	83.0
6	−1	1	0	78.0
7	0	0	0	91.8
8	0	0	0	92.0
9	0	−1	−1	76.8
10	1	0	−1	81.0
11	1	1	0	76.3
12	1	−1	0	84.6
13	−1	0	−1	76.7
14	1	0	1	77.1
15	0	0	−1	79.0

of 15 experimental groups. The experimental design is shown in table 2.

2.7 Validation model

After obtaining optimal process conditions and corresponding results, the optimized results were verified by a triangle bottle test and a small fermentation tank test. Next, 200 mL of 12 °P wort in a 500-mL tripod was used for the shake flask verification experiment. Three parallel verification experiments were carried out. Six L of 12 °P wort in a 10-L fermentor was used for the fermentor validation experiments. A ring of *P. kluyveri var kluyveri* was inoculated into 500 mL of wort medium at 28 °C and cultured in a shaker at 120 rpm for 18 h. It was then added to the fermentor and allowed to stand for fermentation.

2.8 Analytical methods

Ethanol was determined by the gas chromatography-hydrogen ion flame method using a GC-17A gas chromatograph (Shimadzu Analytical Instrument Co., Ltd., Japan). Esters were determined by gas chromatography-mass spectrometry (GC-MS-QP2010) (Shimadzu Analytical Instrument Co., Ltd., Japan). The determination principle of diacetyl is that diacetyl reacts with phthalonium to form 2, 3-dimethyl-quinoxalin to determine A335. The diacetyl distillation unit was from Tianjin Borai Shihan Trading Co., Ltd. Vitamin C was determined by the fluorescence method, and free amino acids were determined by a l-3000 automatic amino acid analyzer (Suzhou Huameichen Instrument Equipment Co., Ltd.).

2.9 Sensorial analysis

Fermented beverage samples were processed prior to their preparation for evaluation. For each evaluation, eight trained professionals and four ordinary consumers were selected. Only six samples were evaluated in each round, and the time for each evaluation was a maximum of 30 min. No food, sugar, or gum was allowed to be consumed 0.5 hours before each round of tasting. The evaluation and scoring scheme are shown in table 3.

2.10 Data analysis

Design-expert 8.0.3 was used for statistical analyses. All of the experiments were conducted in parallel, and the mean values were calculated by the standard deviation method.

3 Results and discussion

3.1 Single-factor experimental results

The test was carried out following a single-factor test design, and the results are shown in figure 2 (see page 106). The sensory score first increased as the original wort concentration increased and then levelled off. The sensory evaluation revealed that the fermented product with 6 °P had a weak taste. As the concentration of the original wort increased, the taste of killing and the sense of mellow increased; the taste of the fermented product with 12 °P and 14 °P was similar. The sensory scores of the fermented products showed an increasing trend as the inoculation quantity increased, followed by a decreasing trend that eventually levelled off. The sensory score of the 3 % inoculation quantity was the highest, and the fermented product had a strong aroma and good taste. The influence of different temperatures on the sensory score first increased and then decreased, and the sensory score of fermentation at 20 °C was the highest, as the fermentation products at 20 °C had a sweet and bitter flavour. The sensory scores of fermented products changed significantly at different fermentation times. The sensory scores first increased and then decreased after 7 d. When the fermentation time was 7 d, the aroma of the product was thick, refreshing, pure, and harmonious. The sensory points were

Table 3 Sensory evaluation of alcohol-free beverages

Evaluation index	Evaluation content	Single score	Total score
Colour and luster	Tonal situation	2	5
	Chroma	3	
Transparency	Static, whether clear, transparent	2	5
	Dynamic, observe the hanging cup and foam status	3	
Aroma	With or without the scent of joy	10	30
	No pungent odour	10	
	Whether the aroma is harmonious	10	
Taste	Good or bad first impression	10	50
	Touch, whether pure, clean, refreshing, harmonious	15	
	Stimulate feeling, whether refreshing, pricked, astringent, smooth	15	
	Aftertaste, aftertaste time, aftertaste size, tail taste clean miscellaneous	10	
Style	Whether the color, aroma, and taste are typical	10	10

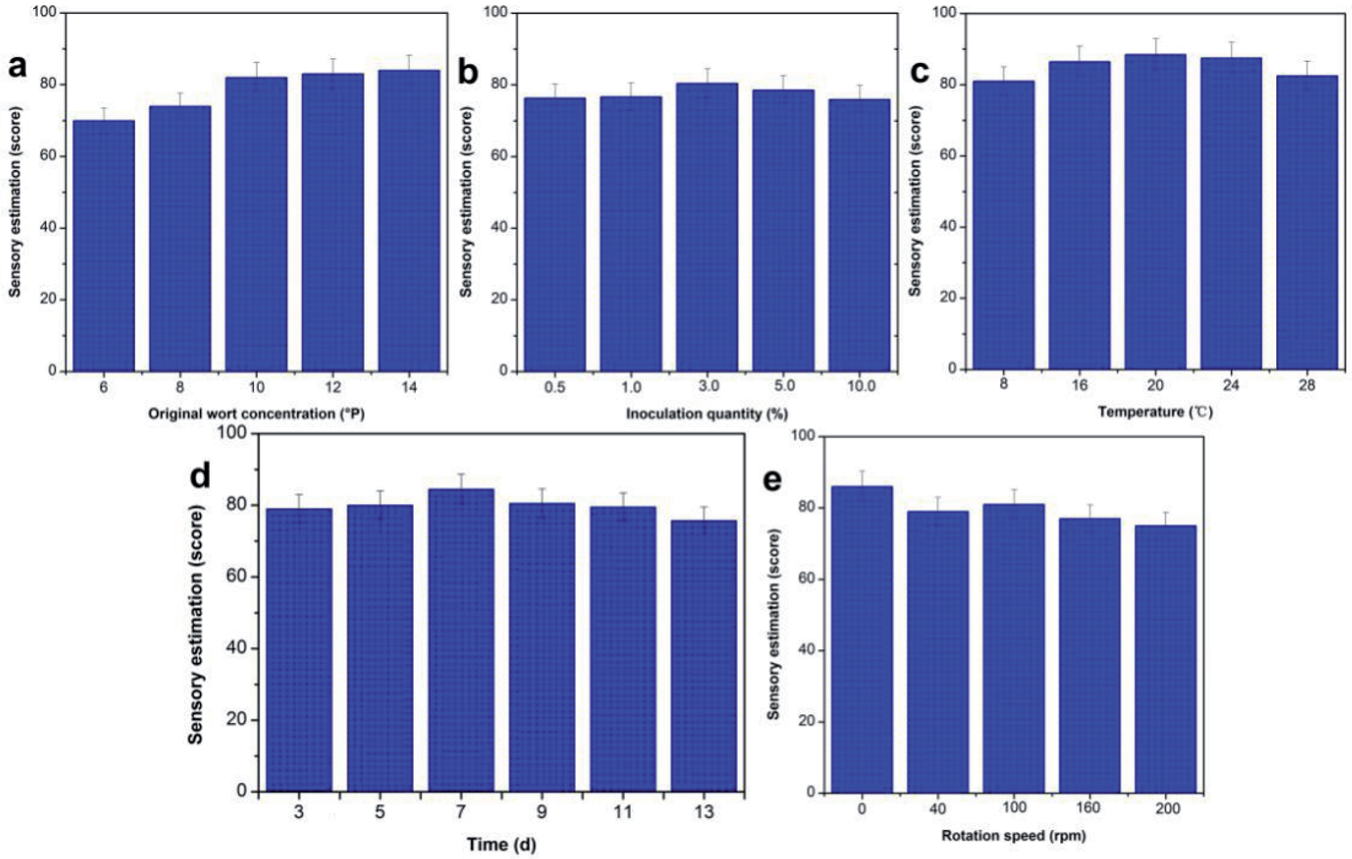

Fig. 2 Effects of original wort concentration. (a) Inoculation quantity, (b) fermentation temperature, (c) fermentation time, and (d) rotational speed of the shaker on (e) the quality of the alcohol-free beverages

the highest in the 0 rpm fermentation. The fermentation product under the 0 rpm condition had a slightly stronger aroma, a long aftertaste, and a clean tail; consequently, this product was selected for non-shaking fermentation.

Inoculation quantity, fermentation time, and fermentation temperature were the main factors affecting the quality of alcohol-free beverages.

Table 4 ANOVA for the response surface quadratic model

Source	df	Sum of squares	Mean square	F value	p-value Prob> F
X_1	1	3.51	3.51	1.32	0.3033
X_2	1	18.30	18.30	6.86	0.0472
X_3	1	14.04	14.04	5.26	0.0703
$X_1 X_2$	1	26.52	26.52	9.94	0.0253
$X_1 X_3$	1	26.01	26.01	9.74	0.0262
$X_2 X_3$	1	26.01	26.01	9.74	0.0262
X_1^2	1	175.15	175.15	65.61	0.0005
X_2^2	1	150.65	150.65	56.43	0.0007
X_3^2	1	118.39	118.39	44.35	0.0012
Model	9	499.97	55.55	20.81	0.0019
Residual	5	13.35	2.67		
Cor Total	14	513.32			

R^2 = 0.9740; Adj R^2 = 0.9272; Adeq Precision = 12.809

3.2 Response surface optimization of the alcohol-free beverage fermentation process

According to the combination of experimental conditions designed by the experimental model, experiments of three levels of the three factors were conducted (Table 2).

The results were then inputted into Design-Expert software. The software was used to perform multiple regression fitting on the experimental data in table 2 and generate a final quadratic regression model. The sensory score of fermented products Y could be obtained from the regression equation of temperature X_1, inoculation quantity X_2, and fermentation time X_3. The multiple quadratic regression equation was as follows:

$$Y= +92.00+0.66X_1-1.51X_2+1.32X_3-2.57X_1X_2-2.55X_1X_3-2.55X_2X_3 -6.89X_{12}-6.39X_{22}-5.66X_{32}$$

ANOVA for the response surface quadratic model revealed that the model was significant (F = 20.81; p = 0.0019) (Table 4). Thus, there was only a 0.19 % chance that the "Model F-Value" would have been obtained by chance. The complex correlation coefficient R^2 was 0.9740, indicating that the regression model was significant and the degree of fit was high; the regression equation could thus be used to determine the fermentation product with the optimal sensory score. The significance test of the coefficient of the regression equation showed that the order of influence of the sensory score on fermented products was inoculation quantity

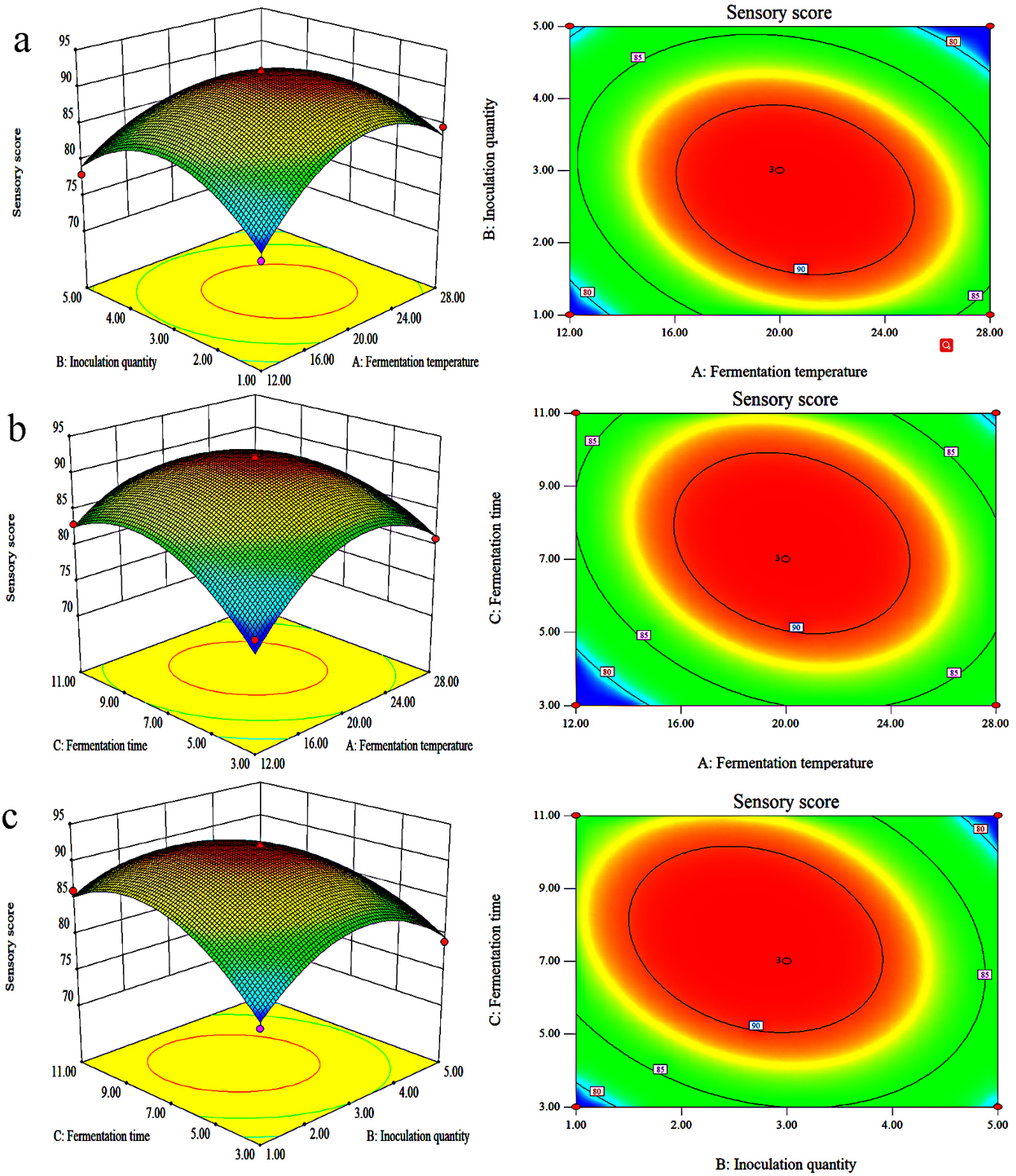

Fig. 3 **Response surface diagram and contour diagram. a) Response surface and contour diagram of the interaction between fermentation temperature and inoculation quantity. b) Response surface diagram and contour diagram of the interaction between fermentation temperature and fermentation time. c) Response surface diagram and contour diagram of the interaction between inoculation quantity and fermentation time**

> fermentation time > fermentation temperature. The influences of X_{12}, X_{22}, and X_{32} on Y were highly significantly different ($P < 0.01$), while the influences of X_2, X_3, X_1X_2, X_1X_3, and X_2X_3 on Y were significantly different ($P < 0.1$). "Adeq Precision" measures the signal-to-noise ratio. A ratio greater than four is desirable. Our ratio of 12.809 indicates that the signal was adequate. This model was used to navigate the design space.

Calculations of the extreme of each independent variable were $X_1 = 20.41$, $X_2 = 2.69$, and $X_3 = 7.56$. In other words, when the

fermentation temperature was 20.41 °C, the inoculation quantity was 2.69 %, and the fermentation time was 7.56 d, or 180.44 hours; the maximum value of the sensory score was 92.23. The response surface diagram and contour map are shown in figure 3. The interaction between fermentation temperature, inoculation quantity, and fermentation time was apparent (Fig. 3).

Temperature is an important variable for the control and management of beverage fermentation. Increases in fermentation temperature generally enhance the metabolic capacity of yeast and accelerate the rate of fermentation to stimulate the formation of volatile metabolic by-products [20]. In contrast, when the fermentation temperature is low, the activity of yeast is weak and the speed of fermentation is slow, producing an aroma of esters that is not ideal. When the fermentation temperature rises, genes encoding alcohol acetyltransferase (*ATF1*, *ATF2*) are up-regulated, and the expression of these genes affects the final concentration of important esters [21]. Aroma production was slow and negligible when the fermentation temperature was below 20 °C. At temperatures above 28 °C, controlling the rate of fermentation is difficult and results in the loss of aromatic compounds [14]. The single-factor experiment and response surface method revealed an optimal fermentation temperature of 20.41 °C, which was consistent with the fermentation temperature (18 – 22 °C) of cider prepared by *P. kluyveri* [22]. The optimized fermentation temperature was also consistent with the main fermentation stage (15 – 35 °C) of the preparation of non-alcoholic waxberry juice by fermentation with *P. kluyveri* [14].

In traditional beer production with wort, the fermentation process is time-consuming; indeed, the main fermentation period before the maturation period takes approximately 1 – 2 weeks. Thus, a major focus of research on current brewing technology is to enhance the economic efficiency of the brewing process by shortening the fermentation time while maintaining the quality of the final product. Previous work suggests that appropriate increases in the inoculation rate shorten the fermentation process and have no major negative effects except that the level of diacetyl is greatly increased [23]. In this study, the optimal inoculation amount was 2.69 %. The fermentation time of wort was shortened (180.44 h), and the content of diacetyl was maintained at a reasonable level (0.05 – 0.07 mg/L), greatly reducing economic costs.

The sensory score of the optimized product was high. The optimized sample had low alcohol content, strong fruit aroma, and good taste; in addition, this sample did not have the peculiar smell of some types of commercial alcohol-free beer and could thus be used as a beer substitute. First, the alcohol content of the optimized samples was consistently lower than 0.5 % (v/v). Because *P. kluyveri* can only use the glucose in wort and consume the sugar to produce flavour compounds, it produces little ethanol [24]. The optimized sample primarily contained six types of esters – butyl isobutyrate, isobutyl butyrate, butyl butyrate, sorbitol hexaacetate, tocopherol acetate, and triethyl 2-butylpropylene ester – which conferred a strong fruit aroma. The diacetyl content of this sample (0.050 to 0.070 mg/L) was acceptable, and this sample was nutritionally rich; consequently, it does not have some of the disadvantages of commercial alcohol-free beer. The optimized samples had a full taste and were rich in vitamin C, various amino acids, and other nutrients. The content of vitamin C in the optimized sample was as high as 17.5 mg/kg. The contents of free amino acids are shown in table 5.

Table 5 Contents of free amino acids in optimized samples

Amino acid	Content (%)	Amino acid	Content (%)
aspartic acid	0.05	tyrosine	0.02
glutamate	0.10	valine	0.02
serine	0.05	methionine	< 0.01
glycine	0.03	cysteine	0.02
histidine	0.02	isoleucine	0.03
arginine	0.04	leucine	0.04
threonine	0.03	phenylalanine	0.47
alanine	0.04	lysine	0.13
proline	0.10	Total amino acid	1.17

3.3 Verification experiment

3.3.1 Shake flask verification experiment.

Three parallel verification experiments were carried out under optimal conditions, and the sensory scores of the sensory evaluation were 92.2, 92.2, and 93.0, which were consistent with the predicted results. The feasibility of the model was thus validated.

3.3.2 Fermentor validation experiments.

The optimal conditions of the response surface model were applied to a 10-L fermentor. The fermentation temperature was 20.5 °C,

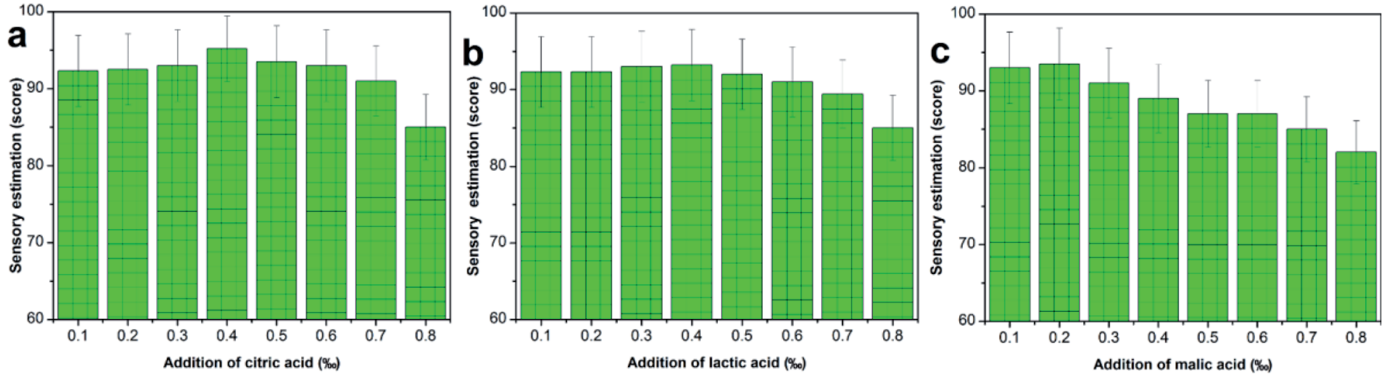

Fig. 4 The effects of different acidity agents on the taste modification of fermented beverages

the concentration of the original wort was 12 °P, the inoculation quantity was 2.7 %, and the fermentation time was 180 h. The initial pH and initial nitrogen content of the wort were used. Three small fermentor experiments were conducted, and the sensory scores of the three batches of fermentation tank samples were 92.5, 92.2, and 92.4, respectively. Given the verification of the optimization results of the corresponding surface, the production process of the alcohol-free beverage could be characterized. The alcohol contents of the three batches of the fermentation tank samples were 0.40 %, 0.39 %, and 0.33 %, respectively, and the contents of diacetyl were 0.050 mg/L, 0.066 mg/L, and 0.061 mg/L, respectively.

3.4 Taste modification after optimization

Because there is no use for the sugar in fermented drinks, fermented drinks often have a slightly sweet taste; consequently, extensive drinking can result in a greasy feeling. Citric acid, lactic acid, and malic acid are commonly used in the preparation of beverages [25]. In this study, citric acid, lactic acid, and malic acid were used to modify the taste of the optimized samples. The experimental results are shown in figure 4. The sensory score was highest for the addition of citric acid. When the amount of citric acid added was 0.4 ‰, the sensory score was 95.2. The taste was relatively fresh, which is a desirable property. The addition of lactic acid resulted in a marked change in the acerbity of the sample, while the addition of malic acid resulted in a sharp sour taste. Therefore, 0.4 ‰ citric acid was added to modify the taste of the fermented beverage.

4 Conclusions

A single-factor test revealed that the significant factors affecting the sensory score of the product were fermentation temperature, inoculation quantity, and fermentation time. Fermentation was also optimized without shaking and when the original wort concentration was 12 °P. The optimal fermentation temperature, inoculation amount, and fermentation time were then used for the BBD experiment on the response surface. This experiment revealed that the optimal fermentation temperature, inoculation quantity, and fermentation time of the alcohol-free beverage were 20.41 °C, 2.69 % (v/v), and 180.44 h, respectively. The feasibility of the optimal fermentation process by the response surface test was fully verified by the triangle bottle test and the fermentor test. Finally, the taste of the fermented beverage was modified, and samples with 0.4 ‰ citric acid during fermentation had superior taste. Thus, the fermentation process and parameters in this study could be used to expand and popularize alcohol-free fermented beverages.

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Declaration of Interest Statement

No potential conflict of interest was reported by the authors.

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