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ORIGINAL PAPER

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PROCESS OPTIMIZATION OF A NOVEL WATER KEFIR DRINK WITH HIGH ANTIOXIDANT ACTIVITY FROM CHINESE JUJUBE (*ZIZIPHUS JUJUBA* MILL.) THROUGH RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Background. Jujube (*Ziziphus jujuba* Mill.) is a widely cultivated crop in China. However, its perishable nature makes it challenging to store over long periods. To avoid spoilage of unsold dates, they are usually processed into beverages and concentrated juice, but there are no jujube drink products fermented with water kefir grains. Therefore, this study aims to explore a new method for deep processing of jujube and provide a reference for the full utilization of jujube.

Methods. The effects of fermentation time, fermentation temperature, and the inoculation amount of water kefir grains on the DPPH free radical scavenging rate (DPPH), sensory evaluation score (SES), pH, Titrable acidity (TA) and soluble solid content (SSC) in jujube water kefir were investigated in a single factor experiment employing response surface methodology.

Results. The optimal fermentation conditions predicted by the model were fermentation at 29°C for 30 hours with an inoculum size of 1%. Under these conditions, the DPPH free radical scavenging rate was 81.06 ±0.29 %, the SES was 88.00 ±2.71, the pH was 3.93 ±0.04, the TA was 3.87 ±0.14 g/L, and the SSC was 11.00 ±0.10 %.

Conclusion. The model's predicted values and actual values correlated well. Fermenting jujube juice with water kefir grains is a good method for the deep processing of jujube, which has acceptable sensory evaluation and high antioxidant activity.

Keywords: jujube, water kefir grains, fermentation, optimization

INTRODUCTION

As a commonly used Chinese medicine, jujube is included in the *Pharmacopoeia of the People's Republic of China*. It is a versatile, nutritious fruit with a delicious taste and a certain medicinal value. With a shared origin in medicine and food, jujube is considered a valuable fruit. Ingredients such as phenol,

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which scour free radicals, can help keep cholesterol levels within normal ranges and reduce the risk of cardiovascular disease (Carocho et al., 2013). Jujube contains eight essential amino acids, including arginine and histidine, which cannot be synthesized by the human body, and is also rich in minerals, vitamins, lipid compounds, polyphenols, flavonoids, saponins and other compounds (Li et al., 2007; Lin et al., 2013). The content of cyclic adenosine monophosphate in jujube is also much higher than that of ordinary animals and plants, and it is also rich in potassium, which plays an important role in cell proliferation and differentiation, regulation of immune responses, stabilization of the vascular environment, nerve activity and protection of neurons (Chen, 2014).

Water kefir grains are composed of a variety of microorganisms. Their primary microbial constituents are lactic acid bacteria, yeast and acetic acid bacteria. These microorganisms secrete a polysaccharide matrix (mainly dextran, and somewhat less levan) as they grow and become embedded in it (Fels et al., 2018), thus cauliflower-like grains are formed (Waldherr et al., 2010; David et al., 2019), ranging in size from a few millimeters to a few centimeters. The color of water kefir grains can change when the fermentation substrate is different (Egea et al., 2020). During the fermentation process, water kefir grains will breed a large number of probiotics, which can produce nutrients such as polysaccharides and organic acids, and have the functions of inhibiting pathogenic microorganisms, improving immunity (Toghyani et al., 2015), and reducing inflammation (Rodrigues et al., 2016). Water kefir is a slightly sweet and slightly sour beverage made by inoculating water kefir grains into non-dairy fermentation substrates such as syrups, fruit and vegetable juices (Lynch et al., 2021). 'Tepache', for instance, a beverage consisting of brown sugar, pineapple and cinnamon and fermented with water kefir grains, is popular in South American countries (Fiorda et al., 2017). However, there are very few studies on water kefir in China.

Jujube juice has many benefits after fermentation. During the fermentation process, probiotics can use the soluble solids to produce enzymes, ketones, amino acids and organic acids which not only improve the nutritional value but also make the product taste softer and smell more fragrant (Tang et al., 2020). Spoilage microorganisms cannot grow in an acidic environment after fermentation, which is more conducive to antiseptic preservation (Zhao et al., 2021). Based on the above advantages, we want to explore the feasibility of fermented jujube juice with water kefir grains.

In this paper, the single factor method and a Box-Behnken experimental design were used to optimize the fermentation process of jujube water kefir for the first time, aiming to develop a low-sugar, high-antioxidant fermented jujube beverage that can be accepted by health-conscious consumers, and make full use of unsold perishable jujube.

MATERIALS AND METHODS

Activation of water kefir grain

The water kefir grains preserved in the laboratory were filtered with a sieve, rinsed with distilled water and then supplemented with 10% brown sugar water, which was replaced every 48 hours. Water kefir grains were considered activated after three consecutive runs.

Preparation of jujube juice

Once the extraction conditions of jujube juice had been determined, we selected Yulin jujube in northern Shaanxi, cleaned and removed the core, and mixed it with water in a ratio of 1:5(w/w). After stirring with a juicer, the Viscozyme L (Novozymes, Tianjin, China) was added (800 μL/L) to the mixture, then it was treated at 54°C for 1.5 hours. After centrifugation at 5000 r/min for 10 min, the supernatant was used as the resulting jujube juice, which had a 14.00 ±0.10% soluble solid content, a juice yield of 64.66 ±0.14%, and a pH of 4.30.

Optimization of fermentation conditions

A single factor experiment was used to explore the effects of inoculation amount (2%, 4%, 6%, 8%, w/v) and fermentation time (24 h, 36 h, 48 h, 60 h, 72 h) on jujube water kefir. The optimal conditions for fermentation were found using a Box-Behnken design and the response surface method. Inoculation dose, fermentation temperature and fermentation time were independent variables, while DPPH radical scavenging rate, sensory evaluation score (SES), pH, Titrable acidity (TA) and soluble solid content (SSC) were response variables.

Antioxidant activity

Antioxidant measurements were based on Mahmoudi et al. (2021) with slight modifications. A newly prepared 0.1 mM/L methanolic DPPH solution was used for each assay. 0.1 mL of the sample was accurately measured, then 3.9 mL of methanolic DPPH solution was added, mixed and placed in the dark for 30 min. The absorbance A_1 was measured at 517 nm, and 0.1 mL of methanol solution was used as a blank group to determine the absorbance A_0. The formula of the DPPH free radical scavenging rate is as follows:

$$\text{DPPH (\%)} = (1 - A_1/A_0) \times 100 \quad (1)$$

pH, TA, SSC

The pH was measured with a pH meter (PHS-3C), and the jujube water kefir sample was titrated with 0.1N NaOH to determine the TA, which was expressed as g/L lactic acid equivalent to organic acid. The content of the soluble solid was determined by a handheld refractometer.

Sensory evaluation

Ten people were selected to form a sensory evaluation group. Samples were randomly coded and evaluated at room temperature. The evaluation indexes were composed of taste, flavor and appearance, accounting for 50%, 40% and 10% respectively. Four grades (dislike extremely, dislike slightly, fair, excellent) were determined by the evaluation of each factor, and the corresponding scores were 60–70, 70–80, 80–90 and 90–100.

Statistical analysis

All experiments were performed in triplicate and data are expressed as the mean and standard deviation of three independent experiments. Data were statistically analyzed using one-way analysis of variance (ANOVA), and Tukey's multiple comparison test was used to determine whether differences were statistically significant ($p < 0.05$).

RESULTS AND DISCUSSION

Effects of time and inoculum size on jujube juice fermented by water kefir grains

Figure 1 shows that the soluble solid content and TA of jujube water kefir decreased and increased, respectively, after fermentation at 30°C for different times at 4% inoculum, indicating that the microorganisms in the water kefir grains had metabolized nutrients in jujube juice to produce acid, and the pH had decreased. The DPPH scavenging rate varied with the fermentation time, reaching its highest value at 36 hours. Corona et al. (2016) reported a decrease of total phenol content and antioxidant activity in the water kefir fermentation of fruits and vegetables, and the same result was found in Randazzo et al. (2016). Due to the synergistic effect

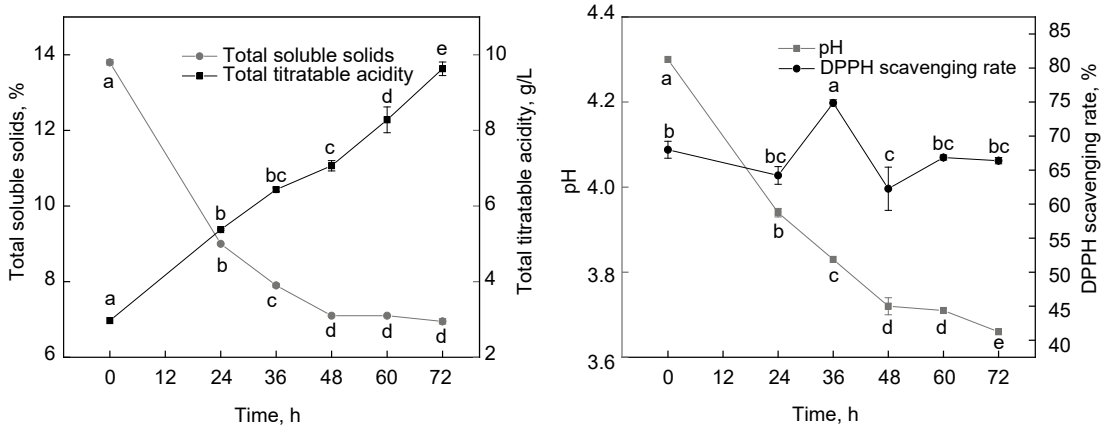

Fig. 1. Effects of time on fermentation of jujube juice: the different letters indicate that the difference between the averages is statistically significant ($p < 0.05$)

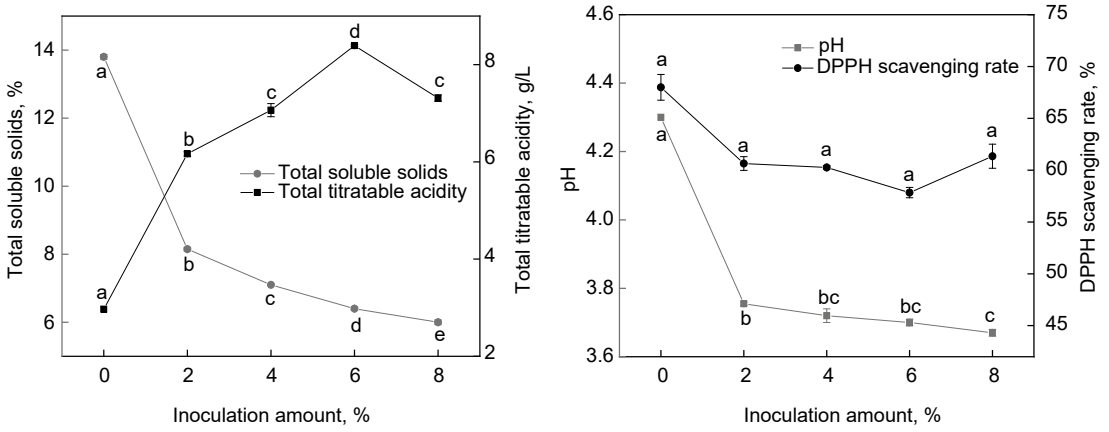

Fig. 2. Effects of inoculum on fermentation of jujube juice: the different letters indicate that the difference between the averages is statistically significant ($p < 0.05$)

of phenolic compounds and other components during the fermentation process, the antioxidant activity of jujube water kefir could not be predicted from the total phenolic content alone. However, jujube contains a large number of phenolic compounds associated with antioxidant activity, especially DPPH radical scavenging (Ali et al., 2019; Narayana et al., 2018). We hypothesize that the antioxidant activity of jujube juice fermented with water kefir grains was influenced by both phases of synthesis and degradation of the total phenolic content. Additionally, some natural phenols and antioxidant compounds are pH-dependent, which means that during the fermentation process, changes in pH can have an impact on the content and structure of the phenolic compounds, which in turn can have an impact on the product's total phenolic content and antioxidant activity (Hur et al., 2014).

The potential of fermentation to increase output and alter the profile of phenolic compounds is mostly attributable to the release of bound phenolic compounds as a result of the degradation of the cell wall structure by microbial enzymes released during fermentation (Huynh et al., 2014; Shen et al., 2021). In addition, depending on the microbial strains and substrates used, the microbial metabolism of phenolic compounds generates a wide variety of new metabolites through various bioconversion pathways like glycosylation, deglycosylation, ring cleavage, methylation, glucuronidation, and sulfate conjugation.

The final DPPH scavenging rate shown in Figure 2 was similar after 48 hours of fermentation at 30°C with an inoculum size between 2% and 8%. Through one-way ANOVA, we found that the amount of inoculation had no significant effect on the clearance rate of DPPH ($p > 0.05$). This is likely because a lower inoculum size corresponds to a smaller volume of water kefir grains. Tiny water kefir grains exhibit a fermentation ability comparable to that of grains with greater volume and mass because they have a greater specific surface area, which is more conducive to the attachment of microorganisms, and a higher quantity of viable bacteria (Laureys et al., 2017). For the consideration of economy and convenience of operation, we chose a 2% inoculation volume for further study.

Fiorda et al (2016) reported that the weight of water kefir grains increased the most at 30°C, which meant that the metabolism of microorganisms was vigorous. Therefore, 30°C was selected when we explored the influence of fermentation time and inoculation amount. However, water kefir grains are composed of a variety of microorganisms, and different temperatures will result in the dominant growth of different microorganisms, whose contribution to flavor is also inconsistent (Patel et al., 2022; Laureys et al., 2018). In order to explore the influence of different fermentation temperatures on the flavor of jujube water kefir, we chose a temperature of 33°C for further study.

Optimization of fermentation conditions for jujube juice by RSM

A Box-Behnken design for a single factor experiment in Design-Expert 12 was used to find the optimal conditions for the fermentation of jujube water kefir by fitting a polynomial model with RSM. The experimental design of three levels of variables is shown in Table 1. The experimental design and results are shown in Table 2 and the response values were: DPPH radical scavenging rate (Y1), SES (Y2), pH (Y3), TA (Y4) and SSC (Y5).

The results in Table 2 show that the DPPH scavenging rate ranged from 72.50 to 82.00%, the SES from 74.50 to 89.50, the pH from 3.48 to 3.87, the TA from 3.74 to 8.55 g/L, and the SSC from 7.00 to 10.00%.

Table 1. Level of variables selected in the Box-Behnken design

Independent variables	Coded variable level		
	–1	0	+1
Inoculation amount (X_1), %	1	2	3
Fermentation temperature (X_2), °C	29	33	37
Fermentation time (X_3), h	30	36	42

The significance of the effects analyzed and the response variables are shown in Table 3. Analysis of variance (ANOVA) was used to evaluate the significance coefficient of the model to determine the effect of different variables. The lower the *p*-value,

Table 2. The Box-Behnken design and the experimental data of jujube water kefir

Runs	Fermentation conditions			Responses				
	X_1 %	X_2 °C	X_3 h	DPPH %	SES	pH	TA g/L	SSC %
1	1	33	30	79.38	89.50	3.76	5.27	10.00
2	3	37	36	77.98	75.50	3.48	8.55	7.60
3	1	29	36	81.07	87.50	3.86	4.28	9.10
4	3	33	30	78.75	81.70	3.61	5.99	8.60
5	2	33	36	71.50	84.00	3.75	5.54	8.00
6	3	33	42	73.52	77.00	3.55	7.25	7.00
7	2	33	36	72.18	82.70	3.77	5.76	8.00
8	2	29	42	75.70	81.10	3.79	5.04	7.00
9	3	29	36	78.73	80.50	3.64	6.30	8.00
10	1	37	36	82.00	80.80	3.58	7.29	8.20
11	2	37	30	76.88	81.80	3.65	6.35	8.40
12	2	33	36	72.93	83.00	3.74	5.63	8.10
13	1	33	42	78.56	80.50	3.64	5.04	7.00
14	2	29	30	77.50	84.00	3.87	3.74	10.00
15	2	33	36	73.07	83.00	3.75	5.67	7.90
16	2	37	42	76.58	74.50	3.52	6.57	7.10
17	2	33	36	71.50	83.10	3.75	5.80	8.00

Table 3. ANOVA of the response surface quadratic polynomial model for DPPH scavenging rate, SES, pH, TA, SSC

Source	DF	DPPH		SES		pH		TA		SSC	
		mean square	F-value	mean square	F-value	mean square	F-value	mean square	F-value	mean square	F-value
Model	9	19.44	31.86***	24.89	47.36***	0.024	96.65***	2.31	64.47***	1.5	220.68***
X_1 – inoculation amount	1	18.11	29.67**	69.62	132.45***	0.039	160.94***	4.82	134.33***	1.2	177.03***
X_2 – temperature	1	0.03	0.04	52.53	99.94***	0.108	443.86***	11.06	308.11***	0.98	144.42***
X_3 – time	1	8.29	13.58*	71.4	135.84***	0.019	78.06***	0.82	22.92**	9.9	1459.13***
X_1X_2	1	0.71	1.17	0.72	1.37	0.0036	14.78**	0.15	4.08	0.06	9.21*
X_1X_3	1	4.87	7.98*	4.62	8.79*	0.0009	3.7	0.55	15.36**	0.49	72.21***
X_2X_3	1	0.57	0.93	4.84	9.21*	0.0006	2.57	0.29	8.13*	0.72	106.47***
X_1^2	1	77.74	127.4***	0.07	0.14	0.034	139.24***	2.02	56.35***	0.07	9.7*
X_2^2	1	48.97	80.26***	16.09	30.62***	0.0021	8.56*	0.23	6.3*	0.04	6.2*
X_3^2	1	4.36	7.14*	3.08	5.86*	0.0021	8.56*	1	27.97***	0.003	0.39
Residual	7	0.61		0.53		0.0002		0.04		0.007	
Lack of fit	3	0.67	1.19	0.9	3.71	0.0004	3.4	0.07	5.92	0.01	1.83
Pure error	4	0.56		0.24		0.0001		0.01		0.005	
Cor total	16	179.27		227.73		0.214		21.07		13.52	
Std. dev.		0.78		0.73		0.016		0.19		0.08	
Mean		76.34		81.78		3.69		5.88		8.12	
CV, %		1.02		0.89		0.42		3.22		1.01	
R-squared		0.98		0.98		0.99		0.99		0.99	
Adj R-squared		0.95		0.96		0.98		0.97		0.99	
Pred R-squared		0.80		0.80		0.90		0.84		0.97	
Adeq precision		16.17		27.85		31.43		32.4		48.67	

*$p < 0.05$, significant; **$p < 0.01$, very significant;***$p < 0.001$, extremely significant.

the more pronounced the effect of the variable. The R-squared value indicates how well the predicted values fit the model. As shown in Table 3, the ANOVA demonstrated that all the models were extremely significant ($p < 0.001$), and no Lack of Fit was significant. Therefore, the quadratic model fitted the experimental data well.

The mean square value in the model reflects the importance of each variable to the response values. The larger the mean square value, the greater the influence on the response value. The Adj R-squared of the five response values is close to the R-squared value, which verifies that the measured value and the predicted value have high fitting accuracy, so the feasibility of the experimental method is proved. Moreover, lower values of the coefficient of variation (CV) clearly show that the deviation between the experimental and predicted values is minimal (Quispe et al., 2021).

The quadratic regression model was established based on the data provided by the Box-Behnken design. The multivariate regression equations used to calculate the correlations between the responses and the independent variables were according to the equations:

$$Y1 = 72.24 - 1.5X_1 + 0.06X_2 - 1.02X_3 - 0.42X_1X_2 - 1.1X_1X_3 + 0.38X_2X_3 + 4.3X_1^2 + 3.41X_2^2 + 1.02X_3^2 \quad (2)$$

$$Y2 = 83.16 - 2.95X_1 - 2.56X_2 - 2.99X_3 + 0.43X_1X_2 + 1.07X_1X_3 - 1.10X_2X_3 - 0.13X_1^2 - 1.96X_2^2 - 0.85X_3^2 \quad (3)$$

$$Y3 = 3.75 - 0.07X_1 - 0.12X_2 - 0.05X_3 + 0.03X_1X_2 + 0.02X_1X_3 - 0.01X_2X_3 - 0.09X_1^2 - 0.02X_2^2 - 0.02X_3^2 \quad (4)$$

$$Y4 = 5.68 + 0.78X_1 + 1.18X_2 + 0.32X_3 - 0.19X_1X_2 + 0.37X_1X_3 - 0.27X_2X_3 + 0.69X_1^2 + 0.23X_2^2 - 0.49X_3^2 \quad (5)$$

$$Y5 = 8.00 - 0.39X_1 - 0.35X_2 - 1.11X_3 + 0.13X_1X_2 + 0.35X_1X_3 + 0.43X_2X_3 + 0.13X_1^2 + 0.10X_2^2 + 0.03X_3^2 \quad (6)$$

The optimization of the extraction process was determined by applying a second-order polynomial equation, which was used to generate a graphical representation of the regression equation simulated by Design-Expert and represented by a 3D response surface (Fig. 3–7).

Effects of the variables on the DPPH scavenging rate

The DPPH radical scavenging rate was most influenced by the amount of inoculation, followed by time and temperature. Notably, the quadratic main effects of inoculation amount, temperature, and time were significant ($pX_1^2 < 0.001$, $pX_2^2 < 0.001$, $pX_3^2 < 0.05$), indicating that a simple linear correlation did not exist

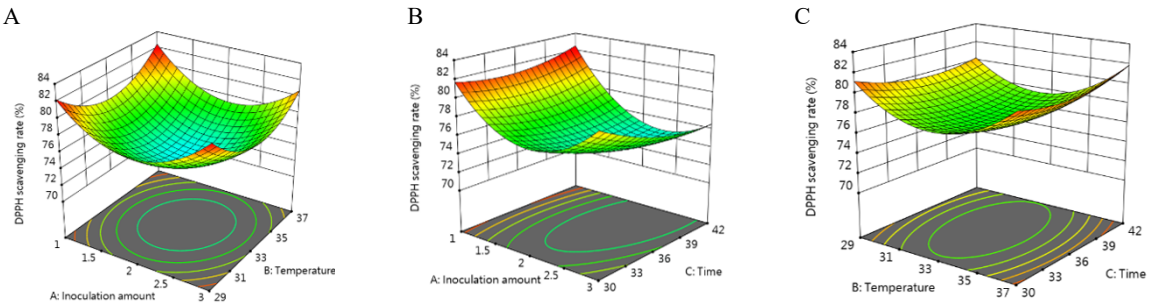

Fig. 3. Response surface of the DPPH as a function of the inoculation amount × temperature (A), inoculation amount × time (B), temperature × time (C)

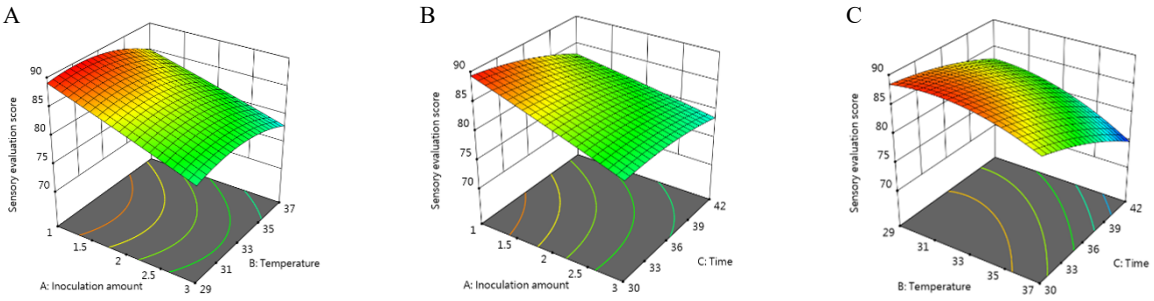

Fig. 4. Response surface of the SES as a function of the inoculation amount × temperature (A), inoculation amount × time (B), temperature × time (C)

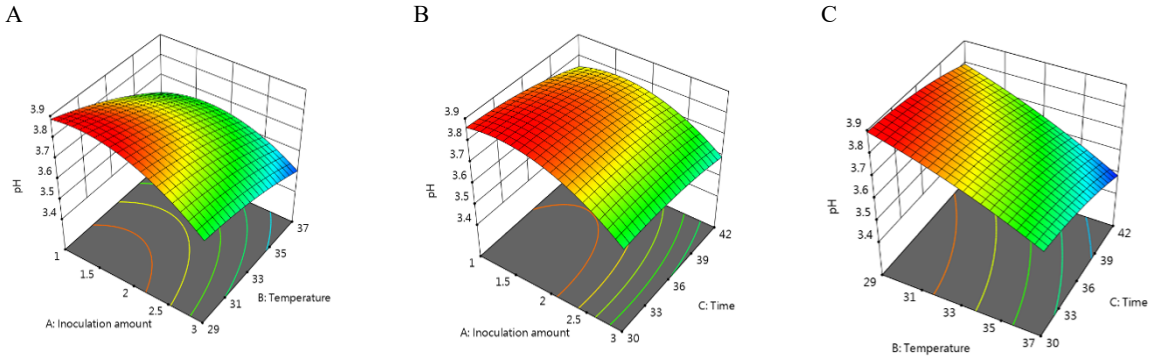

Fig. 5. Response surface of the pH as a function of the inoculation amount × temperature (A), inoculation amount × time (B), temperature × time (C)

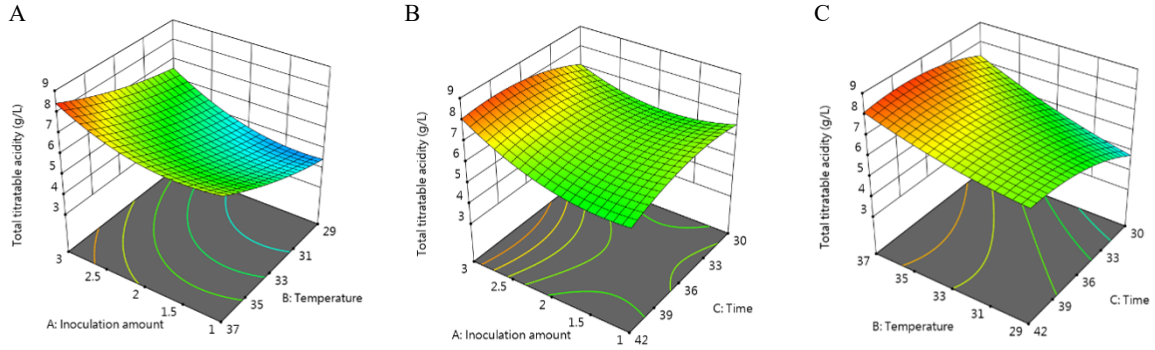

Fig. 6. Response surface of the TA as a function of the inoculation amount × temperature (A), inoculation amount × time (B), temperature × time (C)

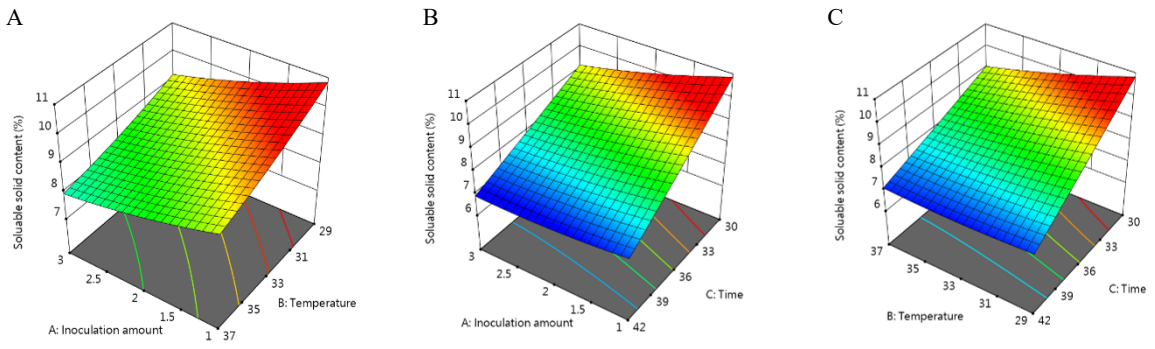

Fig. 7. Response surface of the SSC as a function of the inoculation amount × temperature (A), inoculation amount × time (B), temperature × time (C)

between the variables and DPPH radical scavenging activity. The 3D surface maps generated by the model equation in Figure 3 are curved with varying degrees of curvature. Additionally, the contour plot depicted an elliptical shape, which suggested that the interaction between X_1X_2 and X_2X_3 was not significant.

Darvishzadeh et al. (2021) utilized water kefir grains to ferment Russian olive fruit and determined

that as fermentation temperature increased, the total phenol content and DPPH radical scavenging rate of the beverage decreased, even when the fermentation time remained constant. They assumed that higher temperatures facilitate microbial metabolic activity, thereby causing changes to the structure of phenolic compounds as they are enzymatically converted to other molecules. Ultimately, this transformation results in a lower total phenolic content and reduced antioxidant activity.

Effects of the variables on sensory evaluation score

The color, smell, and taste of beverages are the primary factors that shape consumers' initial impressions. Table 3 shows the extremely significant influences of the three independent variables on the sensory evaluation scores. Additionally, the noteworthy interactions between X_1X_3 and X_2X_3 are also observed to be significant ($pX_1X_3 < 0.05$, $pX_2X_3 < 0.05$).

During the brewing process of red date wine, *Saccharomyces cerevisiae* undergoes autolysis, which results in the production of guanyl and inosinic acid through the action of nuclease. These two umami compounds serve as a crucial source of umami flavor in chicken flavoring and chicken powder flavoring, surpassing even the umami flavor of monosodium glutamate (Tang et al., 2020). Zhao et al. (2021) conducted a study using *Lactobacillus plantarum* TUST-232 to ferment a mixture of jujube and bamboo shoot juice. The resulting mixture produced and released volatile compounds, including acids, aldehydes, ketones, and other substances. This process significantly enhanced the quality and flavor of the beverage. Similarly, the presence of *Lactobacillus* and yeast in water kefir grains accelerates metabolic activity, resulting in improved sensory acceptability.

Effects of the variables on pH, TA and SSC

The three independent variables in this study have been found to have significant effects on pH, TA, and soluble solid content in water kefir grains. This can be attributed to the ability of microorganisms present in water kefir grains to use soluble solids to produce organic acids, thereby reducing pH levels. Currently, there is a consensus on the changes that occur during the water kefir fermentation process. Saccharomyces is responsible for the hydrolysis of sucrose through extracellular glycosidase, producing glucose and fructose. Yeast then utilizes these monosaccharides in glycolysis to produce ethanol and carbon dioxide while providing vital peptides and amino acids to lactic acid bacteria and other microorganisms. Lactobacillus is capable of assimilating fructose for glycolysis or converting it to mannitol through the activity of mannitol dehydrogenase. During the later stage of fermentation, acetic acid bacteria metabolize gluconic and acetic acids under aerobic conditions to enhance the acidic flavor (Lynch et al., 2021; Ras El Gherab et al., 2019). The final pH of the drink was noted to be below 4, representing a crucial feature of the beverage's safety. This pH level effectively inhibits the growth of pathogenic bacteria in the beverage, thereby extending its shelf life and enhancing its overall safety (Phiri et al., 2019).

Validation of the optimized conditions

The analysis of the regression equation conducted using Design-expert 12 helped to determine the optimal fermentation conditions for jujube water kefir. We used the highest DPPH free radical clearance rate and the sensory evaluation scores as the optimization criteria. The optimized conditions included an inoculation amount of 1%, a fermentation temperature of 29°C, and a fermentation time of 30 hours. The resulting beverage displayed a DPPH scavenging rate of 81.06 ±0.29%, a sensory evaluation score of 88.00 ±2.71, a pH of 3.93 ±0.04, a titrable acidity value of 3.87 ±0.14 g/L, and a soluble solid content of 11.00 ±0.10%. The experimental values were found to be similar to the predicted values, highlighting the efficacy of the developed quadratic models.

CONCLUSION

A single factor experiment and a response surface methodology were used to optimize the jujube water kefir fermentation process. The optimized conditions included an inoculation amount of 1%, a fermentation temperature of 29°C, and a fermentation time of 30 hours. The resulting beverage displayed DPPH radical scavenging activity and acceptable sensory evaluation. The quadratic model fitted the experimental data well, and the predicted value of the model was close to the actual value. Therefore, this study provides

a reference for a jujube-based drink using water kefir grains and explores the feasibility of bonding water kefir grains with Chinese native fruits.

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