Optimization of the Fermentation Process of Fermented Soy Milk by Composite Strains Using Response Surface Methodology and Study on Its Physicochemical Properties

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Abstract [**Objectives**] This study was conducted to improve the poor water-holding capacity (WHC) and high syneresis rate of fermented soy milk by optimizing fermentation process conditions. [**Methods**] Based on the results of single factor experiments, response surface methodology was employed to optimize the fermentation temperature, fermentation time, and starter culture addition for enhancing WHC. The physicochemical properties of fermented soybean milk were analyzed. [**Results**] The optimal process parameters were determined as follows: fermentation temperature 36 ℃, fermentation time 8 h, and starter culture addition 5%. Under these conditions, WHC reached (77.18 ± 0.08)%, which was consistent with the theoretical prediction value of (76.75 ± 0.15)%. During fermentation, the pH decreased from (6.6 ± 0.11) to (4.65 ± 0.09), while acidity increased from (16.5 ± 0.04)°T to (81.5 ± 0.08)°T. The viable cell count rose from 1×10^7 to 29×10^7 cfu/ml, and WHC was improved significantly from (10.50 ± 0.18)% to (77.40 ± 0.13)%. [**Conclusions**] This study optimized the fermentation process parameters and revealed physicochemical characteristic changes during soybean milk fermentation, providing a theoretical foundation for industrial production.

Key words Response surface; Fermented soy milk; Physicochemical properties
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Driven by the dual forces of global health awareness upgrading and carbon neutrality strategies, the market of plant-based fermented food is expanding rapidly at a compound annual growth rate of 12.3%, with per capita protein intake reduced by 10% – 15% by 2050. Meanwhile, the proportion of plant-based protein in the diet is expected to increase from 40% to 60% of total dietary protein[1]. As a representative product in this field, fermented soy milk combines the nutritional value of soybeans with probiotic functions through microbial fermentation, emerging as a key growth category in the dairy alternative market[2]. However, industry research reveals that approximately 67% of fermented soy milk producers face rising customer complaints due to batch-to-batch variations in physicochemical properties, with viscosity fluctuations (± 15%), insufficient water-holding capacity (syneresis rate > 8%), and protein aggregation precipitation identified as the three major quality defects[3]. This phenomenon highlights how existing production technologies severely constrain the industry's development.

The physicochemical properties of fermented soy milk are essentially the result of a complex dynamic equilibrium among proteins, polysaccharides and microbial metabolism during fermentation. Studies show that exopolysaccharides (EPS) produced by lactic acid bacteria can interact with soy proteins (such as β-conglycinin) through hydrogen bonds to form a three-dimensional network structure, directly affecting the viscoelasticity and water-holding capacity of products[4]. Meanwhile, the synergistic effect of fermentation temperature and time not only regulates microbial activity but also alters protein isoelectric points by influencing proteolysis, thereby determining gel stability[5]. Although traditional single-factor optimization can identify the main effects of individual variables such as inoculum size, it fails to elucidate the nonlinear kinetic mechanisms underlying multivariate interaction such as temperature-time-strain ratio. For instance, Gholamhosseinpour *et al.*[6] found that the coupling effects of multiple factors make precision quality targeting difficult to achieve through empirical process control.

Therefore, in this study, with mixed bacteria of *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus* as the starter culture, based on single-factor experiments, a response surface model of fermentation temperature, inoculation amount and fermentation time was established by Box-Behnken design (BBD) to investigate the interaction of multiple factors on the water-holding capacity (WHC) and physicochemical stability of fermented soy milk and develop a prediction model, and optimal production conditions were determined through a verification experiment. This study provides theoretical

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support for standardized production of fermented soy milk while offering methodological reference for process optimization innovation in plant-based fermented food.

Materials and Methods

Materials and reagents

GMO-free soybeans from Northeast China were purchased from the Shaoyang Agricultural Product Market in Hunan Province. *L. bulgaricus*, *S. thermophilus*, *L. plantarum*, and *L. rhamnosus* were obtained from Guangdong Institute Of Microbiology. Plate count agar and sodium hydroxide reagents, all of analytical grade, were purchased from Sinopharm Chemical Reagent Co., Ltd.

Instruments and equipment

LS-B50L high-pressure steam sterilizer (Shanghai Huaxian Medical Nuclear Instrument Co., Ltd.); integrated soy milk cooking machine (Beijing Kangdeli Intelligent Technology Co., Ltd.); DHP9140A incubator (Shaoxing Super Instrument Co., Ltd.); acid-base burette (Hunan Aidite Scientific Instruments Co., Ltd.); pH meter (Shanghai Lichen Bangxi Instrument Technology Co., Ltd.).

Experimental methods

Preparation of starter culture The four bacterial strains (*L. bulgaricus*, *S. thermophilus*, *L. plantarum* and *L. rhamnosus*) were, respectively, inoculated into MRS liquid medium and cultured at 37 ℃ for 24 h. They were activated for three successive cycles. The activated cultures were confirmed to have a viable count of 10^7 cfu/ml through plate counting and stored at 4 ℃ for subsequent use.

Design of single factor experiments Using 150 g of soy milk as the substrate, and fixing the addition amount of sucrose at 8% while keeping other conditions constant, the effects of starter culture addition (2%, 3%, 4%, 5%, 6%), fermentation time (6, 7, 8, 9, 10 h), and fermentation temperature (30, 33, 36, 39, 42 ℃) on the viscosity of set-style fermented soy milk were investigated with WHC of set-style fermented soy milk as the evaluation index.

Design of response surface experiment Based on the single factor experiments, a response surface experiment was designed by Box-Behnken Design (BBD) using Design-Expert 8.0.68 RSM software for optimization. The experimental factors, level coding and response values are presented in Table 1. Each group of experiments was conducted in triplicate to optimize the fermentation process conditions.

pH measurement during fermentation The pH values of samples were measured using a pH meter.

Acidity determination during fermentation The acidity was measured according to the method described by Yousefi *et al.* [7].

Analysis of WHC The WHC of soy yogurt was determined by the centrifugation method. The weight of an empty 50 ml centrifuge tube was recorded as W_1. After adding 20 g of soy yogurt into the tube, the total weight was recorded as W_2. The sample was centrifuged at 10 000 × g and 4 ℃ for 20 min. After removing the supernatant, the weight was recorded as W_3. WHC was calculated using following formula:

$$\text{WHC}\ (\%) = [(W_3 - W_1)/(W_2 - W_1)] \times 100\% \qquad (1)$$

Table 1 Factors and levels of the Box-Behnken Design (BBD) experimental design

Factor	Level		
	−1	0	1
A Inoculum size//%	4	5	6
B Fermentation time//h	7	8	9
C Fermentation temperature//℃	33	36	39

Viable cell counting The viable bacterial count was determined according to the method described by Qin *et al.* [8].

Statistical analysis of data

All measurements were performed in three independent replicates, with final results expressed as mean ± standard deviation (Mean ± SD). Statistical analysis was conducted using SPSS 21 software, with $P < 0.05$ considered statistically significant. Graphs were generated using Origin 2021 software.

Results and Discussion

Effects of different fermentation temperatures on WHC of fermented soy milk

The effects of fermentation temperature on the WHC of fermented soy milk are shown in Fig. 1A. From 30 to 36 ℃, WHC gradually increased, and maintained a relatively high level between 33 and 36 ℃. At 39 ℃, WHC decreased but remained lower than those at 30 and 33 ℃. It indicated that increasing temperature within a certain range enhanced WHC, with 36 ℃ being the optimal fermentation temperature. Excessively high temperatures were unfavorable for maintaining WHC.

Effects of different fermentation time on WHC of fermented soy milk

As shown in Fig. 1B, WHC exhibited an increasing trend as fermentation time extended from 6 to 8 h, followed by a declining trend from 8 to 10 h. The highest WHC was achieved in the fermented soy milk at 8 h of fermentation.

Effects of different starter culture addition levels on WHC of fermented soy milk

Fig. 1C demonstrates that WHC initially increased and then decreased as the inoculum size rose from 2% to 6%. The WHC was observed at the starter culture addition level of 5%, indicating that appropriate increases in inoculum size could enhance WHC.

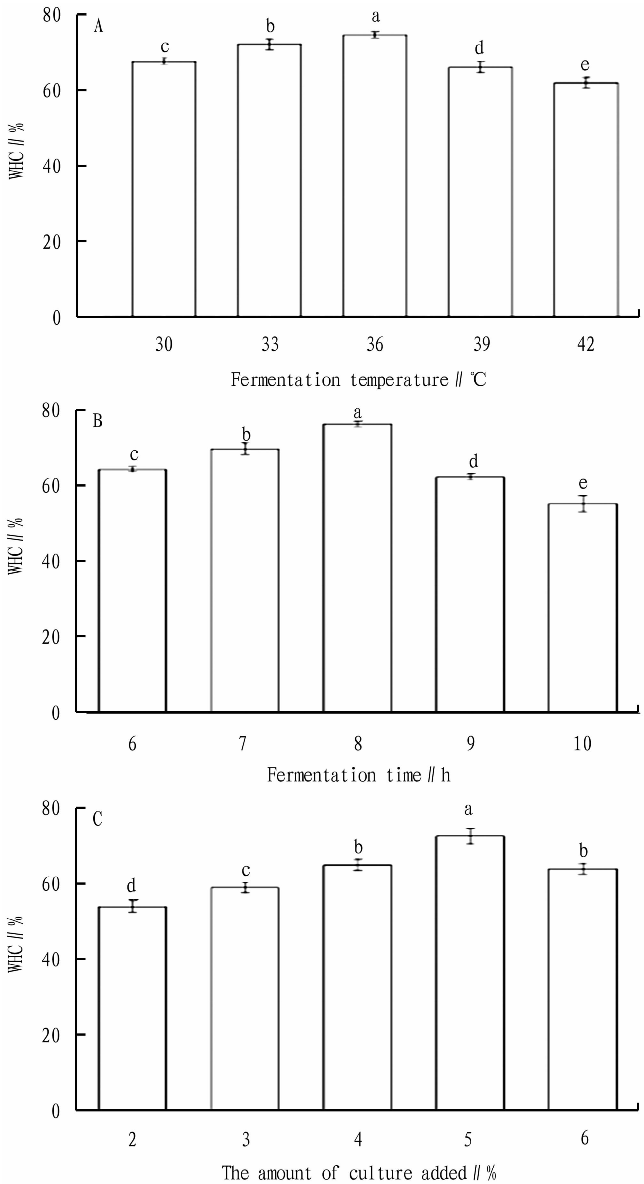

Fig. 1 Effects of different fermentation temperatures (A), fermentation time (B) and starter culture addition (C) on WHC of fermented soybean milk

Response surface optimization results

Optimization on fermentation conditions of fermented soy milk by response surface methodology

Table 2 Experimental design of Box-Behnken Design (BBD) and response values of the WHC of fermented soy milk

Test No.	A Inoculum size	B Fermentation time	C Fermentation temperature	WHC // %
1	5	8	36	78.80 ± 0.15
2	5	8	36	74.39 ± 0.42
3	5	8	36	76.88 ± 0.01
4	5	8	36	73.89 ± 0.37
5	5	8	36	77.90 ± 0.04
6	5	9	33	64.85 ± 0.02
7	5	7	33	67.05 ± 0.16
8	5	9	39	60.01 ± 0.78
9	5	7	39	61.13 ± 0.11
10	6	8	39	63.86 ± 0.47
11	6	7	36	62.27 ± 0.02
12	6	8	33	65.25 ± 0.27
13	6	9	36	61.20 ± 0.21
14	4	7	36	67.88 ± 0.02
15	4	8	33	68.85 ± 0.05
16	4	8	39	63.70 ± 0.18
17	4	9	36	61.92 ± 0.42

Model fitting and result analysis The experimental results according to the response surface BBD design are shown in Table 2. The WHC of fermented soy milk ranged from 60.01% to 78.80%. Using Design-Expert 8.0.6, the experimental data were fitted with a quadratic polynomial regression model, yielding following second-order multiple regression equation: $Y = 76.11 - 1.02A - 1.29B - 2.28C + 1.22AB + 1.18AC + 0.27BC - 5.69A^2 - 7.43B^2 - 5.10C^2$.

Table 3 Analysis of variance for the regression model

Source of variation	Sum of squares	Df	Mean square	F	P	Significance
Model	617.36	9	68.60	15.36	0.000 8	Significant
A Inoculum size	6.96	1	6.96	1.56	0.252 1	
B Fermentation time	13.39	1	13.39	3.00	0.126 9	
C Fermentation temperature	29.30	1	29.30	6.56	0.037 5	
AB	5.98	1	5.98	1.34	0.285 2	
AC	3.02	1	3.02	0.68	0.437 9	
BC	0.29	1	0.29	0.065	0.805 6	
A^2	125.68	1	125.68	28.15	0.001 1	
B^2	208.48	1	208.48	46.69	0.000 2	
C^2	86.57	1	86.57	19.39	0.003 1	
Residual	31.26	7	4.47			
Lack of fit	12.68	3	4.23	0.91	0.511 3	Non-significant
Pure error	18.58	4	4.64			
Total deviation	648.62	16				
$R^2 = 0.952$	$R^2_{adj} = 0.889$					

Analysis of variance (ANOVA) was performed on the multiple regression model, with results shown in Table 3. The established regression equation model showed $P < 0.000\ 1$, indicating the fitted model was highly significant. The regression coefficient $R^2 = 0.951\ 8$ demonstrated good fitting accuracy of the equation, while the adjusted coefficient $R^2_{adj} = 0.889\ 9$ indicated the regression model could explain 88.99% of the variation in the response value. Significance test of the regression equation coefficients revealed that among the linear terms, factor C, and among the quadratic terms, A^2, B^2 and C^2 had significant effects on the WHC of fermented soy milk. It could be seen that the effects of various factors on the WHC of fermented soy milk were not simple linear relationships. After eliminating non-significant terms at $\alpha = 0.05$ significance level, the optimized model was obtained as: $Y = 76.11 - 2.28C - 5.69A^2 - 7.43B^2 - 5.10C^2$.

Effects of interaction between factors on WHC of fermented soy milk Fig. 2 shows the 3D response surface plots and contour plots of the regression equation. In the plots, steeper slopes indicate more significant effects, while gentler slopes suggest less significant effects. The shape of the contour lines also reveal significant interaction between factors. Elliptical contours indicate a significant interaction, whereas circular contours suggest a non-significant interaction. As shown in Fig. 2(A), the effect of starter culture addition on WHC was greater than that of fermentation time, and the elliptical contour lines indicate significant interaction between these two factors on WHC. In Fig. 2(B), while starter culture addition showed greater impact on WHC than fermentation temperature, the elliptical contours suggest relatively weaker interaction between the two factors. Fig. 2(C) demonstrates that fermentation time had greater effect on WHC than fermentation temperature, and the near-elliptical contours imply a moderate interaction effect between the two factors.

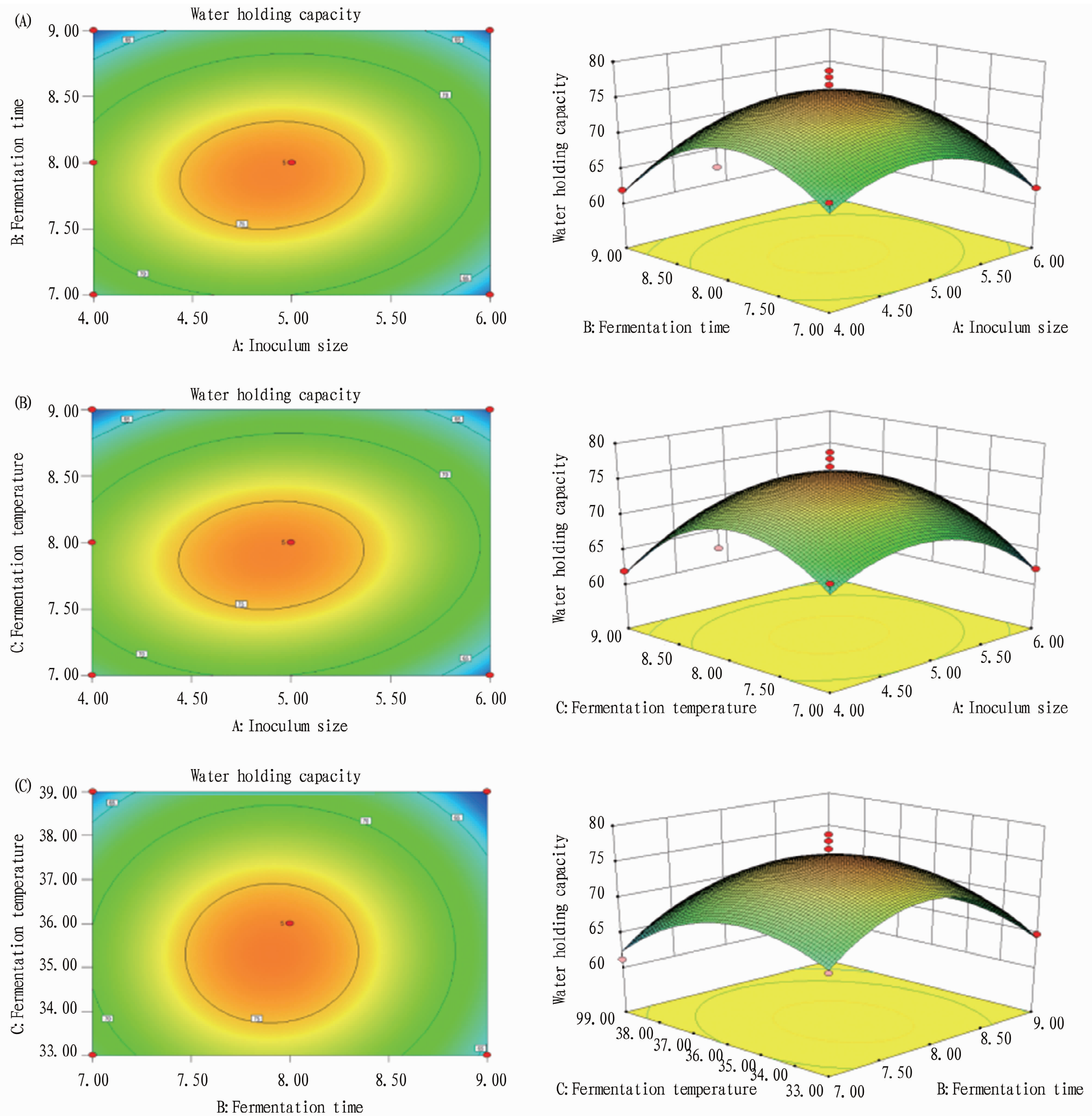

Fig. 2 Response surface and contour plots of the effects of three factors on WHC of fermented soy milk

Model verification Based on response surface optimization analysis, the optimal fermentation conditions for fermented soy milk were determined as: fermentation temperature 35.37 ℃, fermentation time 7.90 h, and starter culture addition 4.86%. For practical operation, these parameters were adjusted as follows: fermentation temperature 35 ℃, fermentation time 8 h, and starter culture addition 5%. Under these optimized conditions, the obtained WHC of fermented soy milk was (77.18 ± 0.08)% (n = 3), which closely matched the theoretical predicted value of (76.75 ± 0.15)%. Therefore, the optimized fermentation process conditions obtained in this study were accurate and feasible.

Changes in pH during fermentation of fermented soy milk

Fig. 3A shows that at the initial fermentation stage (0 h), the pH of the fermentation broth was 6.6 ± 0.11. As the fermentation progressed from 0 to 8 h, the pH exhibited a gradual decreasing trend, reaching 4.65 ± 0.09 at 8 h. This trend indicated the microbial metabolic activity continued throughout fermentation, with increasing production of acidic metabolites, which made acidic substances in the fermentation broth accumulate continuously, leading to consequent pH reduction.

Changes in acidity during fermentation of fermented soy milk

As shown in Fig. 3B, the total acidity of fermented soy milk exhibited a continuous increasing trend with prolonged fermentation time. At the initial stage (0 h), before fermentation began, the acidity remained relatively low at (16.5 ± 0.04) °T. The total acidity progressively increased as fermentation proceeded, reaching approximately (81.5 ± 0.08) °T after 8 h of fermentation. It demonstrated that microbial metabolic activity gradually intensified after fermentation initiation, leading to continuous accumulation of acidic compounds. Extended fermentation duration caused greater production of acidic metabolites by microorganisms, resulting in higher total acidity.

Changes in viable bacterial count during fermentation of fermented soy milk

Fig. 3C demonstrates that during the initial fermentation stage (0, 2 and 4 h), the viable bacterial count remained relatively low and stable at approximately 1×10^7 cfu/ml, indicating slow microbial proliferation at the beginning of fermentation. By 8 h of fermentation, the viable count significantly increased to about 29×10^7 cfu/ml, representing the peak value throughout the fermentation process. It showed that with the extension of fermentation time, microorganisms had adequate time to grow and reproduce using nutrients, and the viable cell number increased accordingly.

Changes in WHC during fermentation of fermented soy milk

Fig. 3D shows that the WHC was approximately (10.50 ± 0.18)% at 0 h of fermentation. Between 0 and 2 h, WHC showed an increasing trend. When fermentation time reached 2 – 6 h, WHC gradually rose to (56 ± 0.15)%. At 8 h of fermentation, WHC remained a high level of (77.40 ± 0.13)%. Overall, WHC exhibited a progressive increase with fermentation time and was stabilized in later stages, demonstrating that the fermentation process effectively enhanced the WHC of the system.

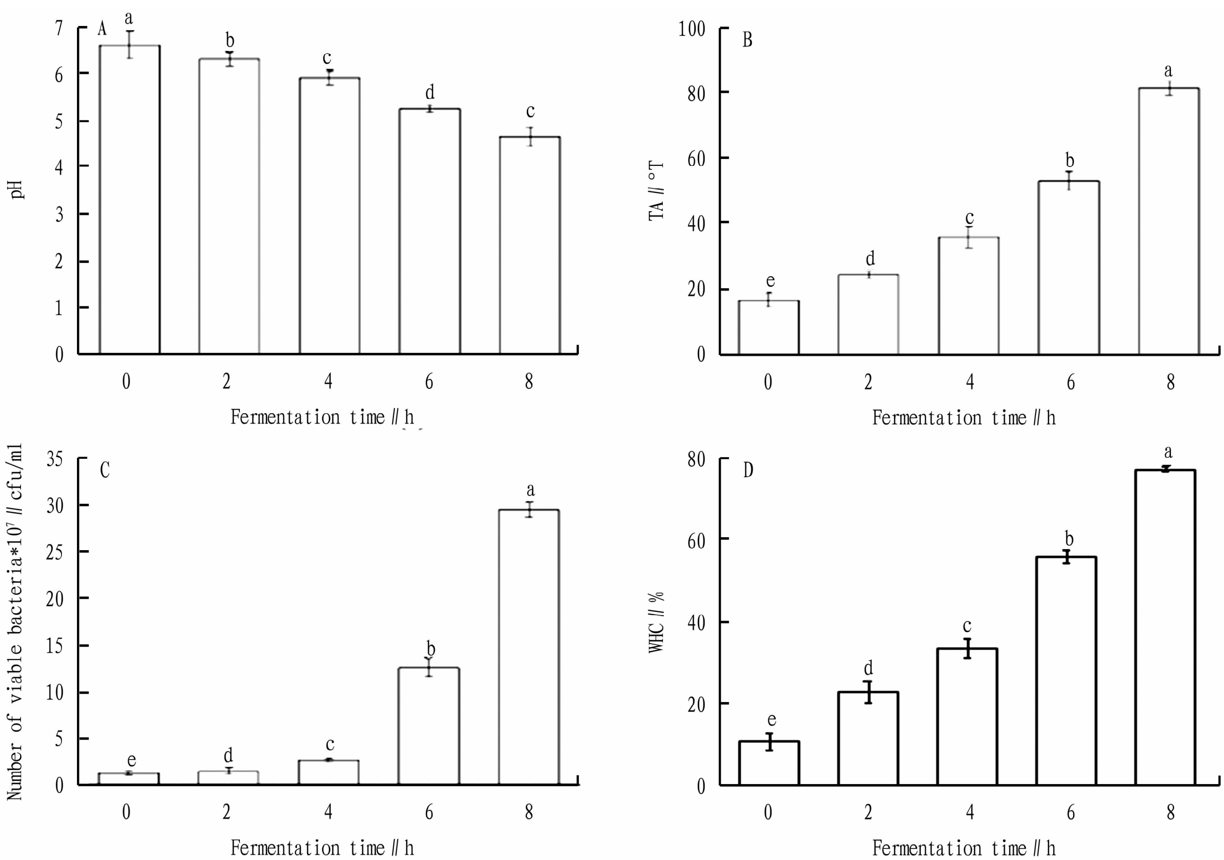

Fig. 3 Changes in pH (A), acidity (B), viable bacteria count (C), and water-holding capacity (D) of fermented soy milk with the extension of fermentation time

Conclusions and Discussion

The results of this study elucidated the effects of fermentation temperature, time, and starter culture addition on the WHC of fermented soy milk. The optimal parameters determined through response surface methodology were as follows: fermentation temperature 35 ℃, fermentation time 8 h, and starter culture addition 5%. Under these conditions, the fermented soy milk achieved superior WHC of (77.18 ± 0.08)%. ANOVA results confirmed the highly significant reliability of the established multiple regression model. Fermentation time was identified as the most significant factor affecting the WHC of fermented soy milk. The study revealed a nonlinear relationship between various process parameters and WHC. During fermentation, the pH value was on the decrease with time, while acidity, WHC and viable bacterial count showed increasing trends. These findings provide both theoretical foundation and practical guidance for industrial production and quality improvement of fermented soy milk.

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(Continued from page 55)

soils and crops, and flexibly adjust nutrient management strategies just as the Red Army responded flexibly to the enemy when crossing the Chishui River four times. ② Using the greenhouse of the school, different soil and crop planting combinations were set up, so that students could practice nutrient regulation according to their own schemes. In practice, students should adjust the nutrient supply in time according to the actual growth of crops, such as symptoms of nutrient deficiency in leaves. Such practical operation could allow students to master the method of precise regulation of soil nutrients, improve their ability of nutrient management in agricultural production, and stimulate their interest in agricultural science and technology and love for agriculture.

Ideological and political objectives: The ideological and political objectives included carrying forward the flexible tactical spirit of Crossing the Chishui River Four Times, cultivating students' innovative thinking and practical ability in soil nutrient regulation, enhancing their love for agricultural production, and making them realize the importance of scientific nutrient management for good harvest in agriculture.

Prospects

In the future reform, it is necessary to further strengthen the exploration and integration of red cultural resources, and continuously enrich the teaching contents of the course. The innovation of teaching methods and means should be strengthened to improve the pertinence and effectiveness of teaching. The improvement of the teaching evaluation system should be strengthened to evaluate students' learning achievements more comprehensively and objectively, which is also an inevitable requirement for the construction of "new agricultural science"[5]. Through continuous exploration and practice, we will better cultivate more new "red heart" young talents with firm ideals and beliefs, solid professional knowledge and strong social responsibility who know and love agriculture.

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