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Research Article

OPTIMIZATION OF PARAMETERS IN THE FERMENTATION OF SOAPNUTS USING *Saccharomyces cerevisiae* VIA RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Soapnuts represent a promising natural source of surface-active compounds for the development of eco-friendly bio-detergents as alternatives to conventional chemical-based cleaning products. Currently, soapnuts are recognized as a major saponin-rich raw material for producing various bio-based cleansing products such as shampoos, soaps, body washes, and dishwashing liquids. Soapnut extracts, which are rich in saponins, have been demonstrated to exhibit multiple biological activities, including anticancer, antifungal, antibacterial, and anti-inflammatory effects. Moreover, saponins from soapnuts also show strong cleansing properties. These characteristics make soapnuts a promising raw material for the development of green detergent products that are safer for human health. This study aimed to optimize several parameters in the fermentation process of soapnuts using *Saccharomyces cerevisiae*, employing response surface methodology (RSM) to obtain a fermented extract with the highest possible saponin-to-total soluble solids (TSS) ratio. The multi-factor experimental plan employed the Box-Behnken planning model with three independent variables corresponding to the following variation ranges: inoculum size (1-15%), fermentation temperature (25-35ºC), and fermentation time (2-8 days), with the objective function being the saponin/TSS content (%). Experimental results revealed that the optimal conditions for achieving the highest saponin/TSS content (85.1%) were an inoculum size of 9%, a fermentation temperature of 32°C, and a fermentation duration of 5 days. The fermented extract exhibited strong antibacterial activity against three pathogenic bacterial strains: *Escherichia coli* VTCC12272, *Bacillus cereus* SH46, and *Staphylococcus aureus* VTCC12275, with mean inhibition zone diameters of 20.5, 15.3, and 28.2 mm, respectively. The fermented extract from soapnut exhibited a high saponin/TSS content. It demonstrated significant antimicrobial activity, indicating its strong potential as a valuable raw material for the development of natural bio-detergent products.

Keywords: Antibacterial activity, optimization, response surface methodology, *Saccharomyces cerevisiae*, soapnuts.

INTRODUCTION

Soapnut (*Sapindus mukorossi* Gaertn.) belongs to the family Sapindaceae and is widely distributed across Asia as well as tropical and subtropical regions. The pericarp of the soapnut fruit is a rich source of saponins, which are natural surface-active compounds. Soapnuts have also been reported to exhibit various pharmacological properties, including antifungal, antibacterial, and anti-inflammatory activities (Wei *et al.*, 2021; Li *et al.,* 2021). Currently, the extraction of saponins from plants for use as surface-active agents in cleaning products has attracted considerable attention due to their safety for human use and environmental friendliness. Traditional fermentation methods, which utilize the native microbial community present on raw materials, have been adopted by several production units. While this approach is simple and cost-effective, it often results in low-quality fermented products with limited consistency and poor process control (Capozzi *et al.*, 2017). Recent studies have explored the application of specific microbial strains, such as the yeast *Saccharomyces cerevisiae* and the mold *Aspergillus oryzae*, in the fermentation of soapnut extracts to enhance saponin purification and improve the sensory and functional quality of the final product (Le *et al.*, 2023; Heng *et al.*, 2015; Wei *et al.*, 2021).

In Vietnam, soapnut is distributed across various provinces, such as Bac Ninh, Thai Nguyen, Phu Tho, Thanh Hoa, and Gia Lai. However, studies focusing on the fermentation-based extraction of saponins from Vietnamese soapnut resources remain limited. This study aims to identify optimal parameters for the fermentation process to extract saponins from soapnut using *S. cerevisiae*, including inoculum size, fermentation temperature, and duration, by employing response surface methodology (RSM). RSM has been widely applied to optimize fermentation processes such as wine and beer production involving *S. cerevisiae*, offering significant cost and time savings while maintaining statistically meaningful results (Phan and Nguyen, 2019; Bărbulescu *et al*., 2021). The findings of this study provide a scientific basis for establishing a standardized fermentation protocol for saponin extraction from soapnut, aiming to produce high-quality, stable fermented products with potential applications in the development of bio-based detergents.

MATERIALS AND METHODS

Materials

Soapnut fruits collected from Gia Lai province were de-seeded, dried at 60ºC to a constant weight, ground, and sieved through a 1 mm mesh for use in subsequent experiments.

The yeast strain *S. cerevisiae* NE2 and the pathogenic bacterial strains *Escherichia coli* VTCC12272, *Bacillus cereus* SH46, and *Staphylococcus aureus* VTCC12275 were obtained from the microbial culture collection of the Center for Biological and Environmental Technology, National Center for Technological Progress, Ministry of Science and Technology.

Fermentation process of soapnut using *S. cerevisiae* NE2

Soapnut powder and sucrose solution (2%) were sterilized separately and then cooled. The fermentation was conducted in 250 mL glass flasks containing 10 g of soapnut powder, 1-15 mL of yeast suspension (10^6 cells/mL), and supplemented with a 2% sucrose solution to a final fermentation volume of 100 mL. The mixture was incubated under shaking conditions at 150 rpm, at the design-specified temperature and time. After fermentation, the broth was centrifuged at 8000 rpm for 10 minutes to obtain the supernatant (Truong *et al*., 2024). The supernatant was then analyzed to determine the saponin content relative to the total soluble solids (TSS).

Experimental design

The optimization of parameters in the fermentation process of soapnut using the yeast strain *S. cerevisiae* NE2 was conducted following RSM using Expert Design 11 software. The multi-factor experimental plan for soapnut fermentation was carried out according to the Box-Behnken design with three independent variables. The optimal ranges of the investigated factors were determined through preliminary exploratory experiments and included: inoculum size X_1 (1-15%), temperature X_2 (25-35ºC), and fermentation time X_3 (2-8 days) (Table 1). The experimental design matrix consisted of 17 runs, as shown in Table 2. These experiments were selected to evaluate the influence of the factors on the objective function, which is the ratio of saponin content to TSS (Y). Based on the results, a second-order polynomial regression model was developed as follows:

$$Y_n = a_0 + \sum_{i=1}^{k} a_i X_i + \sum_{i=1}^{k} a_{ii} X_i^2 + \sum_{i \neq j=1}^{k} a_{ij} X_i X_j$$

Where, Y_n is the objective function, X_i and X_j are the independent variables, a_0 is the constant or the regression coefficient of zero order, a_i and a_{ii} are the first-order and second-order regression coefficients describing the effects of variable X_i on Y_n, a_{ij} is the interaction regression coefficient describing the combined effect of variables X_i and X_j on Y_n (Gong *et al.*, 2012). The saponin/TSS content was determined following the procedure previously reported by the research group (Truong *et al*., 2024). The total saponin content was determined by spectrophotometry. A 1 mL aliquot of the fermented soapnut extract was diluted to 100 mL, and then 0.2 mL of this diluted sample was taken for analysis. To the reaction tube, 0.6 mL of vanillin/glacial acetic acid solution (50 mg/mL) and 1 mL of perchloric acid were added. The tube was sealed, mixed thoroughly, and incubated at 70°C for 20 minutes. After rapid cooling, glacial acetic acid was added to bring the total volume to 10 mL. The mixture was shaken thoroughly, and absorbance was measured at λ = 550 nm. Quantification of saponin was performed using a standard calibration curve prepared with oleanolic acid, expressed as $y = 0.0414x - 0.0233$ ($R^2 = 0.999$). The TSS content was determined by weighing 10 g of the extract and drying it at 80°C to a constant weight.

Table 1. Experimental design.

Investigated factors	Coded levels of variables		
	-1	0	+1
Inoculum size X_1 (%)	1.0	8.0	15.0
Fermentation temperature X_2 (ºC)	25	30	35
Fermentation time X_3 (days)	2	5	8

Table 2. Experimental design matrix.

No.	Inoculum size (%)	Fermentation temperature (ºC)	Fermentation time (days)	Saponin/TSS (%)
1	8	25	2	45.2
2	8	30	5	85.7
3	1	35	5	68.4
4	8	30	5	84.0
5	1	30	2	41.0
6	1	30	8	64.7
7	8	25	8	68.5
8	1	25	5	60.0
9	8	35	2	44.7
10	15	30	8	81.1
11	8	30	5	84.2
12	15	25	5	80.0
13	8	35	8	76.4
14	8	30	5	85.2
15	15	30	2	50.1
16	15	35	5	79.2
17	8	30	5	84.5

Antibacterial activity assessment of the fermented extract

Agar plates containing bacterial strains (*E. coli, B. cereus,* and *S. aureus*) were prepared by spreading 100 µL of bacterial suspension (10^6 cells/mL) onto LB agar medium (composed of 20 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, and 20 g/L agar). Each petri dish (Φ9 cm) contained 15 mL of LB medium. Wells of 8 mm in diameter were created in the agar using a sterile borer. Each well was filled with 50 µL of the soapnut fermented extract. The plates were then refrigerated for 4 hours to allow for uniform diffusion of the extract into the medium, followed by incubation at 37°C for 24 hours. The antibacterial activity of the fermented extract was compared with a control extract (soapnut extract without *S. cerevisiae* NE2 fermentation). Antimicrobial activity was calculated using the following formula: D - d (mm). D: diameter of the inhibition zone (mm); d: diameter of the well (mm) (Hadacek and Greger, 2000). For each bacterial strain, the experiment was conducted on three plates and repeated independently in triplicate.

Data analysis method

Biostatistical analyses were performed using Microsoft Excel 2016 and GraphPad Prism version 8. Data were analyzed using one-way ANOVA followed by Tukey's test to determine significant differences. Statistically significant differences were determined at $p < 0.05$. Experimental data from multi-factor experiments and optimization were processed using Design-Expert 11 software.

RESULTS AND DISCUSSION

Optimization of parameters in the soapnut fermentation process

Results of multi-factor experiments

Based on the experimental design matrix (Table 2) and the objective function data, the data were processed using Design-Expert 11 software. ANOVA analysis was conducted to evaluate the significance of the regression coefficients and the adequacy of the experimental models corresponding to the objective function (saponin/TSS), summarized in Table 3.

Table 3. Regression analysis results of the objective function.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	4050.75	9	450.08	567.11	< 0.0001
A - Inoculum size	396.21	1	396.21	499.23	< 0.0001
B - temperature	28.13	1	28.13	35.44	0.0006
C - time	1504.26	1	1504.26	1895.39	< 0.0001
AB	21.16	1	21.16	26.66	0.0013
AC	13.32	1	13.32	16.79	0.0046
BC	17.64	1	17.64	22.23	0.0022
A^2	159.12	1	159.12	200.50	< 0.0001
B^2	187.46	1	187.46	236.20	< 0.0001
C^2	1576.11	1	1576.11	1985.92	< 0.0001
Residual	5.56	7	0.7936		
Lack of fit	3.53	3	1.18	2.32	0.2170
Pure error	2.03	4	0.5070		
Total Correlation	4056.3	16			
Coefficient of determination R^2	0.9986				
Adjusted R^2 (R^2_{Adj})	0.9969				
Predicted R^2 (R^2_{Pred})	0.9853				

Effect of investigated factors on the objective function

The regression analysis yielded an F-value of 567.11 ($p < 0.05$), indicating that the model is statistically significant with a confidence level of 99.99% ($p = 0.0001$). The significance of the regression coefficients was validated using standard F, where $p < 0.05$ confirms that all regression coefficients are statistically significant. Moreover, the lack-of-fit F-value of 2.32 (p

> 0.05) indicates that the lack of fit of the model is not statistically significant. The coefficient of determination (R^2) of the model was 0.9986, indicating that 99.86% of the variation in the objective function could be explained by the independent variables. Meanwhile, the predicted R^2 (R^2_{Pred} = 0.9853) was in good agreement with the adjusted R^2 (R^2_{Adj} = 0.9969).

Regression equation of the objective function based on coded variables (1a) and actual variables (1b).

$$Y_1 = 84.72 + 7.04A + 1.88B + 13.71C - 2.30AB + 1.82AC + 2.10BC - 6.15A^2 - 6.67B^2 - 19.35\,C^2 \quad (1a)$$

$$Y_1 = -250.70471 + 4.54961X_1 + 16.21471X_2 + 21.17282X_3 - 0.065714X_1X_2 + 0.086905X_1X_3 + 0.140000X_2X_3 - 0.125459X_1^2 - 0.266900\,X_2^2 - 2.14972X_3^2 \quad (1b)$$

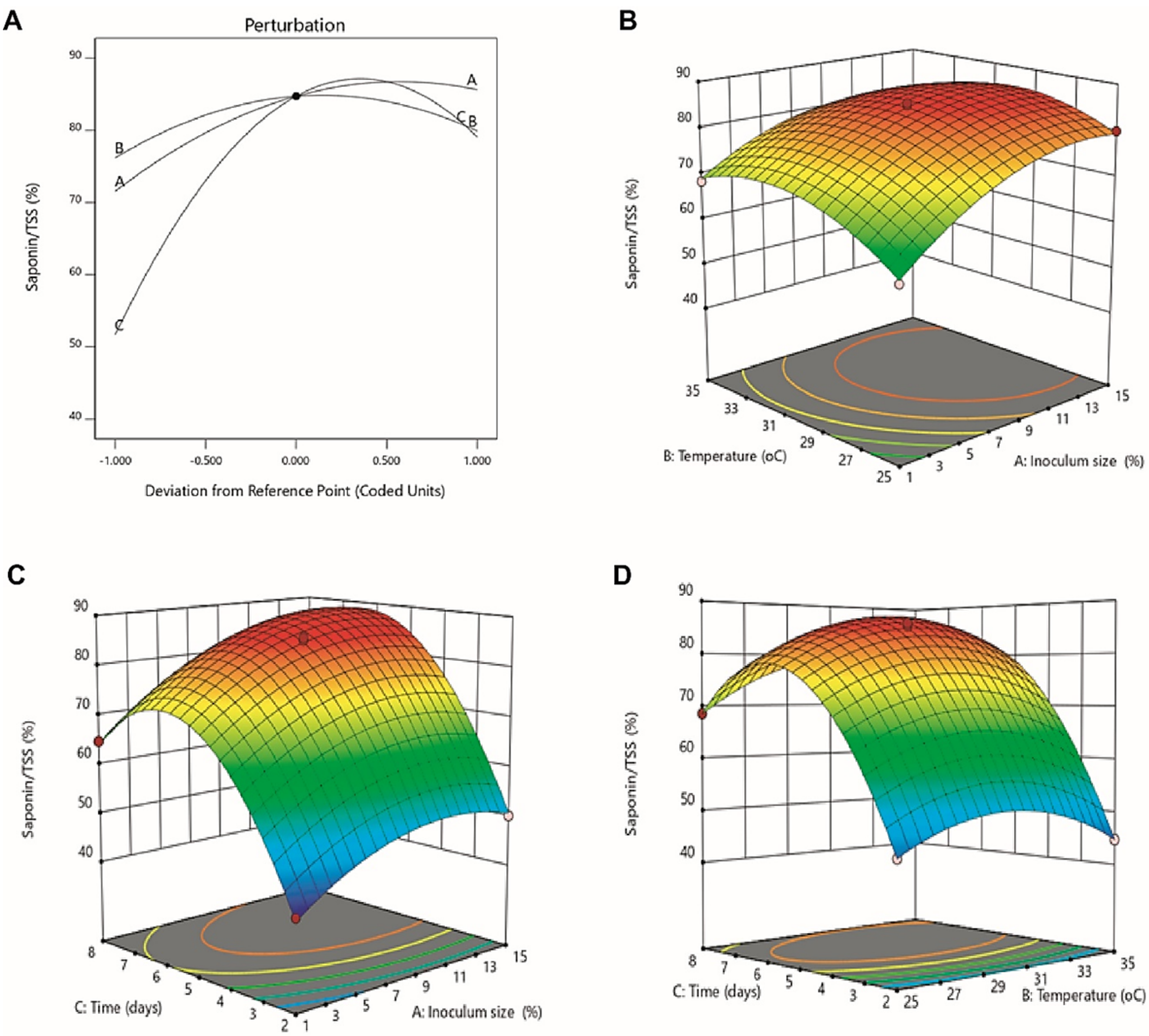

Figure 1. 2D and 3D representations of the relationships between experimental factors and the objective function. (A) Deviation from reference point, (B) Inoculum size – Temperature, (C) Inoculum size – Time, and (D) Temperature – Time.

Observation of Figure 1 and the regression coefficients (1b) indicates that fermentation time is the most influential factor, followed by fermentation temperature and inoculum size, on the saponin/TSS content within the variation range of 68.4-85.2%. As fermentation time increases from 2 to 5 days, the saponin/TSS content shows a sharp increase and then continues to rise more gradually between 6 and 8 days. Meanwhile, when the inoculum size varies from 5% to 10%, the saponin/TSS content increases steadily. However, beyond a certain inoculum threshold, nutrient metabolism in the medium becomes insufficient to sustain yeast growth, leading to a decrease in saponin content. On the other hand, at fermentation temperatures between 30–33ºC, the saponin/TSS content tends to increase markedly. Temperature is one of the critical factors affecting the fermentation process. According to Heng *et al.* (2015), fermentation of soapnuts using *S. cerevisiae* at 30ºC for 4 days increased the saponin/TSS content from 45.71% to 75.50%.

Optimization of soapnut fermentation conditions

RSM is an experimental design technique aimed at minimizing the number of experiments while ensuring reliable results. It has been widely applied in optimizing fermentation processes such as wine and beer fermentation (Phan and Nguyen, 2019; Bărbulescu *et al.*, 2021). The optimal parameters predicted by RSM for soapnut fermentation were as follows: inoculum size of 9.08%, fermentation temperature of 32.3ºC, and fermentation time of 4.9 days. The expected objective of the experimental model is presented in Figure 2.

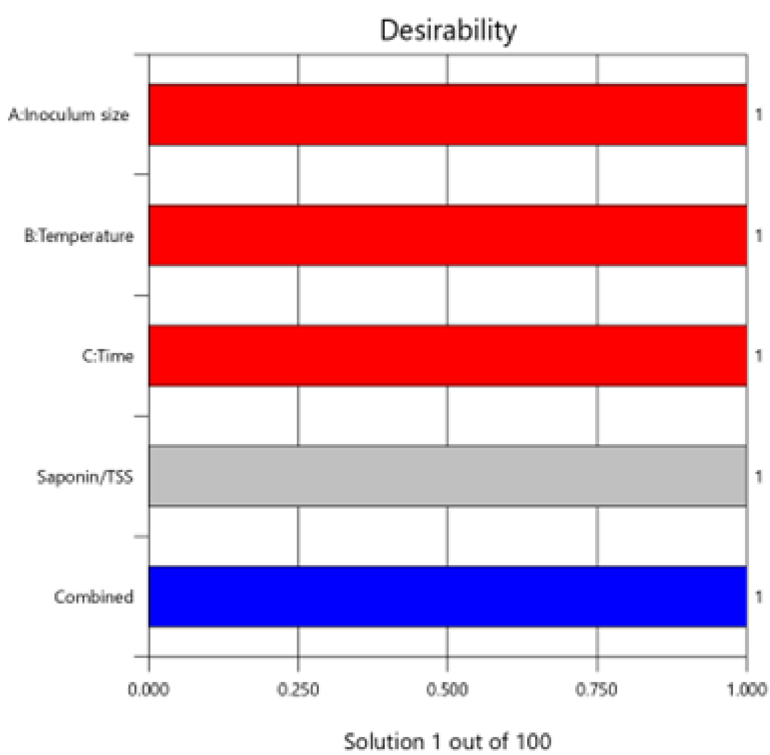

Figure 2. Desirability plot showing the optimal levels of inoculum size, temperature, and fermentation time for maximizing saponin/TSS content using response surface methodology.

With the three experimental factors, namely inoculum size, fermentation temperature, and fermentation time, the expected responses reached 100%, corresponding to the objective function fully meeting the expected targets. This implies that the overall goal of the experimental model satisfies 100% of the anticipated response. Experiments were conducted to validate the compatibility of values within the optimal parameter region obtained from RSM. The experiments were repeated three times under the conditions of 9.0% inoculum size, 32ºC fermentation temperature, and 5 days fermentation time. The experimental results, averaged for the objective function, reached 85.1 ± 1.14%. Statistical tests showed that the experimental results were consistent with the theoretical values predicted by the model, and the deviation was not statistically significant ($p > 0.05$). This confirms the model's accuracy, indicating that it can be used to predict the properties of the fermented soapnut product. Another study also demonstrated that the optimal temperature for fermentation using *S. cerevisiae* is 28-32ºC, with a fermentation time of 4 days, which significantly improves the color and aroma of the fermented soapnut product (Le *et al.,* 2023).

Antibacterial activity of fermented soapnut extract

The fermented soapnut extract obtained under optimal conditions (inoculum size: 9%, fermentation temperature: 32ºC, fermentation time: 5 days), which had a saponin/TSS content of 85.1%, exhibited strong antibacterial activity against all three bacterial strains: *E. coli* VTCC12272, *B. cereus* SH46 and *S. aureus* VTCC12275, with mean inhibition zone diameters of 20.5, 15.3, and 28.2 mm, respectively (Figure 3). Saponin extracts from soapnuts have also been shown to possess antimicrobial activity against various pathogenic microorganisms, including bacteria and yeasts (Wei *et al.*, 2021; Li *et al.,* 2021). Moreover, this study prepared a soapnut extract using a similar process to the fermentation but without *S. cerevisiae* (the 9% yeast inoculum was replaced with sucrose solution). This extract exhibited antibacterial activity against the three bacterial strains (*E. coli* VTCC12272, *B. cereus* SH46, and *S. aureus* VTCC12275), with mean inhibition zone diameters of 8.8, 4.2, and 14.0 mm, respectively (Figure 3). In addition, this study also revealed slight differences in ethanol concentration and pH between the fermented extract (with *S. cerevisiae*) and the non-fermented extract (without *S. cerevisiae*). In the non-fermented extract, ethanol was absent and the pH was 4.85, whereas the fermented extract contained 5.5% ethanol with a pH of 4.4. The ethanol concentration and pH of the non-fermented extract were then adjusted to match those of the fermented extract, and its antibacterial activity was evaluated. The results showed no significant difference in the inhibition zone diameters before and after adjustment ($p > 0.05$) (Figure 3). These findings indicate that minor changes in ethanol concentration and pH do not affect antibacterial activity. The antibacterial activity results indicated that the fermented soapnut extract with *S. cerevisiae* exhibited stronger antibacterial effects compared to the soapnut extract without *S. cerevisiae*, and this difference was statistically significant ($p < 0.05$) (Figure 3). These findings demonstrate that fermented soapnut extract has potent antibacterial properties, and the use of *S. cerevisiae* during fermentation enhances the antibacterial activity of the extract compared to the non-fermented soapnut extract.

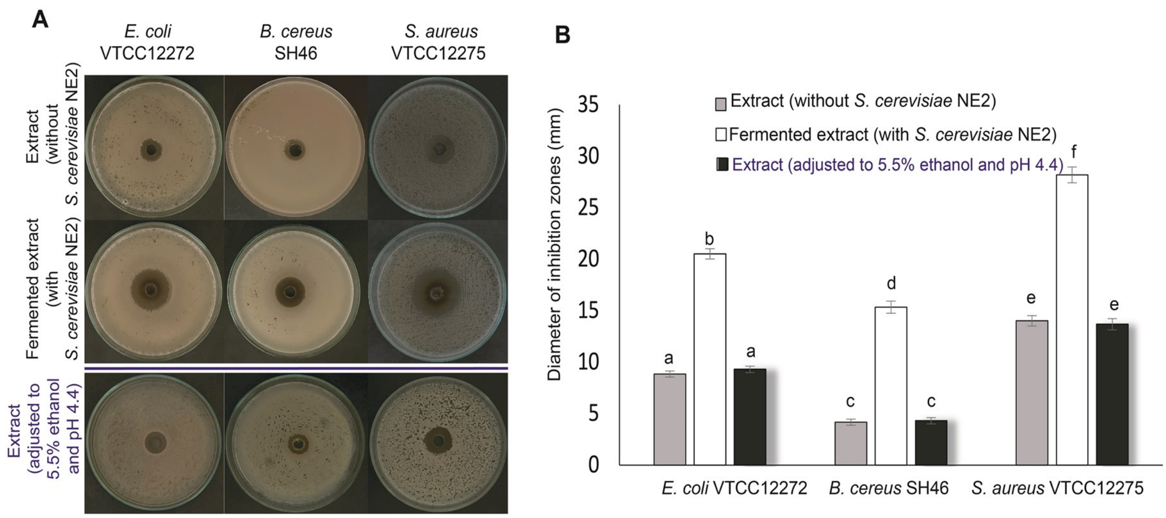

Figure 3. Antibacterial activity of fermented extract and soapnut extract. (A) Inhibition zones on agar plates, and (B) diameter of inhibition zones. Data were analyzed by comparing the three types of extracts for each bacterial strain using one-way ANOVA followed by Tukey's test to determine significant differences ($p < 0.05$). Different letters on the bars indicate statistically significant differences ($p < 0.05$).

CONCLUSION

This study successfully optimized several parameters in the fermentation process of soapnut using *S. cerevisiae* through RSM to produce a fermented extract with a high saponin/TSS content. The constructed response surface model, incorporating fermentation parameters such as inoculum size, temperature, and fermentation time, demonstrated high accuracy, with experimental results consistent with theoretical predictions. Experimental findings showed that at an inoculum size of 9%, fermentation temperature of 32ºC, and fermentation time of 5 days, the fermented extract achieved a high saponin/TSS content of 85.1%. Additionally, the fermented soapnut extract exhibited strong antibacterial activity against *E. coli, B. cereus* and *S. aureus*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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