Article

Optimization of Solid-State Fermentation Process of *Radix Ranunculi ternate* Using Response Surface Method and Addressing Its Antioxidant and Hypoglycemic Activity

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Abstract: Objective: The aim of this study was to optimize the fermentation process of *Radix Ranunculi ternate* via microbial fermentation and analyze the changes in the contents of the main components, the antioxidant and hypoglycemic capacities of the extract before and after fermentation. Methods: The solid-state fermentation process was optimized using single-factor tests and the response surface method, with the yield of the alcohol extract of *R. ternate* as an evaluation index. Results: The best fermentation process was optimized using solid-state endophytic fungus fermentation technology as follows: strain addition ratio of *Chaetomium globosum/Fusarium equiseti* = 1:1, fermentation for 5 d, sieve size of 40 mesh, liquid/material ratio of 0.8:1 mL·g^{-1}, fermentation temperature of 31 °C, and inoculation amount of 7.5%. Under the optimized conditions, the contents of the water-soluble extract and total polysaccharides decreased by 12.71% and 12.95%, respectively. In the fermentation, the contents of the ethanol-soluble extract, flavonoids, saponins, polyphenols, organic acids, and total amino acids of the fermented *R. ternate* increased by 19.77%, 57.14%, 79.67%, 14.29%, 17.63%, and 3.82%, respectively. The scavenging rate for DPPH, ABTS$^+$, and ·OH free radicals and inhibitory rate for α-amylase of the fermented *R. ternate* also increased by 19.02%, 14.17%, 7.53%, and 34.54%, respectively, compared with the unfermented *R. ternate*. Conclusions: Solid-state fermentation opens new avenues for the development and application of *R. ternate* as a natural antioxidant and hypoglycemic food.

Keywords: *Radix Ranunculus ternate*; mixed-strains fermentation; ethanol extract; antioxidant capacity; hypoglycemic activity

1. Introduction

Radix Ranunculus ternate is a dry root of *Ranunculus ternatus* Thunb. Wild *R. ternate* was found in Xinyang, Henan Province, in the 1950s; it has been recorded in the *Chinese Pharmacopoeia* since 1977 and has been listed as one of the three types of Chinese medicinal materials with national priority [1]. *R. ternate* can be used to resolve phlegm and disperse nodules as well as for detoxification and detumescence [2]. Because of its significant anti-tuberculosis and anti-tumor effects, *R. ternate* is mainly used clinically to treat pulmonary tuberculosis, lymph node tuberculosis, lung cancer, liver cancer, lymph cancer, chronic hepatitis B, chronic pharyngitis, and other diseases [3–5]. Research has found that the main chemical components of *R. ternate* are polysaccharides, fatty acids, saponins, alcohols, esters, volatile oils, alkaloids, flavonoids, and trace elements; and its active anti-tumor in-gredients are mainly saponins, polysaccharides, fatty acids, alcohols, and some compounds

in esters [6–8]. The alcohol-soluble extract of *R. ternate* is an important control item in the quality standards of the 2020 edition of the *Chinese Pharmacopoeia*.

Fermentation technology is widely used in the food, chemical, and pharmaceutical industries. Fermentation is also a main process in traditional Chinese medicine. In the process of fermentation, microorganisms may consume some components of Chinese medicine as carbon and nitrogen sources or increase the effective components through secondary metabolism, thus changing the contents of ingredients of Chinese medicine [9]. Based on the changes in the ingredients, their efficacy will change, which can be multifaceted, such as improving efficacy, reducing toxicity, expanding indications, and generating new therapeutic effects. The optimal solid-state fermentation process of *Quercus liaotungensis* by *Bacillus subtilis* was studied in terms of the inoculation amount, the soybean meal addition amount, the fermentation temperature, and the ratio of material to water, and the fermented Quercus liaotungensis extract markedly increased the tannin content and antioxidant effect [10]. However, it has been found that many important biochemical reactions cannot be realized when relying on a single microorganism and can only be achieved with a co-culture of more than two microorganisms, that is, mixed-strains fermentation [11]. Strain selection is an important research topic in the microbial fermentation of traditional Chinese medicine. Endophytes are an important component of a plant's internal environment, as they have formed stable and mutually beneficial relationships with plants during long-term coevolution. Endophytes not only produce special bioactive substances themselves but also induce and promote the synthesis or accumulation of effective ingredients in medicinal materials [12–15]. The fungi isolated from *Changium smyrnioides* were co-cultured with *C. smyrnioides* floating culture cells, and the results showed that the cell growth and polysaccharide content increased by 31.86% and 38.01%, respectively [16]. In the early stage of this study, an endophytic fungus *Fusarium equiseti* was obtained from *R. ternate*, which produced extracellular polysaccharides with strong antioxidant activity [17]. Therefore, endophytic fungi are important potential raw materials for new medicinal active ingredients.

In this experiment, two endophytic fungi of *R. ternate* were used for the solid fermentation of *R. ternate* (FRT), while unfermented *R. ternate* (RT) was used as a control. The optimal fermentation time, sieve size, liquid-to-material ratio, fermentation temperature, and inoculation amount of the strains were selected by optimizing the fermentation process. Additionally, the antioxidant and hypoglycemic activities of the extracts before and after fermentation were compared, which provided an important theoretical basis for the application of *R. ternate*.

2. Materials and Methods

2.1. Reagents and Strains

R. ternate was collected in the Huaibin area of Xinyang, Henan Province. *Chaetomium globosum* (R12) and *Fusarium equiseti* (S4) were isolated from the wild *R. ternate* in the laboratory. DPPH (2,2-Diphenyl-1-Picrylhydrazyl) and ABTS$^+$ (2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). An amino acid reference substance was purchased from Sigma-Aldrich Biotechnology Co., Ltd. (Shanghai, China).

2.2. Preparation of Strain Seed Liquid

The PDA (potato dextrose agar) medium was prepared from 200 g of potato, 20 g of glucose, 15 g of agar, and 1000 mL of distilled water via sterilization at 121 °C for 15 min. Mycelium was inoculated onto the PDA slope medium using the streak plate method on a sterile super-clean bench and cultured in an incubator at 28 °C for 7–10 days. The activated strains were added to the liquid PDA medium for expansion and cultured on a table at 30 °C and 220 r·min^{-1} for 24 h.

The suspensions of endophytic fungi were vacuumized and filtered, and the mycelium was collected. The obtained mycelium was dried to a constant weight at 60 °C using an electric blast drying oven (DHG-9240A-T, Binglin Electronic Technology Co., Shanghai,

China), and the growth curve was drawn via continuous weighing for 7 days. The strain seed liquid was selected after the expansion culture reached the growth index stage, and the concentration of the endophytic fungi was adjusted to 10^8 CFU/mL.

2.3. Screening of Fermentation Strains

R. ternate was washed, dried, crushed, and passed through a 40-mesh sieve. Then, 15 g of *R. ternate* powder was accurately weighed as the substrate for fermenting strains, poured into a 250 mL triangle bottle, and sterilized at 120 °C for 15 min.

Single-strain fermentation: After sterilizing the fermentation substrate, inoculations of 2% activated R12 and S4 were added separately and treated at 28 °C and 150 rpm for 0–7 days. The fermentation products were taken out at the same time every day and dried at 40 °C for 1 h using an electric blast drying oven (DHG-9240A-T, Binglin Electronic Technology Co., Shanghai, China).

Mixed-strains fermentation: Different proportions of the mixed suspensions of endophytic fungi (R12/S4 = 1:1; R12/S4 = 1:2; R12/S4 = 2:1; R12/S4 = 1:3; and R12/S4 = 3:1) were added to the sterilized fermentation substrate. The total inoculation amount was 2%, and the fermentation culture was carried out at 28 °C and 150 rpm for 0–7 days. The fermentation products were taken out at the same time every day and dried at 40 °C for 1 h.

The yield of the ethanol-soluble extract (ESE) of *R. ternate* was calculated according to the following equation [1]:

$$\text{ESE yield (\%)} = \frac{M}{N} \times 100\%$$

where M is the mass of the alcohol-soluble extract (g) and N is the mass of the *R. ternate* powder (g).

2.4. Optimization of Solid-State Fermentation Process of R. ternate

2.4.1. Single-Factor Experiments

Firstly, 15 g of *R. ternate* was accurately weighed, sieved with 20-mesh, 40-mesh, 60-mesh, 80-mesh, and 100-mesh sieves, and added to 250 mL triangle bottles with liquid/material ratios of 0.5:1 mL·g^{-1}, 0.75:1 mL·g^{-1}, 1:1 mL·g^{-1}, 1.25:1 mL·g^{-1}, and 1.5:1 mL·g^{-1}. The additional inoculation amounts were 2%, 4%, 6%, 8%, and 10%, and the fermentation temperatures were 26 °C, 28 °C, 30 °C, 32 °C, and 34 °C, respectively. Five days later, the fermentation products were sterilized again and dried at 40 °C. Using the ESE yield from the fermentation products as the evaluation index, the effects of the sieve size, liquid/material ratio, inoculation amount, and fermentation temperature on the solid-state fermentation of *R. ternate* were investigated using single-factor tests.

2.4.2. Response Surface Method Experiments

On the basis of the single-factor tests, Box–Behnken tests were designed with four factors and three levels, including sieve size (A), liquid-to-material ratio (B), fermentation temperature (C), and inoculation amount (D) (Table 1). The Box–Behnken experiment (BBD) test designs were generated using the Design-Expert 10.0.3 software.

Table 1. Factors and levels.

Levels	Factors			
	A: Sieve Size (mesh)	B: Liquid-to-Material Ratio (mL·g^{-1})	C: Fermentation Temperature (°C)	D: Inoculation Amount (%)
−1	20	0.5:1	28	4
0	40	0.75:1	30	6
1	60	1:1	32	8

2.5. Determination of Active Constituent Content

2.5.1. Total WSE and ESE Content

The water-soluble extract (WSE) was determined according to the extraction method of the Chinese Pharmacopoeia 2020 (General Rule 2201), in which the water used was distilled water. The distilled water was replaced with dilute ethanol as the solvent to determine the ESE.

2.5.2. Total Polysaccharides Content

An amount of 5 g of dried *R. ternate* powder was placed in a triangle bottle. Then, 200 mL of distilled water was added and extracted using ultrasound at 80 °C for 2 h. Extracts were filtered through a filter paper under vacuum (SHB-IIIG, Zhengzhou Century Shuangke Co., Zhengzhou, China), and concentrated to 20 mL at 55 °C in a rotary evaporator (RE5298A, Yarong, Shanghai, China). The filtrate was concentrated and then mixed with 4 volumes of absolute ethanol at 4 °C for 24 h. Subsequently, the precipitates were obtained via centrifugation (DL-5-B, Anting, Shanghai, China) at 4000 rpm for 10 min. The precipitates were dried to give the desired polysaccharides. The crude extract was dissolved in water and diluted to 100 mL in a volumetric flask. According to the phenol–sulfuric acid method [18], D-glucose was used as a standard reference material. The extraction was dissolved in water and fixed to 50 mL in a volumetric flask. The liquid to be tested, 5% phenol, and concentrated sulfuric acid were mixed at a ratio of 1 mL:2.5 mL:10 mL and bathed at 100 °C for 15 min. After cooling, the absorbance of the solution at 490 nm was used to calculate the glucose concentration in the solution according to the standard curve using the linear equation $A_{490} = 0.0596C_g - 0.0027$ ($R^2 = 0.9998$), where A_{490} is the absorbance at 490 nm and C_g is the glucose concentration (mg/mL).

2.5.3. Total Flavonoids Content

An amount of 5 g of dried *R. ternate* powder was placed in a triangle bottle with 200 mL of 60% aqueous ethanol. The flavonoids, saponins, and phenolic acids in the *R. ternate* were extracted using ultrasound at 80 °C for 2 h. According to the $Al(NO_3)_3$-$NaNO_2$-NaOH method, rutin was used as a standard reference material for the determination of total flavonoids. The total flavonoid contents in the fermentation products were determined according to a previously described method, with some modifications [19]. The liquid to be tested, 60% aqueous ethanol, and 5% sodium nitrite were mixed at a ratio of 1 mL:5 mL:1 mL. Then, 1 mL of 10% aluminum nitrate was added 6 min later and diluted to 25 mL with 60% aqueous ethanol. The absorbance of the solution by a spectrophotometer (TU-1901, Puxi General Instrument Co., Beijing, China) at 274 nm was used to calculate the rutin concentration in the solution according to the standard curve using the linear equation $A_{274} = 0.002C_g - 0.0142$ ($R^2 = 0.9996$), where A_{274} is the absorbance at 274 nm and C_g is the rutin concentration (mg/mL).

2.5.4. Total Saponins Content

According to the vanillin–glacial acetic acid method, oleanolic acid was used as a standard reference material for the determination of total saponins. The measurement of total saponins was performed using a modified version of a previously described method [20]. The liquid to be tested was diluted to 10 mL with methanol and then evaporated to dryness at 55 °C using a rotary evaporator. Then, 0.2 mL of a 5% vanillin–glacial acetic acid solution and 0.8 mL of perchloric acid were added, followed by heating in a bath at 60 °C for 10 min and quickly cooling to room temperature using ice crystals. Next, 5 mL of anhydrous acetic acid was added to terminate the reaction. The absorbance of the solution by a spectrophotometer (TU-1901, Puxi General Instrument Co., Beijing, China) at 544 nm was used to calculate the oleanolic acid concentration in the solution according to the standard curve using the linear equation $A_{544} = 17.015C_g - 0.2084$ ($R^2 = 0.9997$), where A_{544} is the absorbance at 544 nm and C_g is the oleanolic acid concentration (mg/mL).

2.5.5. Total Phenols Content

Referring to the Folin–Ciocalteu reagent (FCR) method, gallic acid was used as a standard reference material for the determination of total phenols. A previously described protocol was followed, with some modifications [21]. The liquid to be tested and FCR were mixed at a ratio of 1 mL:5 mL and incubated at room temperature for 5 min. Then, 4 mL of a 6% sodium carbonate solution was added and allowed to stand in the dark at room temperature for 30 min. The absorbance of the solution using a spectrophotometer (TU-1901, Puxi General Instrument Co., Beijing, China) at 725 nm was used to calculate the gallic acid concentration in the solution according to the standard curve using the linear equation $A_{725} = 0.1012C_g + 0.0453$ ($R^2 = 0.9997$), where A_{725} is the absorbance at 725 nm and C_g is the gallic acid concentration (mg/mL).

2.5.6. Total Organic Acid Content

An amount of 5.0 g of *R. ternate* powder was precisely weighed in a triangle bottle with 200 mL of an 80% ethanol solution and extracted using ultrasound for 30 min. The organic acid content was determined according to the potentiometric titration method of the Chinese Pharmacopoeia 2020 (General Rule 0701).

Then, 20 mL of the test solution was accurately measured and placed in a 200 mL beaker. Subsequently, 20 mL of a 0.05 mol·L^{-1} NaOH solution was added. This mixture was placed in a constant-temperature magnetic stirrer and titrated with a 0.05 mol·L^{-1} HCl standard solution. The amount of HCl consumed was recorded. Then, 20 mL of a 0.05 mol·L^{-1} NaOH solution was used as a blank control and titrated with a 0.05 mol·L^{-1} HCl standard solution. The total organic acid content in the sample was calculated via potentiometric titration using the following formula [22] (calculated as citric acid ($C_6H_8O_7$) per dry sample, where 1 mL of HCl titrant is equivalent to 3.302 mg of citric acid):

$$\text{Total organic acid content (mg/g)} = \frac{T \times (V_0 - V)}{W}$$

where V is the volume of the HCl standard solution consumed by the titrating sample (mL), V_0 is the volume of HCl consumed by the blank (mL), and W is the sample mass (g).

2.5.7. Amino Acid Analysis

An amount of 0.1 g of *R. ternate* powder was weighed and placed in a hydrolysis tube, 4 mL of a 6 mol·L^{-1} HCl solution containing 0.1% w/v phenol was added, nitrogen was blown with a nitrogen blower for 15 min, and the tube was tightly closed and hydrolyzed at 110 °C for 24 h. After hydrolysis, the tubes were cracked open and filtered. Then, 0.5 mL of hydrolysate was moved to a 10 mL EP tube and blown dry with nitrogen. The residue was dissolved in 6 mL of diluent (0.2 mol·L^{-1} sodium citrate, pH 2.2, containing 0.1% w/v phenol) and passed through a 0.22 μm filter column. The mixed amino acid standard solution and sample determination solution were injected into the amino acid analyzer, respectively. The composition of the free amino acids in the filtrate was directly measured using an L-8900 ion-exchange HPLC system (HITACHI, Tokyo, Japan), utilizing postcolumn ninhydrin derivatization and detected at wavelength 570 nm (440 nm for proline) [23]. The amino acids content in the sample was calculated from the areas of standards obtained from the integrator using the external standard method.

2.6. Antioxidant Activity

The scavenging ability of DPPH was estimated by a method according to [10] with some modifications. The ESE of *R. ternate* was diluted with ethanol to 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, and 0.625 mg/mL of the test solution, respectively. The DPPH radical solution was produced by gently mixing 100 μL of 0.1 mmol·L^{-1} DPPH solution and 100 μL of the sample solution with various concentrations. This was allowed to stand in the dark for 30 min, and absorbance was measured using a spectrophotometer (TU-1901, Puxi General Instrument Co., Beijing, China) at 517 nm. Ascorbic acid (V$_C$)

solutions with different mass concentrations were used as positive controls. The free radical scavenging activity was calculated as follows:

$$\text{DPPH scavenging rate (\%)} = (1 - \frac{A_1 - A_2}{A_0}) \times 100\%$$

where A_1 is the absorbance of DPPH and the sample, A_2 is the absorbance of the reagent blank without DPPH, and A_0 is the absorbance of the solvent control.

The scavenging ability of ABTS$^+$ was estimated by a method according to [19] with some modifications. The ABTS$^+$ radical solution was produced by adding an equal volume of 7 mmol·L^{-1} ABTS$^+$ solution and 1.4 mmol·L^{-1} potassium persulfate solution. This was allowed to stand in the dark for 24 h. Then, the ABTS$^+$ solution was diluted with ethanol to an absorbance of 0.7 ± 0.01 at 734 nm, and the ABTS$^+$ working solution was obtained. An amount of 100 µL ABTS$^+$ solution was added with 50 µL of the sample solution with various concentrations, and the mixture was allowed to stand at 30 °C for 6 min in the dark. The absorbance was measured using a spectrophotometer (TU-1901, Puxi General Instrument Co., Beijing, China) at 734 nm. V$_C$ solutions with different mass concentrations were used as positive controls. The free radical scavenging activity was calculated as follows:

$$\text{ABTS}^+ \text{ scavenging rate (\%)} = (1 - \frac{A_1 - A_2}{A_0}) \times 100\%$$

where A_1 is the absorbance of ABTS$^+$ and the sample, A_2 is the absorbance of the reagent blank without ABTS$^+$, and A_0 is the absorbance of the solvent control.

The scavenging ability of ·OH was measured according to [18] with some modifications. An amount of 1 mL of the sample solution with different concentrations was mixed with 1 mL of 9 mmol·L^{-1} FeSO$_4$ solution and 1 mL of 9 mmol·L^{-1} salicylic acid solution, and this was kept at room temperature for 10 min. Subsequently, 1 mL of 8.8 mmol·L^{-1} H$_2$O$_2$ solution was added to start the Fenton reaction and the mixture was heated in a bath at 37 °C for 30 min. The absorbance was measured using a spectrophotometer (TU-1901, Puxi General Instrument Co., Beijing, China) at 510 nm. V$_C$ solutions with different mass concentrations were used as positive controls. The free radical scavenging activity was calculated as follows:

$$\text{·OH scavenging rate (\%)} = (1 - \frac{A_1 - A_2}{A_0}) \times 100\%$$

where A_1 is the absorbance of H$_2$O$_2$ and the sample, A_2 is the absorbance of the reagent blank without H$_2$O$_2$, and A_0 is the absorbance of the solvent control.

2.7. Hypoglycemic Activity

The inhibiting ability of α-amylase was measured referring to [24] with some modifications. The ESE of *R. ternate* was diluted with 0.1 mol·L^{-1} sodium phosphate buffer (PBS, pH 5.6) to 64 mg/mL, 32 mg/mL, 16 mg/mL, 8 mg/mL, 4 mg/mL, and 2 mg/mL of the test solution, respectively. An amount of 200 µL of the sample solution with various concentrations was mixed with 100 µL of PBS containing α-amylase (1.5 mg·mL^{-1}), and this was kept at 37 °C for 10 min. Then, 200 µL of 1% starch solution was added to the mixture at 40 °C for 30 min. The reaction was terminated with 400 µL of DNS reagent (1% dinitrosalicylic acid, 12% potassium sodium tartrate), and the mixture was heated in boiling water for 10 min and cooled to room temperature. Next, the reaction mixture was diluted by adding 2 mL of distilled water and the absorbance was measured at 540 nm. Acarbose solution with different mass concentrations was used as the positive control. The α-amylase inhibiting activity was calculated as follows:

$$\alpha\text{-amylase inhibitory rate (\%)} = (1 - \frac{A_1 - A_2}{A_3 - A_4}) \times 100\%$$

where A_1 is the absorbance of the α-amylase, starch solution, DNS reagent, and sample, A_2 is the absorbance of the reagent blank without α-amylase, A_3 is the absorbance of the α-amylase, starch solution, and DNS reagent, and A_4 is the absorbance of the solvent control.

2.8. Model Verification and Statistical Analysis

The Design-Expert 10.0.3 software was used for the regression analysis and the optimization. The model adequacy was tested by calculating the coefficient of determination (R^2_{Pred}, R^2, and R^2_{adj}). The analysis of variance utilized an ANOVA procedure. All experiments were repeated thrice. The data were analyzed using SPSS 20.0, and the results are expressed as means $\pm$ standard deviations. The significance level was $p < 0.05$.

3. Results and Analysis

3.1. The Growth Curves of R12 and S4

Growth curves were measured using the mycelium dry weight method. The growth curves of R12 and S4 are shown in Figure 1. It can be seen that the mycelium dry weight of R12 did not change much over the first two days. On the third day, the mycelium dry weight increased rapidly. After the fourth day, the mycelium dry weight reached its maximum value and remained stable, so it was determined that R12 entered the rapid growth phase on the fourth day. According to the growth curve of S4, it was found that over days 1–4, the strain grew slowly, and the dry weight changed little. After the fifth day, the mycelium dry weight reached its maximum value and remained stable, so it was determined that S4 entered the rapid growth stage on the fifth day. Therefore, for the experiment, we selected R12 and S4 cultured for 4 and 5 days, respectively, as the fermentation seed liquids.
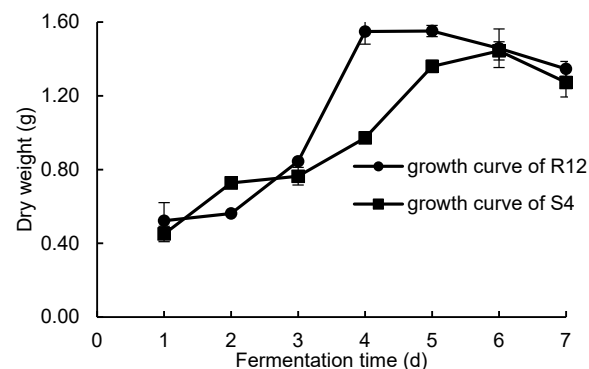

Figure 1. Growth curves of R12 and S4. R12: *Chaetomium globosum*; S4: *Fusarium equiseti*.

3.2. Screening of Fermentative Strains

The ESE yields from *R. ternate* from the single fermentation of two endophytic fungi are shown in Figure 2. The content of the alcohol-soluble extract first increased during the single fermentation of R12, reached its maximum of 42.22% on the third day, and decreased after the third day. The yield of the ESE was lowest on the seventh day. This may have been due to the use of the liquid PDA medium during fermentation. No other nutrients were added, and the fermentation products were consumed by the endophytic fungi as carbon and nitrogen sources during the late fermentation period. When S4 was used alone as a fermentation strain, the yield of the ESE of *R. ternate* first increased, reached its maximum value of 40.66% on the fourth day, and then decreased. Comparing the two groups of data, it was found that the ESE yield and efficiency of R12 as a fermentation strain were higher than those of S4. Therefore, in the fermentation of single strains, R12 should be selected as the superior fermentation strain, and its fermentation time is 3 d.

The ESE yields from *R. ternate* from the fermentation of two endophytic fungi (R12 and S4) are shown in Figure 3. The yields of the ESE of *R. ternate* increased with an increase in the fermentation time with different addition ratios. They generally reached their maximum values on the fourth or fifth day and gradually decreased on the sixth and

seventh days. When R12/S4 = 1:1, the maximum ESE yield was 53.52% on the fifth day. Therefore, compared with the single fermentation, it was found that mixed fermentation could effectively increase the content of the ESE of *R. ternate*.
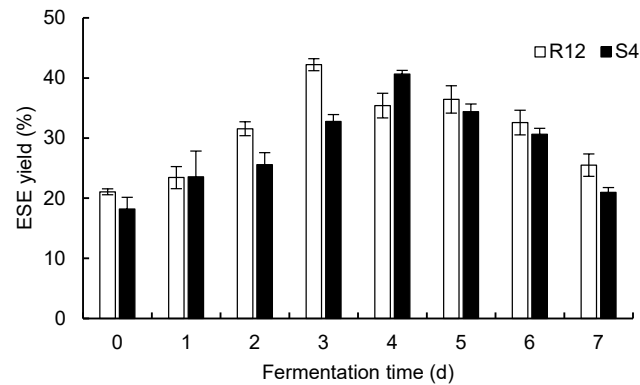

Figure 2. Comparison of the yields of ESE from single strains. ESE: ethanol-soluble extract; R12: *Chaetomium globosum*; S4: *Fusarium equiseti*.
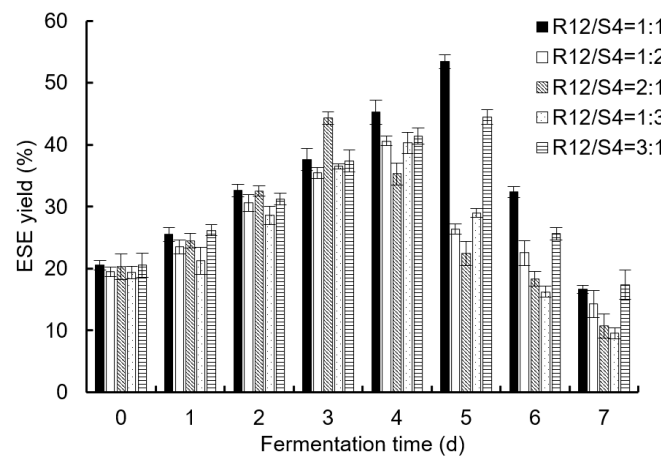

Figure 3. Comparison of the yields of ESE using mixed strains. ESE: ethanol-soluble extract; R12: *Chaetomium globosum*; S4: *Fusarium equiseti*.

3.3. Single-Factor Experiment of Fermentation Process Optimization

3.3.1. Effects of Sieve Size on the Yield of ESE

As shown in Figure 4, the ESE yield of *R. ternate* increased between samples sieved with a 20-mesh sieve and samples sieved with a 40-mesh sieve and decreased between samples sieved with a 40-mesh sieve and samples sieved with a 100-mesh sieve, so the optimal sieve size was 40. Therefore, the sieve size was lower, the aperture of the sieve was larger, and the coarse impurities in the powder could not be completely removed. When the sieve size was larger, the aperture of the sieve was smaller and the effective ingredients may have been lost, so the ESE yield decreased.
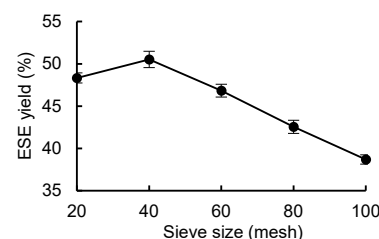

Figure 4. Effect of sieve size on the yield of ESE. ESE: ethanol-soluble extract.

3.3.2. Effect of Liquid-to-Material Ratio on the Yield of ESE

As shown in Figure 5, the ESE yield of *R. ternate* increased between the liquid-to-material ratios of 0.5:1 and 0.75:1 and decreased between the liquid-to-material ratios of 0.75:1 and 1.5:1. The optimal liquid-to-material ratio was 0.75:1. Therefore, it was hypothesized that when the liquid-to-material ratio was low, the amount of water in the substrate was relatively low, which resulted in the slowing down of the metabolism of the mycelium, and that when the liquid-to-material ratio was too large, the oxygen capacity in the substrate was low, which led to the incomplete growth of the mycelium, which in turn affected the yield of the ESE.
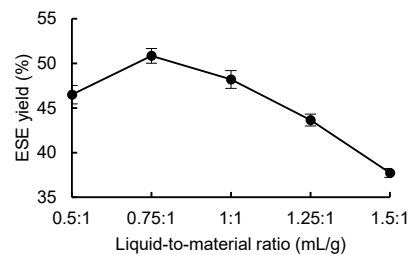
Figure 5. Effect of liquid-to-material ratio on yield of ESE. ESE: ethanol-soluble extract.

3.3.3. Effect of Fermentation Temperature on the Yield of ESE

As shown in Figure 6, the yield of the ESE of *R. ternate* increased continuously when the fermentation temperature was between 26 °C and 30 °C, while it decreased continuously when the fermentation temperature was between 30 °C and 34 °C. Therefore, the optimal fermentation temperature was 30 °C, and it is speculated that the optimal growth temperature of the two endophytic fungi is about 30 °C. When the temperature is lower than the optimal growth temperature, mycelial metabolism is inhibited, and when the temperature is higher than the optimal growth temperature, mycelial metabolic capacity decreases, which affects the yield of the ESE in fermentation.
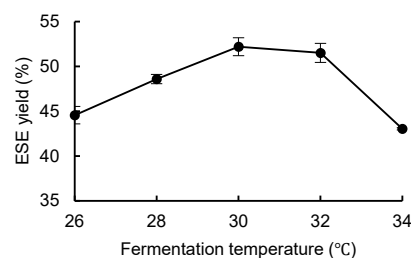
Figure 6. Effect of fermentation temperature on the yield of ESE. ESE: ethanol-soluble extract.

3.3.4. Effect of Inoculation Amount on the Yield of ESE

As shown in Figure 7, between the inoculation amounts of 2% and 6%, the *R. ternate* ESE yield increased, while between the inoculation amounts of 6% and 10%, the ESE yield decreased. Therefore, the optimal inoculation amount was 6%. It is assumed that when the inoculation amount is low, the mycelium in contact with the raw material is less able to maximize the fermentation activity of the microorganisms. With an increase in the inoculation amount, to a certain extent, the raw material in the carbon and nitrogen source is not able to meet the growth of the mycelium, resulting in mycelium metabolism inhibition. Therefore, the ESE yield showed a trend of increasing and then decreasing.
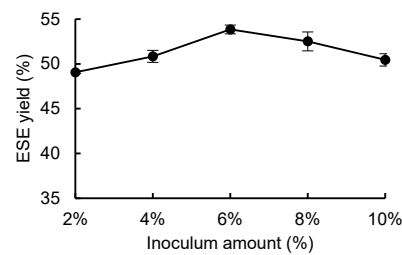
Figure 7. Effect of inoculation amount on the yield of ESE. ESE: ethanol-soluble extract.

3.4. Response Surface Method for Fermentation Process Optimization

On the basis of a one-way test, the sieve size (A), liquid-to-material ratio (B), fermentation temperature (C), and inoculation amount (D) were used as the investigating factors; the yield of the alcoholic leachate of the fermentation product (Y) was used as the response value; and a BBD using response surface methodology (RSM) was designed to optimize the fermentation process of *R. ternate*. The design scheme and the resultant data are shown in Table 2. A multiple quadratic regression model equation was obtained, which demonstrated the relationship between the response variables and the measured variables:
$$Y = -1017.29 + 0.88A + 35.96B + 65.96C + 11.2D - 0.19AB + 0.04AC - 0.096AD + 2.56BC + 4.06BD - 0.1CD - 0.01A^2 - 81.81B^2 - 1.13C^2 - 0.58D^2.$$

Table 2. Box–Behnken design and results of tests.

Run	A: Sieve Size (Mesh)	B: Liquid-to-Material Ratio (mL·g^{-1})	C: Fermentation Temperature (°C)	D: Inoculation Amount (%)	ESE Yield (%)
1	20	0.75:1	30	4	41.22
2	40	0.5:1	32	6	46.73
3	40	0.75:1	30	6	56.7
4	40	0.5:1	30	4	47.26
5	20	0.75:1	30	8	52.92
6	40	0.75:1	28	8	46.76
7	60	0.75:1	32	6	48.22
8	40	0.75:1	32	8	49.36
9	60	0.75:1	28	6	42.37
10	20	0.75:1	32	6	45.62
11	40	0.5:1	30	8	44.25
12	20	0.5:1	30	6	39.38
13	40	0.75:1	28	4	46.05
14	40	0.75:1	30	6	55.11
15	60	0.75:1	30	4	49.68
16	60	0.75:1	30	8	46.04
17	40	0.75:1	30	6	56.5
18	20	0.75:1	28	6	45.53
19	40	1:1	28	6	43.02
20	60	1:1	30	6	46.37
21	40	0.5:1	28	6	44.21
22	40	0.75:1	30	6	54.34
23	40	1:1	30	8	53.83
24	40	1:1	32	6	50.65
25	40	0.75:1	32	4	50.27
26	60	0.5:1	30	6	45.36
27	40	1:1	30	4	48.72
28	20	1:1	30	6	44.24
29	40	0.75:1	30	6	55.15

Note. ESE: ethanol-soluble extract.

According to the variance analysis of the model (Table 3), the B (liquid-to-material ratio) and C (fermentation temperature) in the primary term had an extremely significant

impact on the response value ($p < 0.01$), the AD (sieve size number and inoculation amount) in the interaction term had an extremely significant impact on the response value ($p < 0.01$), the BD (liquid-to-material ratio and inoculation amount) in the interaction term had a significant impact on the response value ($p < 0.05$), and the other terms were not significant ($p > 0.05$). In the square term, A^2, B^2, C^2, and D^2 all had significant effects on the response value ($p < 0.01$). The fitting model obtained from the experimental data had an F-value of F = 14.74 ($p < 0.0001$), indicating that the model fit the data well. The misfit term ($p = 0.1204 > 0.05$) indicated that the misfit was not significant, and the experimental value was in good agreement with the predicted value. The correlation coefficient ($R^2 = 0.9365$), the adjusted determination coefficient ($R^2_{Adj} = 0.8729$), and the coefficient of variation (C.V. = 3.48%) of the model indicated that the linear relationship between the independent variables and the response values of the model was significant and appropriate to predict the ESE yield of *R. ternate* under different fermentation conditions.

Table 3. Analysis of variance.

Parameter	Sum of Squares	Df	Mean Square	F-Value	*p*-Value	Significance
Model	577.77	14	41.27	14.74	<0.0001	**
A	6.95	1	6.95	2.48	0.1376	
B	32.14	1	32.14	11.48	0.0044	**
C	43.74	1	43.74	15.62	0.0014	**
D	8.27	1	8.27	2.95	0.1078	
AB	3.71	1	3.71	1.32	0.2693	
AC	8.29	1	8.29	2.96	0.1073	
AD	58.83	1	58.83	21.01	0.0004	**
BC	6.53	1	6.53	2.33	0.1491	
BD	16.48	1	16.48	5.89	0.0294	*
CD	0.66	1	0.66	0.23	0.6358	
A^2	233.29	1	233.29	83.31	<0.0001	**
B^2	169.60	1	169.60	60.56	<0.0001	**
C^2	132.35	1	132.35	47.26	<0.0001	**
D^2	34.94	1	34.94	12.48	0.0033	**
Residual	39.20	14	2.80			
Lack of fit	35.16	10	3.52	3.48	0.1204	
Pure error	4.04	4	1.01			
Cor total	616.97	28				

Note. **: highly significant ($p < 0.01$); *: significant ($p < 0.05$). A, B, C, and D are sieve size, liquid-to-material ratio, fermentation temperature, and inoculation amount, respectively; A^2, B^2, C^2, and D^2 are secondary factors of A, B, C, and D, respectively; AB, AC, AD, BC, BD, and CD are interaction factors.

Figure 8a–f illustrate the effects of A, B, C, and D on the ESE yield of *R. ternate*. The steeper and more skewed response surface plots and the denser and closer elliptical contour lines indicate that the interaction of the factor has a more significant effect on the yield of the ESE [25].

After fitting using Design-Expert 10.0.3, according to the obtained desirability values (desirability 1.000), the optimal solid-state fermentation process was determined as follows: the sieve size is 36.932 mesh, the liquid-to-material ratio is 0.796:1 mL·g^{-1}, the fermentation temperature is 30.737 °C, and the inoculation amount is 7.532%. Therefore, the yield of the ESE is predicted to be 55.452%. Considering the actual operation, the sieve size was 40 mesh, the liquid-to-material ratio was 0.8:1 mL·g^{-1}, the fermentation temperature was 31 °C, and the inoculation amount was 7.5%. According to the above conditions, three extractions were carried out, and the ESE yield was 55.36%.
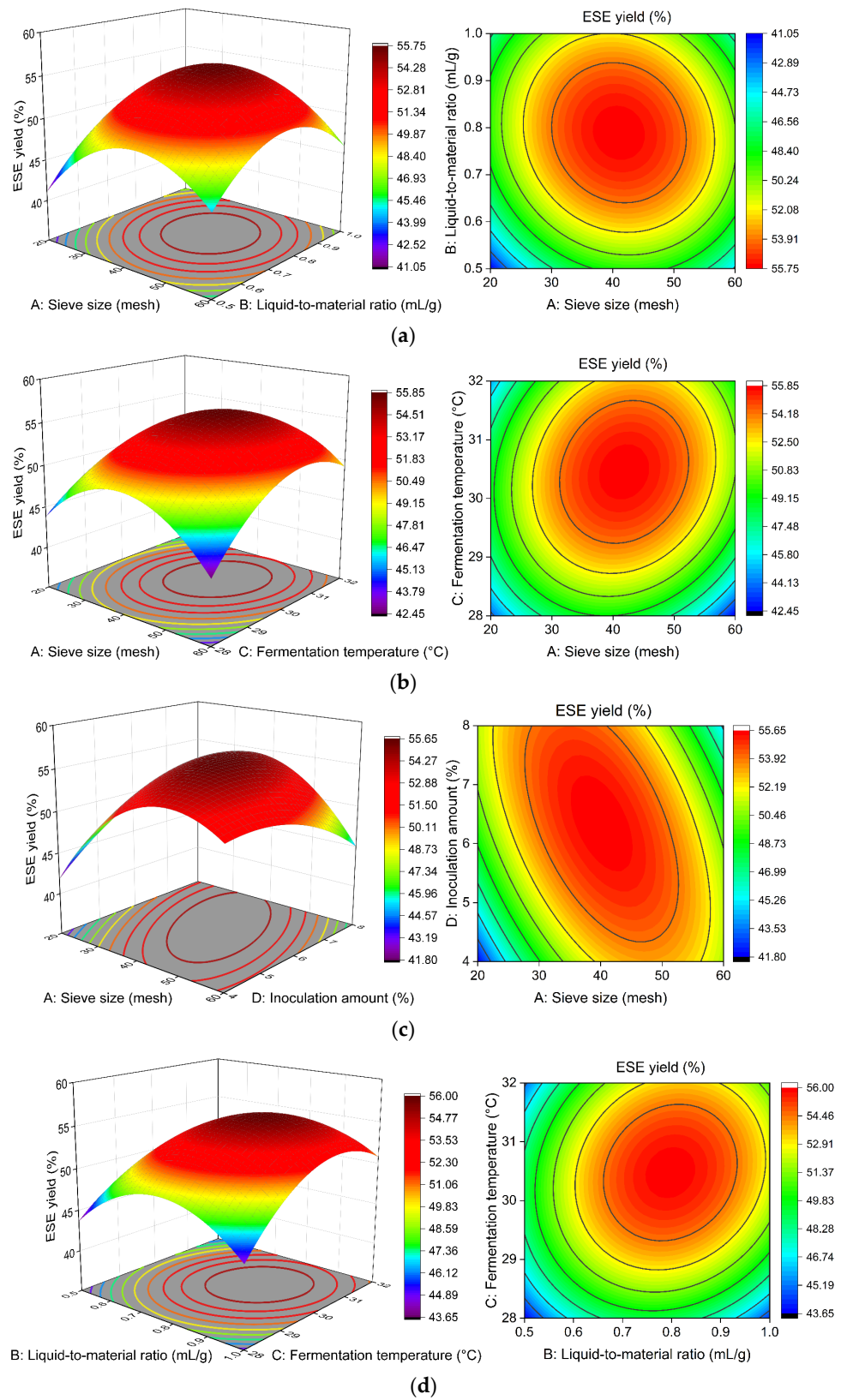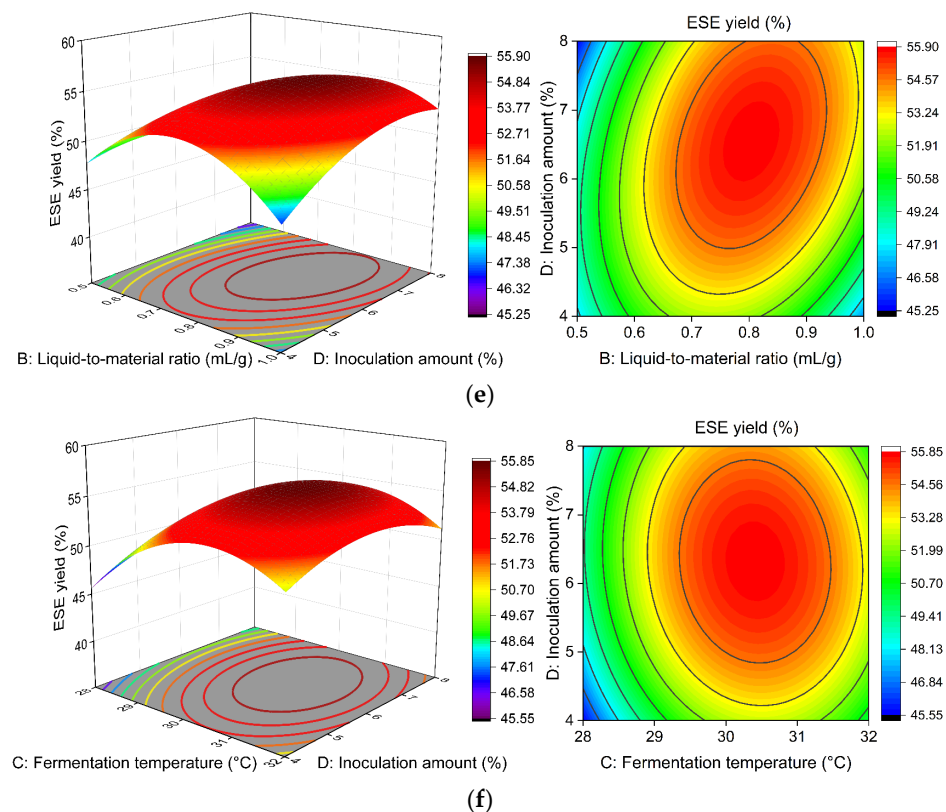
(**a**)

(**b**)

(**c**)

(**d**)

Figure 8. *Cont.*

(**e**)

(**f**)

Figure 8. Response surface plots and contour lines. (**a**) The effects of sieve size and liquid-to-material ratio on ESE yield. (**b**) The effects of sieve size and fermentation temperature on ESE yield. (**c**) The effects of sieve size and inoculation amount on ESE yield. (**d**) The effects of liquid-to-material ratio and fermentation temperature on ESE yield. (**e**) The effects of liquid-to-material ratio and inoculation amount on ESE yield. (**f**) The effects of fermentation temperature and inoculation amount on ESE yield. ESE: ethanol-soluble extract.

3.5. Changes in the Contents of Active Ingredients before and after Fermentation

After the solid-state fermentation of *R. ternate* by endophytic fungus (R12/S4 = 1:1) for 5 d, the contents of the WSE, ESE, polysaccharides, flavonoids, saponins, and polyphenols of *R. ternate* powder were significantly different. As shown in Table 4, the WSE and polysaccharide contents of *R. ternate* showed decreases of 12.71% and 12.95% ($p < 0.05$), respectively, after fermentation, while the content of the ESE, flavonoids, saponins, polyphenols, organic acids, and amino acids increased by 19.77%, 57.14%, 79.67%, 14.29%, 17.63%, and 3.82% ($p < 0.05$), respectively, after fermentation. Except for alanine, lysine, and proline, the contents of the other 14 amino acids increased, of which 12 increased by more than 60%, while the contents of medicinal amino acids (Glu, Asp, Arg, Gly, Phe, Tyr, Met, Leu, and Lys) and essential amino acids (Thr, Val, Met, Phe, Lys, Ile, and Leu) increased by 66.39%, 74.9%, 74.9%, and 75.9% ($p < 0.05$), respectively.

Table 4. Content changes in effective constituents before and after fermentation.

Active Ingredient	RT	FRT
WSE (%)	69.38 ± 1.02 [a]	60.56 ± 0.79 [b]
ESE (%)	65.41 ± 1.38 [b]	78.34 ± 1.22 [a]
Polysaccharides (mg·g^{-1})	60.37 ± 2.18 [a]	52.55 ± 1.25 [b]
Flavonoids (mg·g^{-1})	2.17 ± 0.12 [b]	3.41 ± 0.02 [a]
Saponins (mg·g^{-1})	4.28 ± 0.25 [b]	7.69 ± 0.28 [a]
Polyphenols (mg·g^{-1})	0.33 ± 0.02 [b]	0.40 ± 0.02 [a]
Organic acids (mg·g^{-1})	17.40 ± 0.48 [b]	20.47 ± 0.54 [a]
Total amino acids (mg·g^{-1})	186.48 ± 1.95 [b]	193.61 ± 0.76 [a]

Note. In the same row, values with different small letter superscripts mean significant difference ($p < 0.05$), while those with the same or no letter superscripts mean no significant difference ($p > 0.05$). RT: *R. ternate*; FRT: unfermented *R. ternate*; WSE: water-soluble extract; ESE: ethanol-soluble extract.

3.6. Changes in Antioxidant Capacity of ESE before and after Fermentation

In Figure 9a–c, it can be seen that within a certain range, the ESEs of unfermented and fermented *R. ternate* both had a certain scavenging capacity for DPPH radicals, ABTS$^+$ radicals, and ·OH radicals, and the scavenging rate tended to increase with increasing concentrations of the alcoholic extracts, but none of them had less antioxidant capacity than V$_C$. When the ESE concentration was 20 mg·mL^{-1}, the DPPH, ABTS$^+$, and ·OH free radicals' scavenging rate of FRT reached 87.2%, 81.3%, and 70.31%, respectively, which were higher than the scavenging rate of RT of 68.18%, 67.13%, and 62.78%, respectively. As can be seen in Table 5, the IC$_{50}$ values (the half maximal inhibitory concentration) of DPPH free radicals, ABTS$^+$ free radicals, and ·OH free radicals scavenged by the ESE of fermented *R. ternate* were lower than those unfermented, indicating that the antioxidant capacity of the ESE of *R. ternate* significantly increased after fermentation.
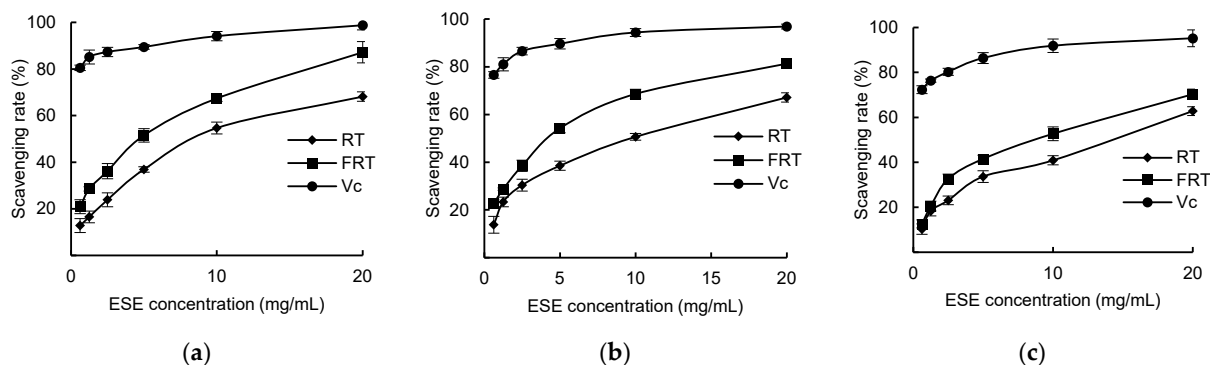

(**a**)　　　　　(**b**)　　　　　(**c**)

Figure 9. Changes in scavenging rate of DPPH free radicals (**a**), ABTS$^+$ free radicals (**b**), and hydroxyl free radicals (**c**) before and after fermentation. ESE: ethanol-soluble extract; RT: *R. ternate*; FRT: unfermented *R. ternate*; V$_C$: ascorbic acid; DPPH: 2,2-Diphenyl-1-Picrylhydrazyl; ABTS$^+$: 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate).

Table 5. IC$_{50}$ values of ESEs determined using different methods before and after fermentation.

IC$_{50}$	RT	FRT	V$_C$	Acarbose
DPPH (mg·mL^{-1})	8.96 ± 0.09 [a]	4.83 ± 0.1 [b]	0.72 ± 0.06 [c]	
ABTS$^+$ (mg·mL^{-1})	7.37 ± 0.20 [a]	3.27 ± 0.18 [b]	0.04 ± 0.01 [c]	
·OH (mg·mL^{-1})	12.46 ± 0.38 [a]	7.33 ± 0.10 [b]	0.11 ± 0.03 [c]	
α-amylase (mg·mL^{-1})	37.70 ± 1.58 [a]	23.79 ± 0.55 [b]		2.24 ± 0.55 [c]

Note. In the same row, values with different small letter superscripts mean significant difference ($p < 0.05$), while those with the same or no letter superscripts mean no significant difference ($p > 0.05$). IC$_{50}$: half maximal inhibitory concentration; RT: *R. ternate*; FRT: unfermented *R. ternate*; V$_C$: ascorbic acid; DPPH: 2,2-Diphenyl-1-Picrylhydrazyl; ABTS$^+$: 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate); ·OH: hydroxyl free radicals.

3.7. Changes in Hypoglycemic Activity of ESE before and after Fermentation

In Figure 10, with the increase in the sample concentration, the inhibitory effect of ESE of unfermented and fermented *R. ternate* on α-amylase gradually increases, indicating a dose–response relationship between the extract and inhibitory activity. When the ESE concentration was 64 mg·mL^{-1}, the inhibitory rate of FRT reached 74.68%, which was higher than the inhibition rate of RT of 40.14%. Moreover, as can be seen in Table 5, the IC$_{50}$ value of the fermented *R. ternate* was lower than the IC$_{50}$ value of the unfermented, indicating that the ESE of *R. ternate* has an enhanced inhibitory effect on α-amylase after fermentation.
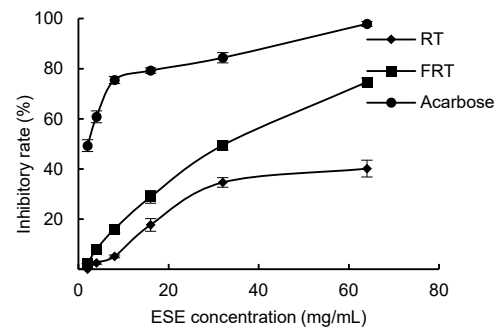

Figure 10. Changes in inhibition rate of α-amylase before and after fermentation. ESE: ethanol-soluble extract; RT: *R. ternate*; FRT: unfermented *R. ternate*.

4. Discussion

Microbial fermentation is an important method for the processing of traditional Chinese medicine. During the microbial fermentation process of traditional Chinese medicine, due to the metabolic effects of microorganisms and the presence of active enzymes, biological transformation reactions occur to modify and transform the active ingredients of traditional Chinese medicine. The transformed products often lead to changes in certain types of Chinese medicine components, increases or decreases in their efficacy, or changes in their properties [26,27].

According to the different types of fermentation, microbial fermentation methods can be divided into solid-state fermentation and liquid fermentation. The process of solid fermentation and the equipment it requires are simple, and it is currently mainly used for the direct fermentation of Chinese medicinal materials. Su found that the polyphenol content in *Acanthopanax senticosus* was increased by 40% after solid-state fermentation [28]. Strain selection is an important research topic in the microbial fermentation of Chinese medicine. Endophytes and host plants use the same or similar pathways for effective ingredient synthesis. Compared with single-strain fermentation, there is usually a good interaction between different mixed strains, which improves the yield of microbial fermentation [29]. During the fermentation process, microorganisms can produce proteases, cellulases, and esterases, promoting the release of active ingredients and increasing the contents of effective ingredients. Therefore, in this study, the fermentation strain and fermentation endpoint were first determined based on the yields of the ethanol-soluble extracts. It was found that the best ratio of strains was 1:1 for *Chaetomium globosum* and *Fusarium equiseti*. After 5 days of fermentation, the yield of the ethanol-soluble extracts was the highest, indicating that the fermentation endpoint was reached. Then, using the yield of the ethanol-soluble extracts as the indicator, single-factor tests and RSM experiments were used to determine the solid-state fermentation conditions of endophytic fungus in *R. ternate*.

Microorganisms can only achieve efficient fermentation at appropriate fermentation temperatures, and high or low growth environment temperatures may cause activity to decrease or be lost, thereby affecting the contents of the fermentation products. The suitable temperature range for microbial growth is 16–30 °C, with a maximum temperature range of 37–43 °C. Therefore, in this study, a temperature suitable for microbial growth was selected to optimize the fermentation temperature. When the temperature is low,

microbial growth is slow and the metabolism is affected. When the temperature is high, it is easy to cause microbial deactivation, leading to a decreasing trend in the contents of fermentation products. Due to the different optimal growth temperatures of different strains, significant differences in fermentation temperatures were reported in a previous study, but temperatures of 28–32 °C had a good fermentation effect [10,30,31]. The research results indicate that the fermentation effect is optimal when the fermentation temperature reaches 31 °C.

The moisture content in the fermentation substrate has an important impact on solid fermentation. When the liquid-to-material ratio is low, there is insufficient water, and the mycelium grows slowly. When the liquid-to-material ratio is too high, it results in poor breathability and heat transfer, which are not conducive to the growth of strains [28]. Due to the use of aerobic endophytic fungi in this study, the results showed that the highest yield of the ESE was achieved when the liquid-to-material ratio was 0.8:1 mL·g^{-1}. The inoculation amount also affects the fermentation effect. When the nutrient substrate is constant, at the beginning, the nutrients are sufficient for the growth of the mycelium, and the yield of the ESE has an upward trend. However, as the inoculation amount increases, the nutrients required for mycelial growth are insufficient, which is not conducive to mycelial growth and leads to a downward trend in the yield of the ESE. The research results indicate that the fermentation effect is best when the inoculation amount is 7.5%. Traditional Chinese medicine sieving refers to the process of screening Chinese medicinal materials through a mesh to remove some coarse and difficult-to-crush fibrous components in order to ensure the quality and purity of Chinese medicinal materials. The number of a sieve refers to the pore size of the sieve, which is the number of holes per inch on the sieve. Gao found that the impurity content varied with the use of different sieve sizes on *Typha angustifolia* [32]. If the number of a sieve is too large and the aperture of the sieve is smaller, it may lead to the loss of effective Chinese medicine ingredients, affecting the efficacy of the medicine. If the number of a sieve is too small and the aperture of the sieve is larger, it may prevent the complete removal of impurities from Chinese medicines, which affects their quality. The research results indicate that 40 mesh is the most suitable sieving number for the fermentation of *R. ternate* to improve the ESE yield.

The contents of the active ingredients of the products of FRT by endophytic fungi were determined. The contents of the WSE and polysaccharides decreased by 12.71% and 12.95%, respectively, but the contents of the ESE, flavonoids, saponins, polyphenols, organic acids, and amino acids increased by 19.77%, 57.14%, 79.67%, 14.29%, 17.63%, and 3.82%, respectively, compared with RT. Yin found the polysaccharides contents of 15 different resources of *Schisandra chinensis* were reduced after fermentation, and the reduction rate was 7.00~16.00%. Conversely, the polyphenol content was increased, and the improvement rate was in the range of 6.00~13.00% [33]. After fermenting fresh *Codonopsis pilosula* with yeast solid fermentation, the content of polysaccharides was decreased by 7.56%, and those of flavonoids, saponin, and total amino acids were increased by 70.27%, 128.00%, 69.90%, respectively, along with enhanced scavenging activities on DPPH and ·OH free radicals [34], which is consistent with the research results of this experiment. During the fermentation process, polysaccharides may be decomposed and consumed by the strains as a fermentation substrate, which may lead to a decrease in the polysaccharide content. Polysaccharides are water-soluble extracts, thus leading to a similar decrease in the yield of the WSE of *R. ternate* after fermentation. Meanwhile, the results of the antioxidant and hypoglycemic capacities experiments found that the scavenging rates of FRT for DPPH free radicals, ABTS^{+} free radicals, and ·OH free radicals and the inhibitory rate of FRT for α-amylase increased compared with RT. Type 2 diabetic mellitus (T2DM) is one of the most common endocrine and metabolic diseases. Inhibition α-amylase can delay the digestion of starch in food in the intestinal tract, thus inhibiting the rise of postprandial blood sugar level, which is helpful for the diet treatment of T2DM. Although chemically synthesized drugs have clear therapeutic effects on lowering blood sugar, most drugs have side effects. Medicinal plants and their phytochemicals have a good therapeutic effect on

T2DM [35], which is not only safer, but also helps improve the body's antioxidant system and insulin resistance [36]. Studies have shown that the antioxidant activity of plant extracts is related to the content of flavonoids, saponins, polyphenols, and other substances [37–39]. Polyphenols and flavonoids can regulate the blood sugar level by promoting the synthesis and secretion of insulin, so as to inhibit glucose uptake and transport [40]. Therefore, the increases in the contents of the above substances in the fermented *R. ternate* contribute to the enhancement in the antioxidant and digestive enzyme inhibitory activities of fermentation products produced by endophytic fungi. However, the antioxidant and hypoglycemic mechanism of the ESE of FRT is not yet clear, and the monomer substances of the ESE require further separation and purification. The protective effects of ESE of FRT need further comparative in vivo examination.

5. Conclusions

In this experiment, we used single-factor tests and an RSM to optimize the process for the solid-state fermentation of *R. ternate*: strain addition ratio of *C. globosum*/*F. equiseti* = 1:1, fermentation for 5 d, sieve size of 40 mesh, liquid-to-material ratio of 0.8:1 mL·g^{-1}, fermentation temperature of 31 °C, and inoculation amount of 7.5%. Under optimal conditions, the ethanol-soluble extract, flavonoid, saponin, polyphenol, organic acid, and amino acid contents, and the antioxidant and hypoglycemic activities of the solid-state endophytic fungus fermentation of *R. ternate* significantly increased, and the product quality was significantly improved. This experiment aimed to investigate the effects of endophytes on the fermentation of traditional Chinese medicine, which helped to explore the synergistic symbiotic effects between plants and microorganisms. Furthermore, the findings of antioxidant and digestive enzyme inhibition studies show that the ethanol-soluble extract of fermented *R. ternate* has the potential to lower blood sugar and may be used as food supplements and in medicinal production. This study can provide a theoretical basis for the development of products with auxiliary hypoglycemic effects for *R. ternate*.

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