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Optimization of Fermentation Culture Medium for *Sanghuangporus alpinus* Using Response-Surface Methodology

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Abstract: The newly identified *Sanghuangporus alpinus* species of the Sanghuang mushroom genus has been found to possess significant medical benefits. However, the current artificial cultivation technology has not reached the requisite maturity. The response-surface methodology (RSM) was used to optimize the *Sanghuangporus alpinus* culture medium formulation and evaluate the functional activity of *S. alpinus* exopolysaccharides. First, a single-factor experiment was conducted to screen for optimal carbon and nitrogen sources for *S. alpinus*. Then, using Box–Behnken's central composite design, a response-surface experiment was conducted to determine optimal culture parameters. Finally, the rationality of those parameters was assessed in a shaking flask experiment. The optimal culture parameters, determined through regression analysis, were 20.20 ± 0.17 g/L fructose (carbon source), 7.29 ± 0.10 g/L yeast extract (nitrogen source), and 0.99 ± 0.01 g/L dandelion. With optimization, the *S. alpinus* yield increased to 12.79 ± 1.41 g/L, twice that obtained from the initial culture medium. The *S. alpinus* exopolysaccharide exhibited an excellent antioxidant capacity, with the strongest scavenging effect noted on ABTS free radicals (lowest half-inhibitory concentration: 0.039 mg/mL). Additionally, this exopolysaccharide effectively inhibited various cancer cells, exhibiting the strongest activity against human glioma cells U251 (half-inhibitory concentration: 0.91 mg/mL). The RSM used to optimize the fermentation culture parameters of *S. alpinus* significantly increased the mycelial biomass. The improvement of *Sanghuangporus alpinus* yield through liquid fermentation and optimizing the fermentation medium could fill the existing gap in the cultivation of *Sanghuangporus alpinus*, as well as provide valuable data for the large-scale production of *S. alpinus*.

Keywords: *Sanghuangporus alpinus*; extracellular polysaccharide; response-surface optimization; antioxidant activity

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1. Introduction

Sanghuangporus alpinus is a medicinal fungus belonging to the family Hymenochaetaceae. It usually grows in broad-leaved forests or coniferous forests at high altitudes. It is found in cold climates and is distributed in southwest China [1]. *Sanghuangporus* has various medicinal functions. *S. alpinus* exhibits a good anticancer activity [2], and it also has substantial antioxidant and anti-inflammatory properties [3]. At present, successful cultivation techniques for *Sanghuangporus alpinus* have not been reported. Domestication cultivation of wild *Sanghuangporus alpinus* faces obstacles in fruiting, making it difficult for mass production. At the same time, many studies have shown that the effective substances of Sanghuang mushrooms come more from the mycelium than from fruiting bodies [4]. The mycelium of this fungus is rich in histones and has been proven to exhibit a good sleep-improving activity [5]. The *Sanghuangporus* exopolysaccharide is an extensively

studied active product obtained from the *Sanghuangporus* spp. extract [6–8]. It is one of the important active ingredients of *Sanghuangporus alpinus* [9]. According to research, the *Sanghuangporus* exopolysaccharide exhibits various activities, such as anticancer [10], blood sugar-lowering [11], antioxidant [12], immunity-boosting [13] properties, and has broad research prospects. Studies have shown that exopolysaccharides fight cancer by increasing immune cell activity and apoptosis [14–17].

Culturing in liquid medium is a method used for the large-scale growth of fungal mycelia. The liquid culture medium contains nutrients and trace elements required for fungal growth. Compared with other culture media, after inoculation in liquid culture media, fungal clumps can fully come in contact with the culture medium, which promotes nutrient absorption and enables the rapid release of metabolites. The liquid culturing method is simple to operate, flexible, and convenient in formula adjustment. It is the basis for large-scale fungal production, as well as the collection and extraction of fungal metabolites [18]. Therefore, the formulation was optimized for the liquid culture medium by using the response-surface methodology (RSM), based on one-factor analysis; we implemented the experimental design based on statistics, calculated optimal nutritional conditions, and verified the culture medium formulation. Given that alterations to the medium composition may affect the bioactive components of Sanghuang to some extent, the impact of polysaccharides was elected to assess the most renowned and effective mushroom derivatives as a proxy for its bioactivity [19]. Furthermore, although the relationship between mycelial biomass production and extracellular polysaccharide yield in *Sanghuangporus alpinus* remains unclear, studies on other fungi have indicated a positive correlation [20–22]. After the culturing was complete, the antioxidant and anticancer activities of the extracted exopolysaccharides were evaluated. In this experiment, using the medium suitable for growing *S. alpinus*, the Box–Behnken design and RSM were employed to screen, optimize, and determine the ideal carbon and nitrogen sources, as well as the amount of growth enhancement factors to be added for the large-scale cultivation of *S. alpinus* and the preparation of exopolysaccharides. The findings hold substantial implications for guiding future research and applications.

2. Materials and Methods

2.1. Materials

2.1.1. Strains

S. alpinus specimens with serial numbers MS-10, MS-11, MS-12, and MS-13 were collected from Tibet at an altitude of 2704.13–2729.10 m.

2.1.2. Cells

The human liver cancer cells HepG-2, human breast cancer cells MCF-7, and human prostate cancer cells PC-3 were purchased from Cellverse Bioscience Technology Co., Ltd. (Shanghai, China). The human colon cancer cells HCT-116, human brain glioma cells T98G, and human glioma cells U251 were purchased from Wuhan Pricella Biotechnology Co., Ltd. (Wuhan, China).

2.1.3. Reagents

The PDA-enriched medium was prepared by mixing the juice extracted from 200 g potatoes (peeled), 20 g glucose, 2% agar, 5 g peptone, 1.5 g $MgSO_4$, 2 g KH_2PO_4, and 0.01 g vitamin B1, The mixture was diluted with deionized water calibrated to 1 L and sterilized at 121 °C for 30 min. To prepare the PDB-added rich medium, the juice extracted from 200 g potatoes (peeled), 20 g glucose, 5 g peptone, 1.5 g $MgSO_4$, 2 g KH_2PO_4, and 0.01 g vitamin B1 were mixed. The mixture was diluted with deionized water calibrated to 1 L and sterilized at 121 °C for 30 min.

The cancer cell culture medium was composed of 89% standard cell culture medium (PC-3RPMI 1640 cell culture medium, DMEM cell culture medium, and MEM cell culture

medium), 1% penicillin–streptomycin solution, and 10% fetal bovine serum. This medium was stored at 4 °C in a refrigerator.

To prepare PBS buffer (pH 7.4), 8.5 g NaCl, 0.2 g KCl, 2.85 g $Na_2HPO_4 \cdot 12H_2O$, and 0.27 g KH_2PO_4 were mixed. The mixture was diluted to 1 L with deionized water and sterilized at 121 °C for 30 min.

2.2. Method

2.2.1. Species Cultivation

According to Ding Qiaolian et al.'s method [23], different *S. alpinus* strains were inoculated into PDA-enriched medium using the streak plate method. The culture dish was wrapped with a sealing foil to prevent contamination and cultured at a constant temperature of 25 °C until the mycelia grew to completely cover the surface of the culture dish.

2.2.2. Determination of Biomass

According to Jia Jiao's method [24], each shake culture flask containing 100 mL of culture medium was inoculated with five fungal discs (each size: 7 mm × 2 mm) which were removed using a hole punch. The flasks were then incubated at 25 °C and 160 rpm, protected from light, for 15 days. After 15 days, the liquid in the flask was separated from the mycelia through vacuum filtration, which was repeated three times. The mycelia obtained through filtration were washed with distilled water and dried in an oven at 65 °C to a constant weight. All the mycelial biomass was weighed and recorded.

2.2.3. Impact of Different Carbon Sources

In the PDB-enriched medium, maltose, sucrose, glucose, fructose, xylose, mannitol, mannose, lactose, galactose, etc., were used to replace the original carbon source, with a mass concentration of 20 g/L. No additional carbon source was added to the CK group formula. As a carbon source, 5 *S. alpinus* discs (each size: 7 mm × 2 mm) were inoculated and cultured in a shaker at 25 °C and 150 r/min, protected from light, for 15 days. Changes in the mycelial biomass were measured to investigate the effect of various carbon sources on *S. alpinus* growth.

2.2.4. Impact of Different Sources

In the PDB-enriched medium, yeast extract, peptone, ammonium tartrate, ammonium sulfate, ammonium nitrate, urea, beef extract, etc., were used to replace the original nitrogen source. No additional nitrogen source was added to the CK group formula. Five *S. alpinus* discs (each size: 7 mm × 2 mm) were inoculated and cultured on a shaker in the dark at 25 °C and 150 r/min for 15 days. Changes in the mycelial biomass were measured to study the effect of various nitrogen sources on *S. alpinus* growth.

2.2.5. Effect of Varying Addition Levels of Carbon and Nitrogen Sources

Based on the results of single-factor carbon and nitrogen source screening, we conducted experiments on the optimal conditions for each *S. alpinus* by adding different amounts of carbon and nitrogen sources. Three replicates were maintained for each group. According to the carbon source gradient of 10, 15, 20, 25, and 30 g/L, and the nitrogen source gradient of 2.5, 5, 7.5, 10, and 15 g/L, separate culture media were prepared and 5 pieces of *S. alpinus* discs (each size: 7 mm × 2 mm) were inoculated into all the media. The fungus was cultivated on a shaker in the dark at 25 °C and 150 r/min for 15 days. Changes in the mycelial biomass were measured to determine the impact of added carbon and nitrogen sources on *S. alpinus* growth.

2.2.6. Impact of Growth Factors

In preliminary experiments, the dandelion powder significantly promoted mycelial growth on plates and served as a growth factor for *S. alpinus*. When included in the culture medium, this powder also increased the quality of mycelium in the fermentation broth, as

well as biomass, biological activity, etc., to a certain extent. Therefore, the PDB-enriched culture medium was configured according to the addition gradient of 0, 0.5, 0.75, 1, 1.25, and 1.5 g/L dandelion powder. Then, 5 *S. alpinus* discs (each size 7 mm × 2 mm) were inoculated into the media. The media was incubated on a shaking table at 25 °C and 150 r/min for 15 days. Changes in the mycelial biomass were measured to determine the impact of growth factors on *S. alpinus* growth.

2.2.7. Analysis of Single-Factor Experimental Data

Single-factor experimental data were analyzed using SPSS 25.0 statistical software. The measured data ($x \pm s$) are expressed as the mean ± standard deviation. The *t*-test was employed to analyze the significance of the two groups. $p < 0.05$ indicates a significant difference, while $p < 0.01$ indicates an extremely significant difference.

2.2.8. Optimizing Culture Medium for *S. alpinus* Using the RSM

Based on the results of exploration of optimal carbon and nitrogen sources, the RSM was used to optimize the amounts of various factors added. According to the Box–Behnken design principle, a 3-factor and 3-level response-surface experiment on carbon source addition, nitrogen source addition, and dandelion powder addition was conducted using Design expert 11 software (Stat-ease Co., Minneapolis, MN, USA) to achieve the maximum mycelial biomass. Table 1 presents the factors and levels of the response-surface experiment.

Table 1. Factors and levels of Box–Behnken test of *S. alpinus*.

Level	Factor		
	A: Carbon Source (g/L)	**B: Nitrogen Source (g/L)**	**C: Dandelion Powder (g/L)**
−1	15	5	0.75
0	20	7.5	1
1	25	10	1.25

To validate whether the RSM results were accurate, the predicted formulation was used for verifying the liquid fermentation culture. After fermentation, the mycelium was collected, dried, and weighed, and the results obtained were compared with those obtained under non-optimized conditions.

2.2.9. Extraction of Exopolysaccharides

The fermentation broth was collected and subjected to vacuum filtration. This operation was repeated three times. The filtered fermentation broth was centrifuged at 10,000 rpm for 3 min to remove fine mycelium. Through rotary evaporation, the broth was concentrated to one-fifth of its original volume. Ethanol was added at a 1:4 volume ratio, and the mixture was left to precipitate at 4 °C overnight. The precipitate was collected through centrifugation at 10,000 rpm for 3 min and redissolved in water, and the protein was removed using the Sevega method. The solution was treated with diethyl ether and methanol to remove impurities. This process was repeated multiple times to ensure no protein layer was present. Finally, the crude polysaccharide obtained was dialyzed against running water for 48 h, and the polysaccharide samples were collected through lyophilization and stored at −20 °C for later use.

2.2.10. Antioxidant Activity of Exopolysaccharides

Free radical scavenging activity of ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)): The free radical scavenging activity of ABTS was determined according to Li Yuhong et al.'s method [25] with slight modifications. The exopolysaccharide extract and V_C were diluted to 0.025, 0.5, 0.25, 0.5, 1, 2, and 5 mg/mL concentrations and mixed with the ABTS solution (7 mM). This mixture was added to a 96-well plate at a 1:1 ratio. In

the blank group, distilled water was used instead of the polysaccharide solution. The ABTS solution was also added at a 1:1 ratio. The solutions reacted at 25 °C for 20 min. Absorbance was measured at 734 nm. Using the following formula, the antioxidant performance was calculated based on the calibration curve of the mean value of five repeated groups of exopolysaccharides and V_C:

$$\text{ABTS free radical scavenging rate (\%)} = [1 - (A_x - A_{x0})/A_0] \times 100$$

A_x, A_0, and A_{x0} represent the absorbance values of the experimental, blank, and control groups, respectively.

Free radical scavenging activity of DPPH (2,2-Diphenyl-1-picrylhydrazyl): The free radical scavenging activity of DPPH was determined using Luo Qin et al.'s method [26] with slight modifications. The exopolysaccharide extract and V_C were diluted to 0.025, 0.5, 0.25, 0.5, 1, 2, and 5 mg/mL concentrations and mixed with the DPPH solution (0.2 mM of 80% ethanol solution). This mixture was added to a 96-well plate at a 1:1 ratio. In the blank group, absolute ethanol was used instead of the polysaccharide solution. Similarly, the DPPH solution was also added at a 1:1 ratio. The solutions reacted at 25 °C for 20 min. Absorbance was measured at 517 nm. Using V_C as the positive control group, the DPPH free radical scavenging activity was calculated as follows:

$$\text{DPPH free radical scavenging rate (\%)} = [1 - (A_x - A_{x0})/A_0] \times 100$$

A_x, A_0, and A_{x0} represent the absorbance values of the experimental, blank, and control groups, respectively.

Ferric reducing antioxidant properties: The ferric reducing antioxidant properties were determined using Li Wen et al.'s method [27] with slight modifications. The exopolysaccharide extract was diluted to concentrations of 0.025, 0.5, 0.25, 0.5, 1, 2, and 5 mg/mL. Then, 30 µL polysaccharide solution was added to the extract. This mixture was added to a 96-well plate and treated with 180 µL of the ferric reducing antioxidant properties (FRAP) solution at 25 °C for 20 min. The blank group contained 30 µL deionized water and 180 µL FRAP. The standard curve was configured from a 1:6 mixture of 0.025, 0.1, 0.2, 0.4, 0.8, and 1 mmol/L $FeSO_4$ solution and FRAP solution. At the end of the reaction, absorbance was measured at 593 nm. Each reaction was repeated five times, and the average value was calculated using the formula given below:

$$\text{FRAP value} = A_x - A_0 - A_{x0}$$

A_x, A_0, and A_{x0} represent the absorbance values of the experimental, blank, and control groups, respectively.

The experimental data of antioxidant activity were subjected to a one-way analysis of variance and the LSD test by using SPSS 25.0 statistical software (IBM, Armonk, NY, USA). $p < 0.05$ indicates a significant difference, while $p < 0.01$ indicates an extremely significant difference.

2.2.11. Demonstration of Anticancer Activity

Anticancer activity was determined according to Liu Chengyi's method [28], with slight modifications. The drug administration experiment consisted of the experimental, blank, and control groups. The cancer cells were inoculated into a 96-well plate at 200 µL volume. To decelerate the evaporation of the cell culture medium, PBS buffer was added to the outer ring. The prepared 96-well plate was cultured at 37 °C in a 5% CO_2 incubator for 24 h. After the adherent growth of the cells was evident, the culture medium was removed. In the experimental group, exopolysaccharides extracted from fermentation broth were added to the cell culture medium at 0.025, 0.05, 0.25, 0.5, 1, 2, and 5 mg/mL concentrations, thereby making the volume 200 µL in each well, which was repeated 5 times. In the blank group, the cells were not plated, but only the cell culture medium was added. The control

group included both the cell culture medium and cells in a 96-well plate. The plate was cultured at 37 °C in a 5% CO_2 incubator for 24 h.

Before the experiment, an MTT solution was prepared with PBS buffer at a concentration of 5 mg/mL, the solution filtered, protected from light, and stored at 4 °C in the refrigerator for later use. After 24 h of growth in the 96-well plate containing the drug, the cell culture medium in each well was discarded. Then, 10 µL MTT solution and 90 µL cell culture medium were added to each well and incubated for 4 h. The culture medium in each well was carefully discarded and 150 µL DMSO was added. The mixture was shaken and mixed for 15 min to completely dissolve the formazan, and the absorbance value of each well at 490 nm was measured using the microplate reader.

2.2.12. Data Processing and Analysis

Data were processed using SPSS 25.0 statistical software (IBM, Armonk, NY, USA), and different groups were compared using ANOVA. The significance level was set at $p < 0.05$ for significant differences and $p < 0.01$ for highly significant differences.

3. Results

3.1. Impact of Carbon Source on Growth

Based on the PDB medium, maltose, sucrose, glucose, fructose, xylose, mannitol, mannose, lactose, galactose, etc., were used as additional carbon sources. Figure 1 presents the growth situation after 15 days of culture.

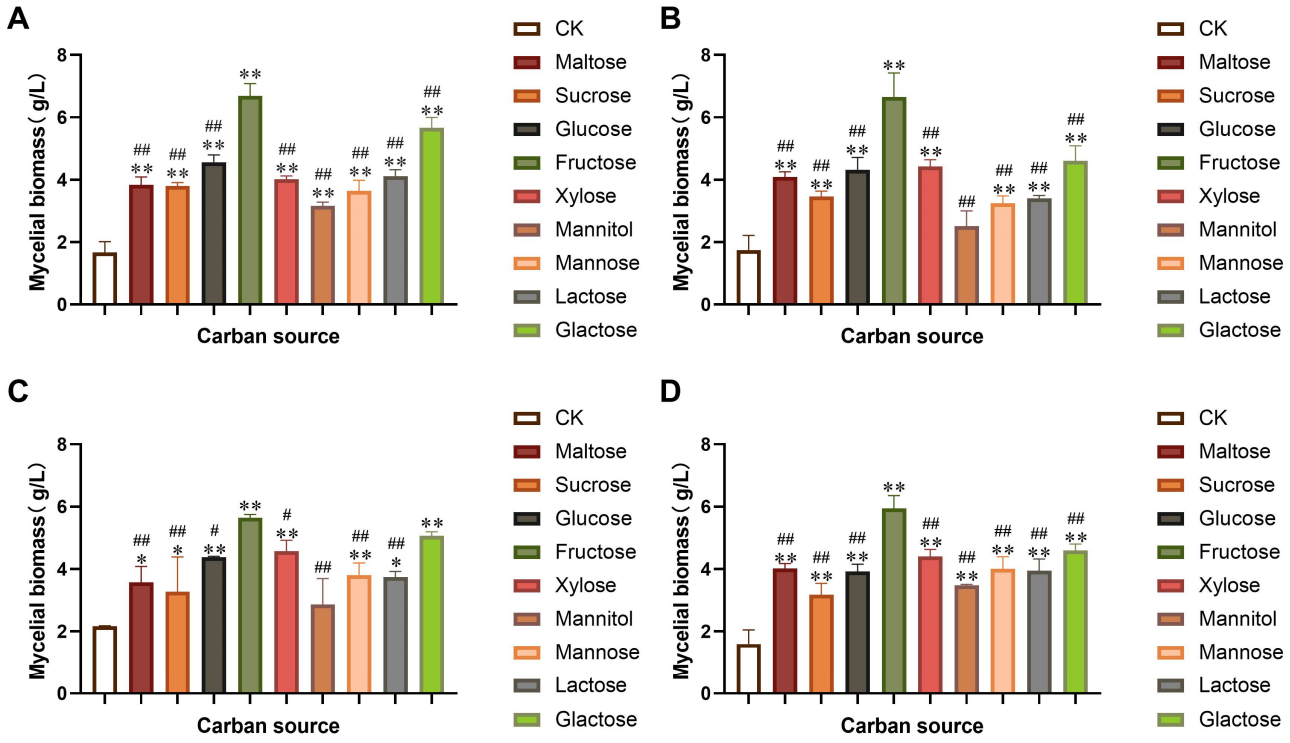

Figure 1. Effects of different carbon sources on the mycelial biomass of *Sanghuangporus alpinus*. (**A**) presents the effect of different carbon sources on MS-10 growth; (**B**) displays the effect of different carbon sources on MS-11 growth; (**C**) shows the effect of different carbon sources on MS-12 growth; and (**D**) shows the effect of different carbon sources on MS-13 growth. * indicates significance at the 0.05 level ($p < 0.05$), ** indicates significance at the 0.01 level ($p < 0.01$) compared with the CK group; # indicates significance at the 0.05 level ($p < 0.05$), ## indicates significance at the 0.01 level ($p < 0.01$) compared with the optimal group.

Figure 1 illustrates that *S. alpinus* proliferated most rapidly with fructose, followed by galactose, xylose, glucose, maltose, lactose, mannose, sucrose, and mannitol (Data shown

in Tables S1, S3, S5 and S7, Supporting Information). When no additional carbon source was added, the growth of the *S. alpinus* biomass was the slowest. The carbon sources exhibited similar effects on the four *S. alpinus* strains, with fructose exerting the best effect. Therefore, fructose was used as the carbon source in the subsequent fermentation using *S. alpinus* medium.

3.2. Impact of Nitrogen Source on Growth

Various nitrogen sources, such as yeast extract, peptone, ammonium tartrate, ammonium sulfate, ammonium nitrate, urea, and beef extract, were used as an additional nitrogen source in the PDB medium. *S. alpinus* growth was observed after 15 days of culture (Figure 2).

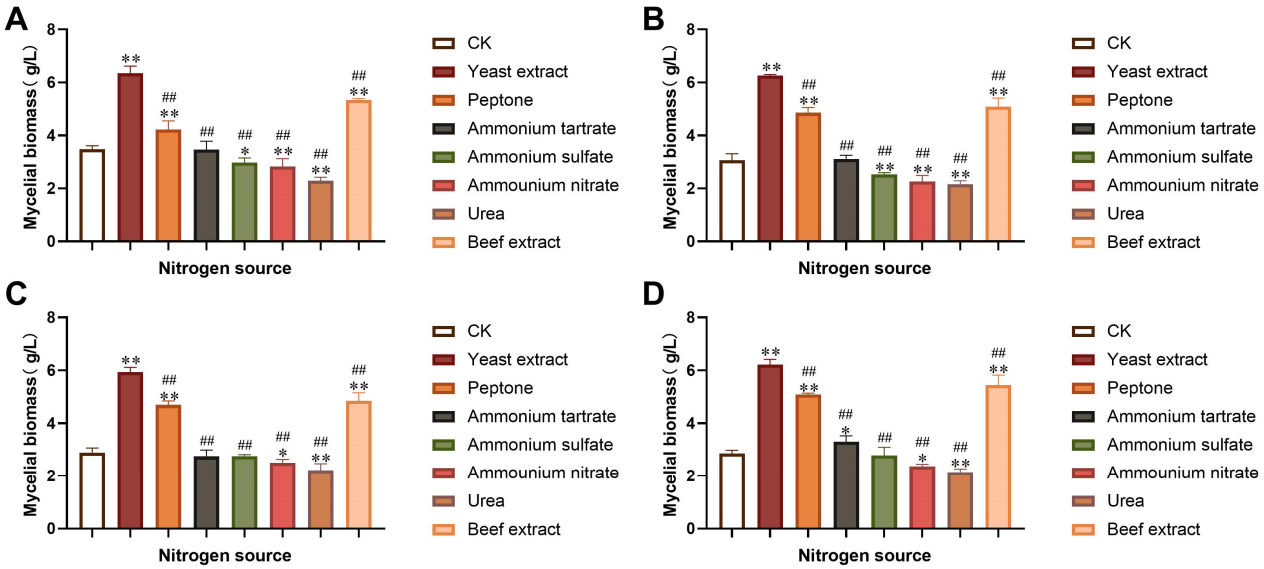

Figure 2. Effects of different nitrogen sources on the mycelial biomass of *Sanghuangporus alpinus*. (**A**) displays the effect of different nitrogen sources on MS-10 growth; (**B**) presents the effect of different nitrogen sources on MS-11 growth; (**C**) displays the effect of different nitrogen sources on MS-12 growth; and (**D**) shows the effect of different nitrogen sources on MS-13 growth. * indicates significance at the 0.05 level ($p < 0.05$), ** indicates significance at the 0.01 level ($p < 0.01$) compared with the CK group; ## indicates significance at the 0.01 level ($p < 0.01$) compared with the optimal group.

Figure 2 illustrates that *S. alpinus* growth decreased in the following descending order: yeast extract, beef extract, peptone, ammonium tartrate, CK, ammonium sulfate, ammonium nitrate, and urea (Data shown in Tables S2, S4, S6 and S8, Supporting Information). The *S. alpinus* strains exhibited high nitrogen source requirements, with yeast extract being the most effective. *S. alpinus* was almost unable to use ammonium sulfate, ammonium nitrate, and urea as nitrogen sources. The yeast extract was used as the nitrogen source for the subsequent medium for *S. alpinus* fermentation.

3.3. Selection of Carbon Source, Nitrogen Source, and Growth Factor Addition Level

3.3.1. Selection of Fructose Amount to Be Added

According to Figure 3, the maximum mycelial biomass of *S. alpinus* was achieved at a fructose concentration of 20 g/L. At this concentration, the *S. alpinus* growth rate was the fastest and the liquid culture effect was the best. At a fructose concentration of <20 g/L or >20 g/L, the mycelial biomass decreased (Data shown in Tables S9–S12, Supporting Information).

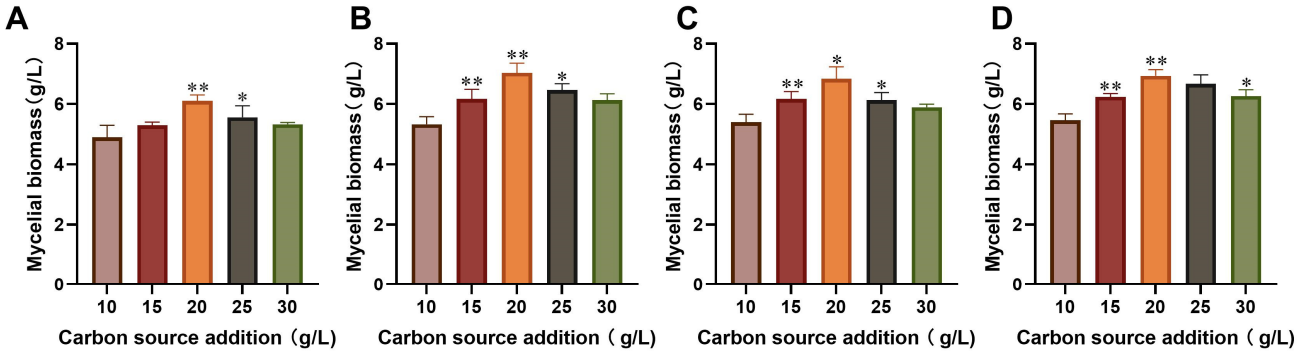

Figure 3. Effects of carbon source addition on the mycelial biomass of *Sanghuangporus alpinus*. ** indicates significance at the 0.01 level ($p < 0.01$), representing extreme significance. * indicates significance at the 0.05 level ($p < 0.05$), representing significance. (**A**) presents the effect of carbon source addition on MS-10 growth. (**B**) shows the effect of carbon source addition on MS-11 growth. (**C**) displays the effect of carbon source addition on MS-12 growth. (**D**) illustrates the effect of carbon source addition on MS-13 growth.

3.3.2. Selection of Yeast Extract Amount to Be Added

As the yeast extract concentration continued to increase, the mycelial biomass of *S. alpinus* also increased (Figure 4). When the yeast extract concentration reached 7.5 g/L, the growth reached the maximum. The mycelial biomass decreased with a decrease in the yeast extract concentration (below the aforementioned concentration), while the mycelial biomass gradually decreased with an increase in the yeast extract concentration (above the aforementioned concentration). Therefore, 7.5 g/L was selected as the optimal addition amount (Data shown in Tables S13–S16, Supporting Information).

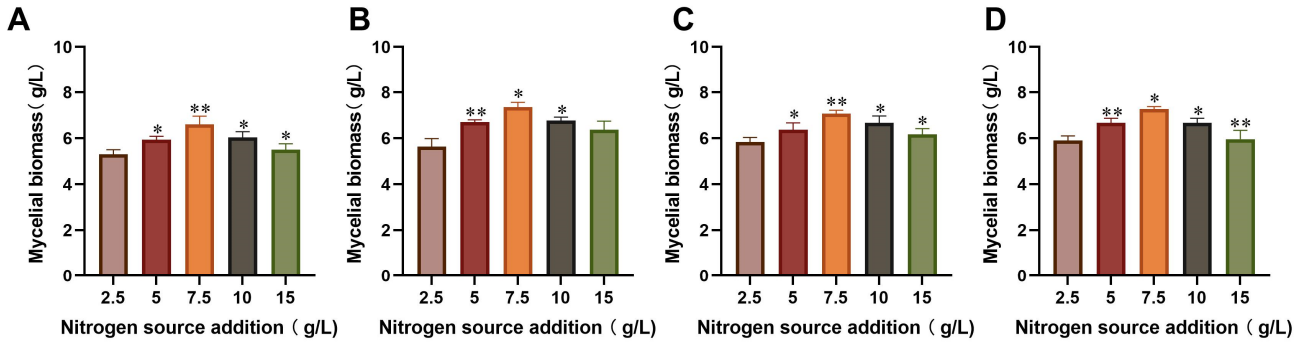

Figure 4. Effects of nitrogen source addition on the mycelial biomass of *Sanghuangporus alpinus*. ** indicates significance at the 0.01 level ($p < 0.01$), representing extreme significance. * indicates significance at the 0.05 level ($p < 0.05$), representing significance. (**A**) shows the effect of nitrogen source addition on MS-10 growth. (**B**) displays the effect of nitrogen source addition on MS-11 growth. (**C**) presents the effect of nitrogen source addition on MS-12 growth. (**D**) shows the effect of nitrogen source addition on MS-13 growth.

3.3.3. Selection of Growth Factor Amount to Be added

Based on the optimal dosage of carbon and nitrogen sources, dandelion powder was included in the culture medium as a growth factor. Figure 5 presents the growth after 15 days of culture.

At a dandelion concentration of 1.0 g/L, the *S. alpinus* biomass was the maximum. At a dandelion concentration of <1.0 g/L, the biomass increased as the dandelion concentration increased and gradually leveled off. When the dandelion concentration exceeded 1.0 g/L, the *S. alpinus* biomass decreased rapidly, resulting in a reduction in its growth (Data shown in Tables S17–S20, Supporting Information). A high dandelion powder concentration was unsuitable for the multiplication and cultivation of *S. alpinus*, and the

optimal dandelion concentration to be added was 1 g/L (Data shown in Tables S17–S20, Supporting Information).

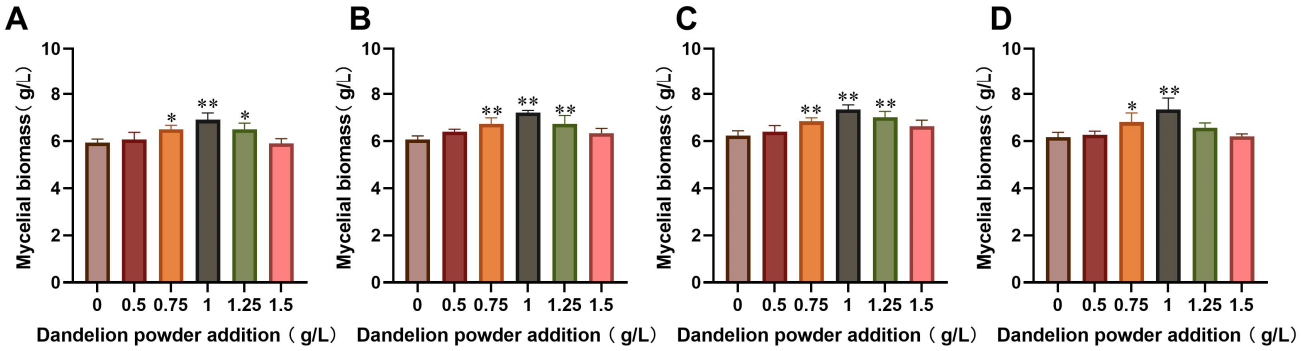

Figure 5. Effects of dandelion powder addition on the mycelial biomass of *Sanghuangporus alpinus*. ** indicates significance at the 0.01 level ($p < 0.01$), representing extreme significance. * indicates significance at the 0.05 level ($p < 0.05$), representing significance. (**A**) presents the effect of dandelion powder addition on MS-10 growth. (**B**) displays the effect of dandelion powder addition on MS-11 growth. (**C**) exhibits the effect of dandelion powder addition on MS-12 growth. (**D**) shows the effect of dandelion powder addition on MS-13 growth.

3.4. RSM Optimization Test Results

3.4.1. Experimental Results with Box–Behnken Design

A response-surface analysis test was conducted by using glucose (A), yeast extract (B), and dandelion powder (C) concentrations as independent variables and the *S. alpinus* mycelial biomass Y as the response value. The test analysis plan and results are presented in Appendix A.

3.4.2. Multiple Quadratic Regression Fitting and Variance Analysis Results

In this study, the Box–Behnken central combination design experiment and Design Expert software were used to conduct regression analysis on the measured mycelial biomass of *S. alpinus*. The obtained variance analysis results are given in Appendix B.

As shown in Appendix B, a model with a p value of <0.05 and the coefficient of determination R^2 of >0.985 fitted the actual situation well. However, a p value of >0.05 for the lack of fit indicated that the lack of fit was nonsignificant and the model was accurate. Based on the aforementioned analysis, the present model was sufficiently accurate and could be used to analyze test results and predict response values. The obtained multiple regression equation Y of the *S. alpinus* mycelial biomass on the independent variables glucose (A), yeast extract (B), and dandelion powder (C) is given as follows:

$$Y_{MS\text{-}10} = 15.194 - 0.08125\,A - 0.4125\,B + 0.0025\,C - 0.4425\,AB - 0.0555\,AC - 0.305\,BC - 1.86825\,A^2 - 1.71825\,B^2 - 2.94705\,C^2$$

$$Y_{MS\text{-}11} = 12.316 + 0.16625\,A - 0.22625\,B + 0.005\,C - 0.3025\,AB + 0.35\,AC - 0.365\,BC - 1.94425\,A^2 - 1.72425\,B^2 - 1.72675\,C^2$$

$$Y_{MS\text{-}12} = 12.87 + 0.27875\,A - 0.245\,B - 0.01125\,C - 0.325\,AB - 0.2825\,AC - 0.47\,BC - 2.06125\,A^2 - 1.85375\,B^2 - 1.93125\,C^2$$

$$Y_{MS\text{-}13} = 12.122 - 0.0075\,A - 0.1825\,B - 0.2325\,C - 0.2375\,AB - 0.2425\,AC - 0.1075\,BC - 1.482255\,A^2 - 1.51725\,B^2 - 1.70225\,C^2$$

3.4.3. Significance Test Analysis Results

To test the validity of the equation, variance analysis was performed on the mathematical model of the measured mycelial biomass of *S. alpinus*, and the partial regression coefficients of each factor were assessed (Appendix B). The regression coefficient of B in the first-order term was highly significant, which indicated that the yeast extract concentration exerted a highly significant influence on *S. alpinus* growth. The partial regression coefficients of A, B, and C in the quadratic term all reached extremely significant levels. The regression coefficient of the interaction term BC was relatively small. Other factors were significant, which revealed that the interaction term between the yeast extract and dandelion powder was significant for *S. alpinus* growth, and the lack of fit term was non-significant, indicating that the model was appropriate with a fitting degree of $R > 0.985$. The model's values closely matched the real data points. This indicated that the equation was effective for the test and had a good fit. The quality of the fit is shown in Table 2.

Table 2. Fit statistics of Y.

	Predictive Model			
	MS-10	**MS-11**	**MS-12**	**MS-13**
RMSE	0.0292	0.0288	0.0398	0.0305
R-square	0.9972	0.9958	0.9951	0.9941
Adjusted R-square	0.9937	0.9904	0.9888	0.9865
Coefficient of variation	0.1778	0.1772	0.1861	0.1518

3.4.4. Response-Surface Analysis of Factor Interactions

Based on the aforementioned regression equation, a response-surface analysis was performed to examine the shape of the fitted response surface. The analysis determined the effects of different concentration combinations of glucose, yeast extract, and dandelion powder on *S. alpinus* growth. The results are presented in Figure 6.

To achieve the maximum growth of *S. alpinus*, glucose and yeast extract levels in the enrichment medium need to be in a suitable ratio (Figure 6). Therefore, according to the aforementioned figure and variance analysis, the order of influence on *S. alpinus* growth was $C^2 > A^2 > B^2$. (1) For MS-10, the concentrations of the added carbon source, nitrogen source, and dandelion powder were 20.18, 7.19, and 1 g/L, respectively. Under these conditions, the MS-10 mycelial biomass reached 15.22 g/L. (2) For MS-11, the concentrations of the added carbon source, nitrogen source, and dandelion powder were 20.24, 7.32, and 1 g/L, respectively. Under these conditions, the MS-11 mycelial biomass reached 12.32 g/L. (3) For MS-12, the concentrations of the added carbon source, nitrogen source, and dandelion powder were 20.37, 7.31, and 1 g/L, respectively. Under these conditions, the MS-12 mycelial biomass reached 12.88 g/L. (4) For MS-13, the concentrations of the added carbon source, nitrogen source, and dandelion powder were 20, 7.35, and 0.98 g/L, respectively. Under these conditions, the MS-13 mycelial biomass reached 12.14 g/L.

To verify the accuracy of model prediction, the culture medium composition was optimized using the response-surface test, and a verification test was conducted based on the results of the test. Three sets of repeated tests were performed, and the average value was taken (Figure 7). The mycelium after MS-10 optimization weighed 15.12 $\pm$ 0.28 g/L, which was 2.45 times higher than that before optimization (6.16 $\pm$ 0.45 g/L) but slightly lower than the predicted value (15.22 g/L). The mycelium after MS-11 optimization weighed 11.88 $\pm$ 0.32 g/L, which was 2.18 times higher than that before optimization (5.43 $\pm$ 0.3 g/L) but slightly lower than the predicted value (12.32 g/L). The mycelium after MS-12 optimization weighed 12.22 $\pm$ 0.27 g/L, which was 2.22 times higher than that before optimization (5.5 $\pm$ 0.45 g/L) but slightly lower than the predicted value (12.88 g/L). The mycelium after MS-13 optimization weighed 11.94 $\pm$ 0.27 g/L, which was 2.29 times higher than that before optimization (5.2 $\pm$ 0.36 g/L) but slightly lower than the predicted value (12.14 g/L). Thus, a good fit was noted between the model values and measured values. Therefore, the fermentation medium optimized using the corresponding

surface method can be considered to provide the necessary theoretical basis for the future cultivation, development, and utilization of *S. alpinus*.

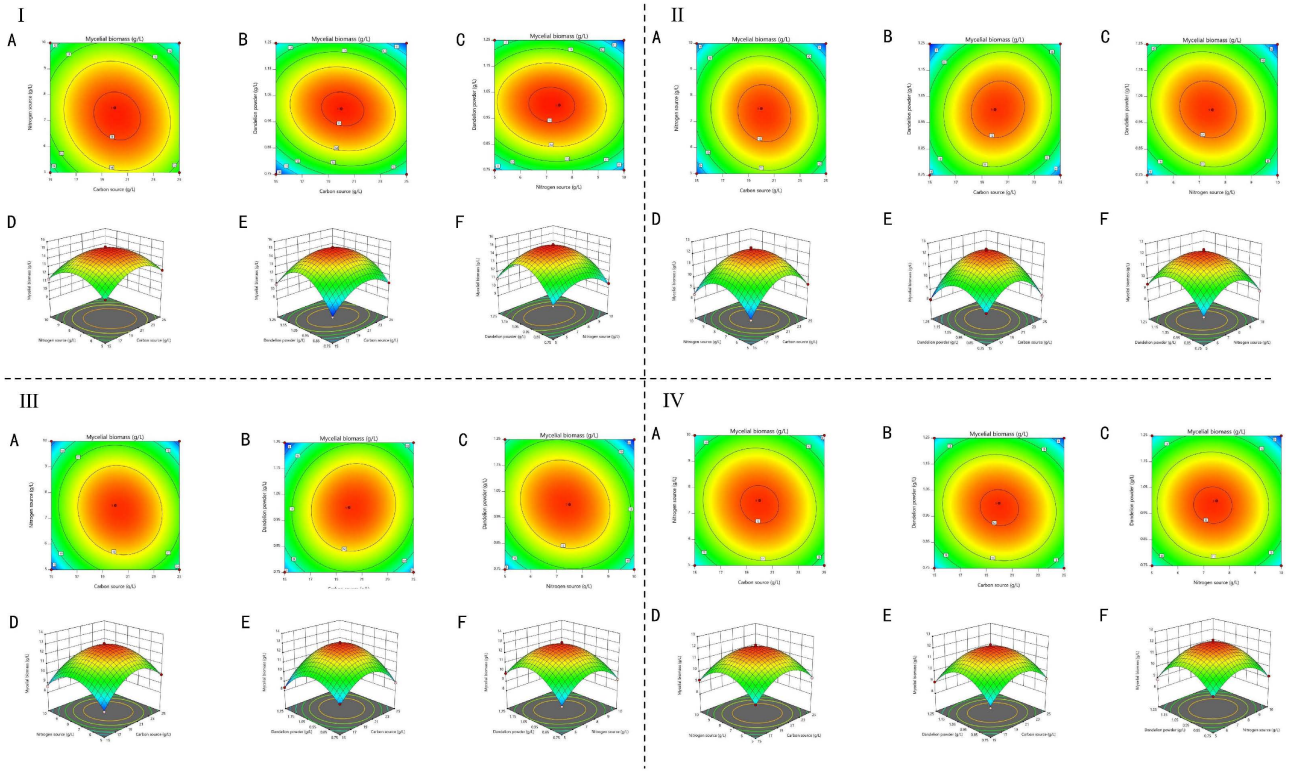

Figure 6. Response-surface plot of *Sanghuangporus alpinus*. (**I**–**IV**) are the response-surface plots and contour plots of MS-10, MS-11, MS-12, and MS-13, respectively. (**B**): Interaction between carbon source addition and nitrogen source addition, and (**A**) illustrates the corresponding contour value. (**D**): Interaction between carbon source addition and dandelion powder addition, and (**C**) presents the corresponding contour value. (**F**): Interaction between nitrogen source addition and dandelion powder addition, and (**E**) shows the corresponding contour value.

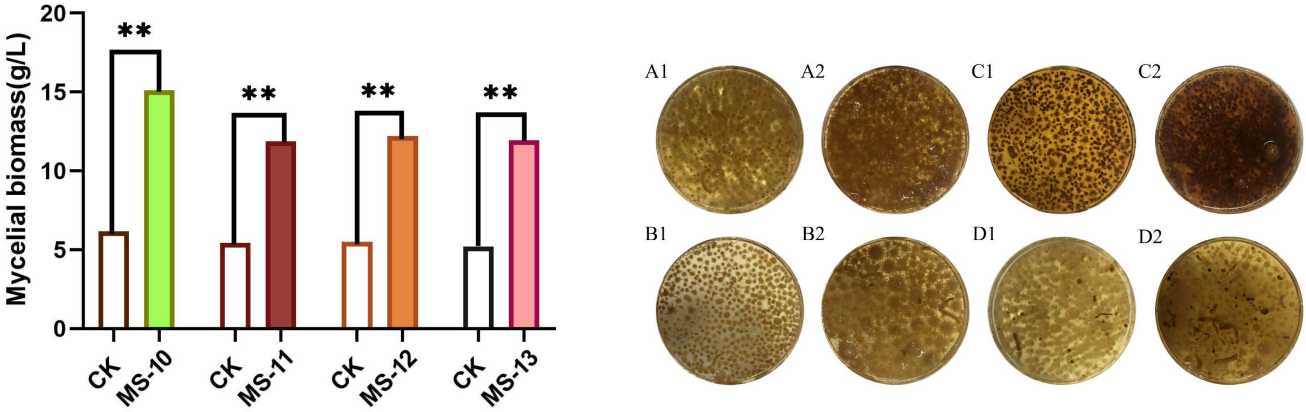

Figure 7. Validation experiment of optimization of Sanghuangporus *alpinus* liquid fermentation formulation. ** indicates significance at the 0.01 level ($p < 0.01$), representing extreme significance. (**A1**) shows MS-10 growth in PDB medium, and (**A2**) shows MS-10 growth in modified medium. (**B1**) presents MS-11 growth in PDB medium, and (**B2**) illustrates MS-11 growth in modified medium. (**C1**) displays MS-12 growth in PDB medium, and (**C2**) exhibits MS-12 growth in the improved medium. (**D1**) illustrates MS-13 growth in PDB medium, and (**D2**) shows MS-13 growth in the improved medium.

3.5. Evaluation of Antioxidant Activity of S. alpinus Extracellular Polysaccharide

The four *S. alpinus* strains all exhibited strong antioxidant activities (Figure 8). Among them, the scavenging effect of *S. alpinus* on ABTS free radicals was more significant at higher concentrations and was close to that in the Vc control group when the concentration was >1 mg/mL. The scavenging effect of *S. alpinus* on DPPH free radicals was generally weak, and only MS-12 exhibited a relatively higher scavenging ability, which was approximately 75% of that of the Vc control group. In addition, the total antioxidant capacity of the exopolysaccharides of *S. alpinus* was weak, and at the highest concentration, the capacity was still less than half of that of the Vc control group.

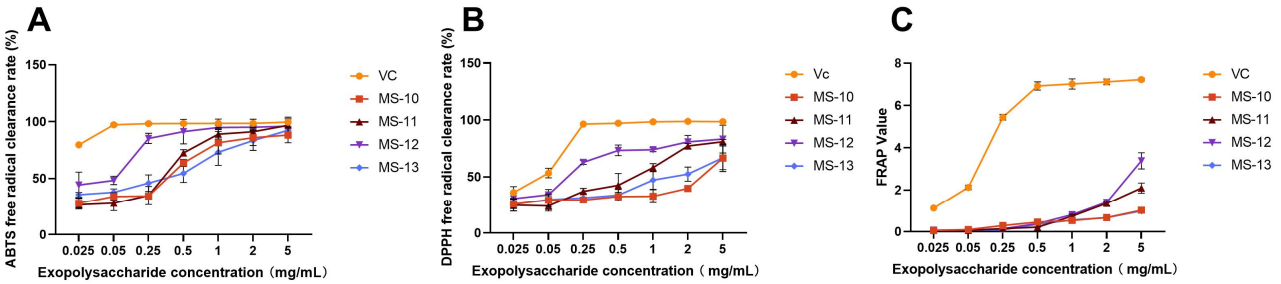

Figure 8. Antioxidant activity of *Sanghuangporus alpinus* exopolysaccharide. (**A**) presents the ABTS radical scavenging ability. (**B**) shows the DPPH radical scavenging ability. (**C**) presents the total antioxidant capacity.

The four *S. alpinus* strains exhibited different scavenging abilities for the two free radicals. In the ABTS free radical scavenging experiment, MS-12 exhibited the strongest scavenging ability (EC_{50} = 0.039 mg/mL). The scavenging abilities of the other three strains were lower than that of MS-12 at a low concentration, which were MS-10 (EC_{50} = 0.21 mg/mL), MS-11 (EC_{50} = 0.17 mg/mL), and MS-13 (EC50 = 0.151 mg/mL). In the DPPH free radical scavenging experiment, the scavenging ability of MS-12 (EC_{50} = 0.039 mg/mL) was still ranked first and was stronger than those of MS-11 (EC_{50} = 0.438 mg/mL) and MS-13 (EC_{50} = 1.396 mg/mL). The strain with the weakest scavenging ability was MS-10 (EC_{50} = 3.21 mg/mL). In the total antioxidant capacity experiment (FRAP method), the antioxidant capacity of *S. alpinus* was generally low. At a polysaccharide concentration of 5 mg/mL, the maximum FRAP values of MS-12, MS-11, MS-10, and MS-13 were 3.38 ± 0.48, 2.09 ± 0.24, 1.02 ± 0.02, and 0.98 ± 0.098, respectively.

3.6. Evaluation of Antioxidant Activity of S. alpinus Polysaccharide

Figure 9 presents the in vitro anticancer activity of the exopolysaccharides of *S. alpinus* against six types of cancer cells. The exopolysaccharide extract had a strong inhibitory effect on the growth of all the cancer cells. The inhibitory effects were ranked in descending order as follows: HCT-166, U251, PC-3, T98G, HepG-2, and MCF-7.

S. alpinus exerted a good pro-apoptotic effect on HCT-116 cells. At a treatment concentration of 5 mg/mL, the survival rate of the cancer cells reduced to 32.62–43.37%. Among the strains tested, MS-11 had the best effect, with a half-effective concentration of 1.37 mg/mL. Of the other strains, MS-10 (IC_{50} = 1.89 mg/mL) and MS-13 (IC_{50} = 1.541 mg/mL) exhibited relatively similar antitumor effects. Furthermore, MS-12 (IC_{50} = 2.676 mg/mL) was slightly less effective.

The four *S. alpinus* strains had similar pro-apoptotic effects on human glioma cells U251. The cell survival rate in the highest concentration group ranged was 32.81–41.46%. Of the strains, MS-10 exerted strong anti-apoptotic effects. Its half-effective concentration was 0.91 mg/mL. MS-11 (IC_{50} = 1.854 mg/mL) and MS-13 (IC_{50} = 1.478 mg/mL) exerted better pro-apoptotic effects. MS-12 (IC_{50} = 2.615 mg/mL) exhibited a poor anticancer activity, and the survival rate of the cells was high even after they were treated at the maximum concentration.

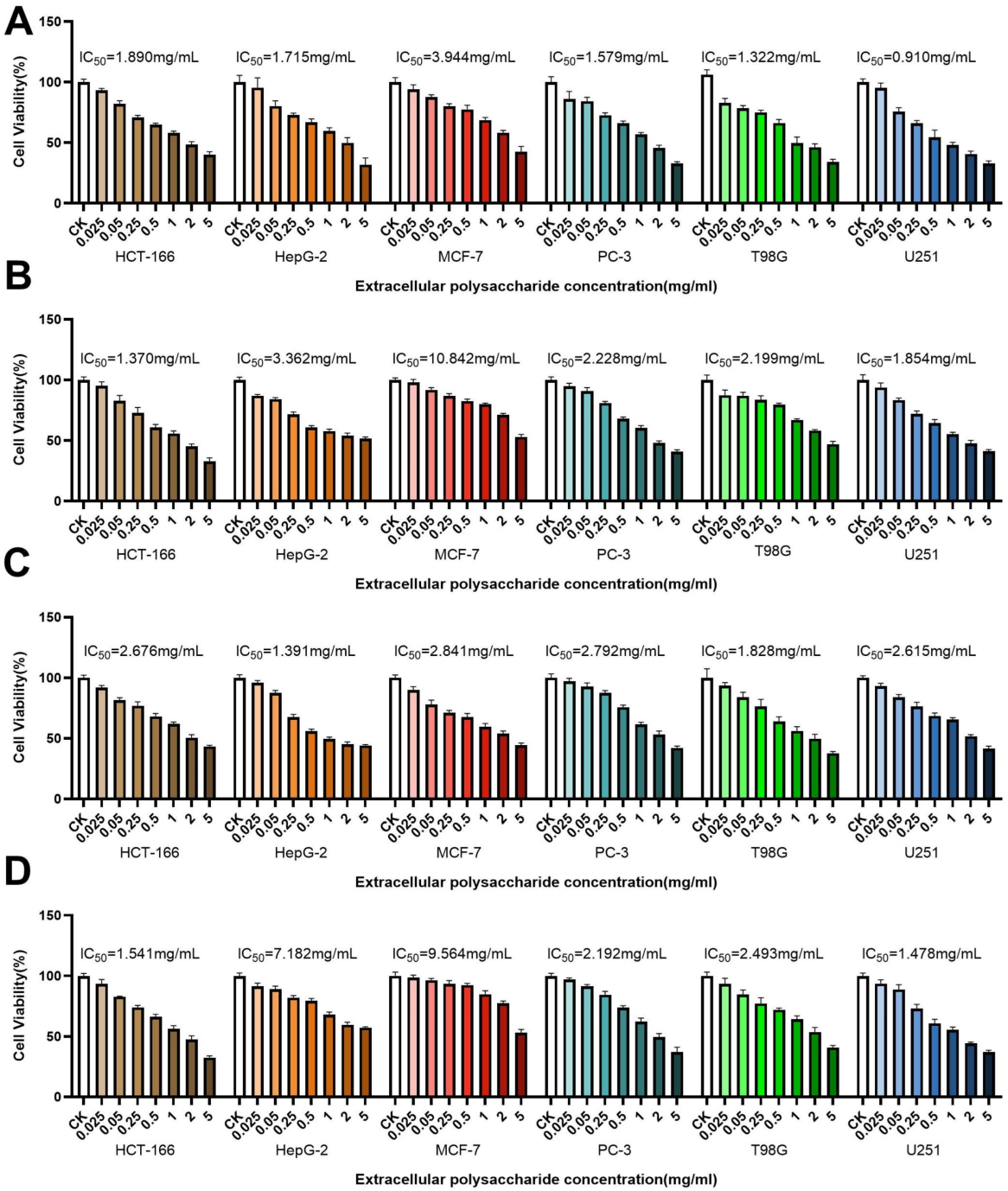

Figure 9. Effects of exopolysaccharides of *Sanghuangporus alpinus* on cancer cell growth. (**A**) shows the effect of the extracellular polysaccharide produced by MS-10 on cancer cell growth. (**B**) displays the effect of the extracellular polysaccharide produced by MS-11 on cancer cell growth. (**C**) illustrates the effect of the extracellular polysaccharide produced by MS-12 on cancer cell growth. (**D**) presents the effect of the extracellular polysaccharide produced by MS-13 on cancer cell growth.

The four *S. alpinus* strains effectively promoted the apoptosis of prostate cancer PC-3 cells. In the highest dose treatment group, the survival rate of the cancer cells was 32.84–42.18%. Among the strains, MS-10 exerted the best pro-apoptotic effect, with a half-effective concentration of 1.579 mg/mL. The anticancer activities of the other three strains were similar. The IC_{50} values of MS-11, MS-12, and MS-13 were 2.228, 2.792, and 2.192 mg/mL, respectively.

S. alpinus exhibited certain differences in anticancer activities in human glioblastoma cells T98G. In the maximum concentration treatment group, the survival rate of the T98G cells was 34.04–46.95% and exhibited large fluctuations. Of the strains tested, MS-10 (IC_{50} = 1.322 mg/mL) and MS-12 (IC_{50} = 1.828 mg/mL) exhibited stronger anticancer activities, whereas MS-11 (IC_{50} = 3.608 mg/mL) and MS-13 (IC_{50} = 2.493 mg/mL) exhibited weak anticancer activities.

S. alpinus had a general apoptosis-promoting effect on the liver cancer cells HepG-2, and the pro-apoptotic activities of the different strains varied considerably. At a treatment concentration of 5 mg/mL, the cell survival rates corresponding to MS-10 and MS-12 were 31.83% and 44.24%, respectively, while those corresponding to MS-11 and MS-13 were less effective at 51.71% and 57.37%, respectively. Among the strains, MS-12 exhibited the strongest pro-apoptotic effect, with a half-inhibitory concentration of 1.391 mg/mL. The IC_{50} values of MS-10, MS-11, and MS-13 were 1.715, 3.362, and 7.182 mg/mL, respectively.

The pro-apoptotic effects of the four *S. alpinus* strains on the breast cancer cells MCF-7 were relatively weak, and the differences between the strains were large. Of the strains, MS-12 (IC_{50} = 2.841 mg/mL) exhibited the best pro-apoptotic effect. MS-10 (IC_{50} = 3.944 mg/mL) also exhibited a strong anticancer activity, whereas MS-11 and MS-13 exhibited poor anticancer activities. At the maximum treatment concentration, the cell survival rates were still high, reaching 53.02% (MS-11) and 53.08% (MS-13).

4. Discussion

A suitable growth medium is essential for producing Sanghuangporus commercially at a mass level. A high-quality liquid fermentation medium for Sanghuangporus should allow the easy acquisition of raw materials, be of low cost, and enable the easy collection of products. To meet these requirements, the commonly used culture medium must be modified or supplemented to ensure that it provides the nutrients necessary for fungal growth.

According to this study, the optimal carbon source for *S. alpinus* liquid fermentation is fructose, and the optimal nitrogen source is yeast extract. Fructose and yeast extract significantly improved the yield and efficiency of fermentation under the liquid culture conditions of *S. alpinus*. *S. alpinus* is commonly cultivated using glucose and peptone as carbon and nitrogen sources, respectively although these sources may not offer the optimal growth and nutritional conditions. This difference is likely the reason for the difficulty in cultivating *S. alpinus* [29].

Moreover, some researchers add external substances as growth factors to the Sanghuangporus culture medium to enhance functional substance production. Promoting growth factors are uncommon additives to culture media, apart from basic nutrients. Several growth factors for *Sanghuangporus* have now been identified, but the mechanisms by which each promotes growth remain unclear.

Although there is currently no specific research on *Sanghuangporus alpinus*, various growth-promoting factors have been identified for other *Sanghuangporus* varieties. For example, Jiang proposed that magnesium acetate significantly promotes *Sanghuangporus* fermentation to produce flavonoids [30]. Huo's research also demonstrated that mulberry extracts can significantly increase the biomass of *Sanghuangporus vaninii* [31]. In the present study, adding the Chinese herbal medicine, dandelion, to the optimized fermentation broth doubled the yield before optimization, thereby significantly increasing the yield of *S. alpinus* liquid fermentation. This discovery offers vital theoretical guidance for producing *S. alpinus* and can effectively augment the quality and yield of its fermentation products.

Furthermore, under the culture conditions of this study, the exopolysaccharide extracted from the culture medium of *S. alpinus* was observed to have strong antioxidant activity and anticancer activity. It is postulated based on past research on other *Sanghuangporus* varieties that *S. alpinus* may inhibit the damage caused by free radicals by increasing antioxidant enzyme activity [32]. With regard to the anticancer activity of exopolysaccharides, it may be the case that the structure of these compounds plays a role in the anticancer effect, which is thought to fight cancer by enhancing immune cell activity or

directly inhibiting cell growth [33]. Therefore, further analysis of the composition and structure of *S. alpinus* exopolysaccharides may help to shed light on the antioxidant or anticancer mechanism of exopolysaccharides.

5. Conclusions

This study analyzed the carbon and nitrogen sources and growth factors of the *S. alpinus* culture medium. The growth of *S. alpinus* is contingent upon a specific environment, and the fruiting bodies of this species are exceedingly difficult to cultivate under general culture conditions. Consequently, liquid culture represents a efficacious method for the production of active *S. alpinus* products. By conducting a single-factor experiment, the optimal level range of each substance concentration was determined, and a three-factor and three-level RSM experimental design was implemented to optimize the formula of the *S. alpinus* fermentation medium. Based on the RSM results, a mathematical model was established, with *S. alpinus* mycelial biomass being considered as the target value and fructose, yeast extract, and dandelion powder as factors. The variance analysis indicated a good fit for the model. The optimal concentrations of the material combination for *Sanghuangporus* growth were evaluated by optimizing the regression equation: fructose 20.20 ± 0.17 g/L, yeast extract 7.29 ± 0.10 g/L, and dandelion 0.99 ± 0.01 g/L. Under these conditions, the established mathematical model was experimentally verified, and the mycelial biomass reached a maximum value of 12.79 ± 1.41 g/L, which was approximately twice that of the biomass before. Wang et al., using yeast dextrose medium for the cultivation of *Sanghuangporus alpinus*, achieved a maximum mycelial biomass increment of approximately 5.5 g/L under inoculum levels higher than those used in this study [34]. Our optimized formulation, compared to theirs, significantly enhances the mycelial yield of *Sanghuangporus alpinus*. This result offers relevant reference data for the large-scale production of *S. alpinus*.

The composition of the Sanghuang culture medium can influence the functional components of Sanghuang by affecting the structure of its secretions [35]. Therefore, we additionally assessed the antioxidant and anticancer activities of Sanghuang extracellular polysaccharides to demonstrate that the optimized medium does not have adverse effects on the functionality of *S. alpinus*. The experiments revealed that the ABTS free radical scavenging ability of the exopolysaccharide was stronger than its DPPH free radical scavenging ability, but the total antioxidant capacity was weak.

Differences were noted in the anticancer effects of *S. alpinus* exopolysaccharides on different types of cancer cells. A strong inhibitory effect of the exopolysaccharides was noted on HCT-166, U251, and PC-3 cells. However, a weak inhibitory effect was observed on the other two types of cancer cells (HepG-2 and MCF-7). Further studies are warranted to determine the mechanism underlying the antitumor effect of the exopolysaccharide of *S. alpinus*.

The objective of this research is to enhance the unit volume yield of *S. alpinus* through laboratory-scale studies. Although the results of this preliminary study may not be directly applicable to large-scale equipment, it can be reasonably concluded that S. alpinus liquid fermentation has the potential to become an effective process in large-scale settings. Sanghuang, as a large fungus with robust mycelium cell walls, may exhibit greater resistance to shear forces, facilitating growth in fermentation tanks. Additionally, initial pH appears to have minimal impact on Sanghuang fermentation, which supports its feasibility [34]. However, further verification is needed to confirm this, as the heat and shear resistance of functional substances like Sanghuang polysaccharides may be lower in larger fermentation tanks [36].

Transitioning research from the lab to production requires addressing technical and equipment limitations. One way to bridge this gap is through tailored training programs and standard operating procedures aligned with manufacturing settings. The proactive identification and resolution of these challenges will ensure a more seamless transition, reducing disruption and optimizing manufacturing efficiency.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10050474/s1, Table S1 significance tests of (MS-10 for carbon source; Table S2 significance tests of (MS-10 for nitrogen source; Table S3 significance tests of (MS-11 for carbon source; Table S4 significance tests of (MS-11 for nitrogen source; Table S5 significance tests of (MS-12 for carbon source; Table S6 significance tests of (MS-12 for nitrogen source; Table S7 significance tests of (MS-13 for carbon source; Table S8 significance tests of (MS-13 for nitrogen source; Table S9 significance tests of (MS-10 for carbon source addition amount; Table S10 significance tests of (MS-11 for carbon source addition amount; Table S11 significance tests of (MS-12 for carbon source addition amount; Table S12 significance tests of (MS-13 for carbon source addition amount; Table S13 significance tests of (MS-10 for nitrogen source addition amount; Table S14 significance tests of (MS-11 for nitrogen source addition amount; Table S15 significance tests of (MS-12 for nitrogen source addition amount; Table S16 significance tests of (MS-13 for nitrogen source addition amount; Table S17 significance tests of (MS-10 for dandelion addition amount; Table S18 significance tests of (MS-11 for dandelion addition amount; Table S19 significance tests of (MS-12 for dandelion addition amount; Table S20 significance tests of (MS-13 for dandelion addition amount.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A. Shows the Table of Response-Surface Analysis of *S. alpinus*

Table A1. Response-surface design and test results of MS-10.

No.	A: Carbon Source (g/L)	B: Nitrogen Source (g/L)	C: Dandelion Powder (g/L)	Mycelial Biomass (g/L)
1	20	7.5	1	15.2
2	20	5	1.25	11.13
3	15	7.5	1.25	10.83
4	15	10	1	11.5
5	25	5	1	12.6
6	20	10	0.75	10.55
7	20	10	1.25	9.93
8	25	7.5	1.25	9.96
9	25	10	1	10.7
10	20	7.5	1	15.35
11	15	7.5	0.75	9.7
12	20	7.5	1	14.96
13	20	5	0.75	10.53
14	20	7.5	1	15.13
15	20	7.5	1	15.33
16	25	7.5	0.75	11.05
17	15	5	1	11.63

Table A2. Response-surface design and test results of MS-11.

No.	A: Carbon Source (g/L)	B: Nitrogen Source (g/L)	C: Dandelion Powder (g/L)	Mycelial Biomass (g/L)
1	15	7.5	0.75	8.96
2	20	7.5	1	12.33
3	25	7.5	1.25	9.03
4	20	7.5	1	12.53
5	25	5	1	9.4
6	25	7.5	0.75	8.46
7	25	10	1	8.36
8	20	5	1.25	9.53
9	15	10	1	8.5
10	15	7.5	1.25	8.13
11	20	10	0.75	8.93
12	15	5	1	8.33
13	20	7.5	1	12.06
14	20	5	0.75	8.67
15	20	7.5	1	12.23
16	20	7.5	1	12.43
17	20	10	1.25	8.33

Table A3. Response-surface design and test results of MS-12.

No.	A: Carbon Source (g/L)	B: Nitrogen Source (g/L)	C: Dandelion Powder (g/L)	Mycelial Biomass (g/L)
1	25	7.5	0.75	8.86
2	20	7.5	1	12.63
3	15	7.5	1.25	8.33
4	20	10	1.25	8.43
5	20	7.5	1	12.8
6	20	7.5	1	13.06
7	25	7.5	1.25	9.26
8	15	7.5	0.75	9.06
9	25	10	1	8.76
10	25	5	1	9.9
11	20	7.5	1	13.1
12	20	10	0.75	9.25
13	20	5	0.75	8.8
14	20	5	1.25	9.86
15	15	5	1	8.5
16	15	10	1	8.66
17	20	7.5	1	12.76

Table A4. Response-surface design and test results of MS-13.

No.	A: Carbon Source (g/L)	B: Nitrogen Source (g/L)	C: Dandelion Powder (g/L)	Mycelial Biomass (g/L)
1	20	10	0.75	9.16
2	20	5	0.75	9.36
3	20	7.5	1	11.96
4	20	5	1.25	8.86
5	25	10	1	8.67
6	20	10	1.25	8.23
7	25	7.5	1.25	8.63
8	15	7.5	1.25	9.03
9	20	7.5	1	12.13
10	25	7.5	0.75	9.33
11	20	7.5	1	12.06
12	20	7.5	1	12.3

Table A4. *Cont.*

No.	A: Carbon Source (g/L)	B: Nitrogen Source (g/L)	C: Dandelion Powder (g/L)	Mycelial Biomass (g/L)
13	25	5	1	9.46
14	20	7.5	1	12.16
15	15	7.5	0.75	8.76
16	15	10	1	9.26
17	15	5	1	9.1

Appendix B. Shows the Table of Variance Analysis and Significance Test of *S. alpinus*

Table A5. Variance analysis and significance tests of MS-10.

Source	Sum of Squares	Df	Mean Squares	F-Value	*p*-Value
Model	74.12	9	8.24	281.89	<0.0001
A	0.0528	1	0.0528	1.81	0.2207
B	1.29	1	1.29	44.09	0.0003
C	0.0001	1	0.0001	0.0017	0.9682
AB	0.7832	1	0.7832	26.81	0.0013
AC	1.23	1	1.23	42.18	0.0003
BC	0.3721	1	0.3721	12.74	0.0091
A^2	14.7	1	14.7	503.06	<0.0001
B^2	12.43	1	12.43	425.52	<0.0001
C^2	36.41	1	36.41	1246.43	<0.0001
Residual	0.2045	7	0.0292		
Lack offit	0.1028	3	0.0343	1.35	0.3779
Pure error	0.1017	4	0.0254		
Cor Total	74.32	16			
	R^2 = 0.9972; Adjusted R^2 = 0.9937; Predicted R^2 = 0.9757				

Table A6. Variance analysis and significance tests of MS-11.

Source	Sum of Squares	Df	Mean Squares	F-Value	*p*-Value
Model	47.81	9	5.31	184.59	<0.0001
A	0.2211	1	0.2211	7.68	0.0276
B	0.4095	1	0.4095	14.23	0.007
C	0	1	0	0	1
AB	0.366	1	0.366	12.72	0.0091
AC	0.49	1	0.49	17.03	0.0044
BC	0.5329	1	0.5329	18.52	0.0036
A^2	15.92	1	15.92	553.07	<0.0001
B^2	12.52	1	12.52	434.99	<0.0001
C^2	12.55	1	12.55	436.25	<0.0001
Residual	0.2014	7	0.0288		
Lack offit	0.0695	3	0.0232	0.7027	0.5981
Pure error	0.1319	4	0.033		
Cor Total	48.01	16			
	R^2 = 0.9958; Adjusted R^2 = 0.9904; Predicted R^2 = 0.9725				

Table A7. Variance analysis and significance tests of MS-12.

Source	Sum of Squares	Df	Mean Squares	F-Value	*p*-Value
Model	56.43	9	6.27	157.69	<0.0001
A	0.6216	1	0.6216	15.63	0.0055
B	0.4802	1	0.4802	12.08	0.0103
C	0.001	1	0.001	0.0255	0.8777
AB	0.4225	1	0.4225	10.63	0.0139

Table A7. *Cont.*

Source	Sum of Squares	Df	Mean Squares	F-Value	*p*-Value
AC	0.3192	1	0.3192	8.03	0.0253
BC	0.8836	1	0.8836	22.22	0.0022
A^2	17.89	1	17.89	449.93	<0.0001
B^2	14.47	1	14.47	363.9	<0.0001
C^2	15.7	1	15.7	394.97	<0.0001
Residual	0.2783	7	0.0398		
Lack offit	0.1147	3	0.0382	0.935	0.502
Pure error	0.1636	4	0.0409		
Cor Total	56.71	16			
	R^2 = 0.9951; Adjusted R^2 = 0.9888; Predicted R^2 = 0.9631				

Table A8. Variance analysis and significance tests of MS-13.

Source	Sum of Squares	Df	Mean Squares	F-Value	*p*-Value
Model	36	9	4	131.26	<0.0001
A	0.0005	1	0.0005	0.0148	0.9067
B	0.2664	1	0.2664	8.75	0.0212
C	0.4325	1	0.4325	14.19	0.007
AB	0.2256	1	0.2256	7.41	0.0297
AC	0.2352	1	0.2352	7.72	0.0274
BC	0.0462	1	0.0462	1.52	0.2578
A^2	9.25	1	9.25	303.62	<0.0001
B^2	9.69	1	9.69	318.13	<0.0001
C^2	12.2	1	12.2	400.43	<0.0001
Residual	0.2133	7	0.0305		
Lack offit	0.15	3	0.05	3.16	0.1477
Pure error	0.0633	4	0.0158		
Cor Total	36.21	16			
	R^2 = 0.9941; Adjusted R^2 = 0.9865; Predicted R^2 = 0.9310				

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