

Article

Optimization of Polysaccharide Extraction from *Termitomyces albuminosus* by Ultrasound-Assisted Extraction and Comparative Analysis of Structural Characteristics and Antioxidant Activity

Zhenjiang Li ^{1,2}, Youpeng Tuo ³, Xiaofang Tang ³, Li Ye ³, Jing Chen ², Lan Chen ², Fangyuan Zeng ² and Changsheng Qiao ^{1,*} 

¹ College of Bioengineering, Tianjin University of Science and Technology, Tianjin 300457, China

² Chengdu Jinkai Biology Engineering Co., Ltd., Chengdu 610041, China

³ Sichuan Food and Fermentation Industry Research & Design Institute Co., Ltd., Chengdu 610041, China

* Correspondence: qiaochangsheng@tust.edu.cn

Abstract

Edible mushroom polysaccharides have attracted considerable attention because of their diverse biological activities, particularly their antioxidant potential. However, efficient extraction of these polysaccharides remains challenging due to the rigid chitin- β -glucan network of fungal cell walls. In this study, polysaccharides from *Termitomyces albuminosus* were extracted using hot water extraction (HWE), ultrasound-assisted extraction (UAE), and ultrasound-assisted aqueous two-phase extraction (UA-ATPE). Extraction conditions for each method were optimized using Box–Behnken response surface methodology, and the effects of different extraction strategies on polysaccharide yield, physicochemical properties, and antioxidant activity were systematically compared. Among the three methods, UAE produced the highest polysaccharide yield (110.32 ± 3.68 mg/g). The extraction strategy significantly influenced the molecular weight distribution and monosaccharide composition of the crude polysaccharides. The crude UAE extract was further purified by DEAE-52 anion-exchange chromatography, yielding the major antioxidant-active fraction eluted with 0.1 M NaCl (designated ATPs-0.1M), which was identified as an acidic heteropolysaccharide with an average molecular weight of 6.37 kDa and composed primarily of glucose, mannose, galactose, xylose, glucuronic acid, rhamnose, and fucose. In vitro antioxidant assays demonstrated that ATPs-0.1M exhibited stronger DPPH radical scavenging, hydroxyl radical scavenging, and ferric reducing activities than the other purified fractions under the tested conditions. The results indicate that different extraction strategies are associated with distinct physicochemical characteristics and antioxidant activities of *T. albuminosus* polysaccharides. These findings provide a practical basis for selecting appropriate extraction methods and support the further development of *T. albuminosus* polysaccharides as natural antioxidant ingredients.

Keywords: *Termitomyces albuminosus*; fungal polysaccharides; ultrasound-assisted extraction; response surface methodology; structural characterization; antioxidant activity



Academic Editors: Andrea Ragusa and Michele Maffia

Received: 26 May 2026

Revised: 13 July 2026

Accepted: 14 July 2026

Published: 25 July 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

Polysaccharides, as the third major class of biological macromolecules after proteins and nucleic acids, play essential roles in energy storage, cell recognition, and signal transduction [1]. Over the past three decades, research in natural product chemistry

has primarily focused on plant-derived polysaccharides (e.g., those from *Astragalus membranaceus*, jujube (*Ziziphus jujuba*), and *Lycium barbarum*), establishing their pharmacological significance in antioxidant, immunomodulatory, and antitumor activities [2,3]. Edible and medicinal mushrooms represent an important source of structurally diverse polysaccharides. Numerous studies have demonstrated that fungal polysaccharides possess considerable antioxidant activities by scavenging reactive oxygen species (ROS), reducing oxidative damage, and modulating endogenous antioxidant defense systems [4–6]. Among edible fungi, *Termitomyces albuminosus* (*T. albuminosus*) is highly valued in East Asia because of its desirable flavor, nutritional value, and medicinal properties. Previous studies have reported that polysaccharides isolated from *T. albuminosus* exhibit antioxidant, immunomodulatory, and anti-inflammatory activities, indicating considerable potential for the development of functional food ingredients and natural health products [7–10]. Nevertheless, the physicochemical properties and biological activities of fungal polysaccharides are strongly influenced by their extraction procedures, making the development of efficient extraction strategies an essential prerequisite for both fundamental research and industrial utilization.

The extraction of fungal polysaccharides is particularly challenging because fungal cell walls are composed of a highly cross-linked chitin- β -glucan matrix that forms a rigid physical barrier, restricting the release of intracellular polysaccharides. Conventional hot water extraction (HWE) remains the most widely adopted industrial method because of its simplicity and low cost; however, it generally requires prolonged extraction times and elevated temperatures, which may reduce extraction efficiency and alter polysaccharide structure through thermal degradation or hydrolysis [11–13]. Consequently, considerable efforts have been devoted to developing more efficient and environmentally friendly extraction technologies.

Among these emerging technologies, ultrasound-assisted extraction (UAE) has attracted considerable interest because acoustic cavitation enhances solvent penetration, disrupts fungal cell walls, and accelerates mass transfer under relatively mild conditions. Numerous studies have demonstrated that UAE can significantly improve extraction efficiency while shortening extraction time and reducing energy consumption [14–17]. In parallel, aqueous two-phase extraction (ATPE) has emerged as a green separation technology capable of integrating extraction and preliminary purification into a single process. By exploiting differences in the partition behavior of biomolecules between two immiscible aqueous phases, ATPE offers advantages including low solvent consumption, mild operating conditions, and improved selectivity [18–20]. More recently, combining ultrasound with aqueous two-phase systems (UA-ATPE) has been proposed as an integrated strategy that combines efficient extraction with simultaneous preliminary purification.

Beyond extraction efficiency, increasing evidence suggests that extraction conditions can substantially influence the structural characteristics of polysaccharides, including molecular weight distribution, monosaccharide composition, branching degree, and conformational features. These structural characteristics are believed to affect the biological properties of polysaccharides, including their antioxidant activities [21–24]. However, despite these advances, systematic comparisons of HWE, UAE, and UA-ATPE using identical biological materials and optimized extraction conditions remain scarce. Furthermore, the extent to which different extraction strategies influence polysaccharide structural characteristics and their associated antioxidant activities has not been comprehensively investigated for *T. albuminosus*.

Accordingly, the present study aimed to systematically compare three extraction strategies—HWE, UAE, and UA-ATPE—for the extraction of polysaccharides from *T. albuminosus*. Extraction conditions for each method were optimized using Box–Behnken

response surface methodology (BBD-RSM), and the resulting crude polysaccharides were comparatively evaluated with respect to extraction yield, chemical composition, molecular weight distribution, monosaccharide composition, and in vitro antioxidant activity. Furthermore, the major antioxidant-active polysaccharide fraction was purified and structurally characterized to further investigate the physicochemical characteristics associated with antioxidant activity. By integrating process optimization with comparative structural and functional analyses, this study provides a comprehensive evaluation of three extraction strategies for *T. albuminosus* polysaccharides and establishes a practical foundation for their efficient extraction and future utilization as natural antioxidant ingredients.

2. Materials and Methods

2.1. Strain Isolation and Purification

Fruiting bodies of *T. albuminosus* were collected from a natural habitat in July 2025 (Figure 1A). Samples were immediately placed in sterile bags and transported to the laboratory, where tissue isolation was completed within 4 h to minimize microbial contamination and tissue degradation. The samples were first thoroughly washed with running tap water to remove surface debris, followed by wiping with 75% (*v/v*) ethanol and air drying. Under a Class II biosafety cabinet, sterile operations were performed. The fruiting body was longitudinally dissected using a sterilized scalpel, and inner tissue (approximately 5 × 5 mm) from the junction between the pileus and stipe was excised, as this region is considered to have high mycelial activity and low contamination risk. The tissue was inoculated onto 90-mm-diameter Petri dish containing modified potato dextrose agar (PDA) medium composed of potato infusion (200 g/L), glucose (20 g/L), peptone (5 g/L), KH₂PO₄ (5 g/L), MgSO₄ (3 g/L), and agar (18 g/L), without pH adjustment. The medium was autoclaved at 121 °C for 20 min and cooled before use. Plates were incubated at 23 °C in the dark. After mycelial outgrowth, actively growing hyphal tips at the colony edge were subcultured by successive streaking (3–5 generations) until a morphologically uniform and contamination-free strain was obtained. The purified strain was maintained on PDA slants at 4 °C and subcultured periodically to preserve viability.

2.2. Morphological Identification

Purified strains were incubated on PDA plates at 23 °C for 14 days before morphological characterization. Macroscopic parameters included colony diameter, growth rate (mm/day), margin morphology, mycelial density, and pigmentation changes. All measurements were performed in triplicate. For microscopic observation, hyphae were mounted on glass slides, stained with lactophenol cotton blue for 10–15 min, and observed under a light microscope (×400–1000). Morphological features including septation, branching pattern, branching angle, and hyphal uniformity were recorded for taxonomic identification [25,26].

2.3. Molecular Identification

2.3.1. Genomic DNA Extraction and PCR Amplification

Genomic DNA was extracted using a fungal DNA extraction kit (TIANGEN Biotech, Beijing, China). DNA quality and concentration were assessed by agarose gel electrophoresis and spectrophotometry prior to polymerase chain reaction (PCR) amplification. The internal transcribed spacer (ITS) region was amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR amplification was performed in a 25 µL reaction system containing 1.0 µL genomic DNA (~50 ng/µL), 1.0 µL each primer (10 µmol/L), 12.5 µL Max PCR Master Mix, and 9.5 µL nuclease-free water. The amplification program consisted of initial denaturation at 95 °C

for 5 min, followed by 32 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

2.3.2. Sequencing and Phylogenetic Analysis

PCR products were verified by 1.5% (*w/v*) agarose gel electrophoresis (120 V, 30 min) and sequenced by Sanger sequencing. The obtained ITS sequences were assembled and edited using standard sequence analysis software before being compared with reference sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST, version 2.17.0) to identify closely related taxa. To further confirm the taxonomic identity of the isolate, representative ITS sequences of closely related *Termitomyces* species were retrieved from GenBank for phylogenetic analysis.

2.4. Total Sugar Determination and Standard Curve

Total polysaccharide content was determined using the phenol–sulfuric acid method. Briefly, 100 µL of sample solution was mixed with 100 µL of 6% (*w/v*) phenol solution, followed by rapid addition of 500 µL concentrated sulfuric acid (98%). After vortexing, the mixture was incubated in a boiling water bath at 100 °C for 5 min, then cooled to room temperature. Absorbance was measured at 490 nm using a UV–Vis spectrophotometer (UV-2600, Shimadzu, Kyoto, Japan). A standard curve was constructed using D-glucose (0–100 µg/mL). The extraction yield of crude polysaccharides was calculated as follows:

$$\text{Yield} = (C \times V \times D)/M \quad (1)$$

where C is sugar concentration (mg/mL), V is extracting volume (mL), D is dilution factor, and M is dry fungal powder mass (g).

2.5. Comparison of Extraction Methods: HWE, UAE, and UA-ATPE

Three extraction methods were systematically compared. All extracts were centrifuged at 12,000 rpm for 3 min at 4 °C, and supernatants were collected for analysis. (1) HWE: Fungal powder was suspended in distilled water at a fixed solid–liquid ratio and extracted in a thermostatic water bath shaker at 150 rpm under specified temperatures. (2) UAE: A probe sonicator (JY92-IIN, Scientz, Ningbo, China) was used with different power settings. Pulse mode (3 s on/2 s off) was applied, with external cooling to prevent thermal degradation. (3) UA-ATPE: A green aqueous two-phase system (30% ethanol/16% salt, *w/w*) was constructed. ZnSO₄, Na₂CO₃, K₂CO₃, (NH₄)₂SO₄, NaH₂PO₄, and K₂HPO₄ were evaluated. Na₂S₂O₃ was not included because preliminary experiments indicated interference with the phenol–sulfuric acid assay. After optimization, ultrasound was combined with ATPS to enhance polysaccharide partitioning.

2.6. Single-Factor Experimental Design

Key variables affecting extraction efficiency were evaluated. (1) Temperature and time: 30–70 °C for all methods. Extraction time ranged from 30–150 min (HWE and UAE) and 60–180 min (UA-ATPE). (2) Solid–liquid ratio and ultrasonic power: 10–50 mL/g for solid–liquid ratio. Ultrasound power (UAE and UA-ATPE) ranged from 240–400 W.

2.7. Response Surface Methodology (RSM)

Box–Behnken design (Design-Expert 13.0, Stat-Ease Inc., Minneapolis, MN, USA) was used for three-factor three-level optimization. The design space was selected based on single-factor results: (1) HWE: 40–60 °C, 60–120 min, 20–40 mL/g; (2) UAE: 50–70 °C, 90–150 min, 20–40 mL/g; (3) UA-ATPE: 50–70 °C, 120–180 min, 20–40 mL/g. Quadratic regression models were fitted and analyzed by analysis of variance (ANOVA), including

the significance of the regression model, lack-of-fit test, coefficients of determination (R^2 and adjusted R^2), and regression coefficients. The optimum extraction conditions predicted by the models were subsequently validated experimentally.

2.8. Purification of Polysaccharides

Crude extracts obtained under optimal conditions were concentrated to one-quarter volume at 65 °C under reduced pressure. Proteins were removed using Sevag reagent (chloroform/n-butanol, 4:1 *v/v*) with vigorous shaking for 20 min, followed by centrifugation (4500 rpm, 10 min). This step was repeated more than 10 times until no interfacial precipitate remained. The deproteinized solution was dialyzed (MWCO 3500 Da) against distilled water for 48 h. After concentration, polysaccharides were precipitated with four volumes of ethanol at 4 °C for 24 h. Precipitates were freeze-dried and designated as H-TAPs, UH-TAPs, and UT-TAPs. Further purification of the crude polysaccharides was performed by DEAE-52 cellulose anion-exchange chromatography (2.5 × 60 cm). Samples (30 mg/mL) were loaded onto the column and sequentially eluted with distilled water followed by stepwise NaCl solutions (0.1, 0.3, and 0.5 mol/L) at a flow rate of 1.5 mL/min. Fractions (10 mL per tube) were collected automatically, and carbohydrate-containing fractions were identified using the phenol–sulfuric acid assay. Fractions corresponding to the same chromatographic peak were pooled, dialyzed extensively against distilled water, freeze-dried, and stored at −20 °C for subsequent analyses.

2.9. Chemical Composition and Structural Characterization

The chemical composition of the crude polysaccharides was determined by measuring total carbohydrate and protein contents. Total carbohydrate content was quantified using the phenol–sulfuric acid method with D-glucose as the standard, whereas protein content was determined using the Coomassie Brilliant Blue G-250 method with bovine serum albumin (BSA) as the reference standard. Molecular weight distribution was analyzed by HPGPC using an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector and a PL aquagel-OH column (7.5 × 300 mm, Polymer Laboratories Ltd., Church Stretton, UK). Samples (5 mg/mL) were eluted with 0.1 mol/L NaCl at 35 °C. Flow rates were 0.5 mL/min for crude and 1.0 mL/min for purified samples. Calibration was performed using dextran standards (T10–T500). Monosaccharide composition was determined by high-performance liquid chromatography following 1-phenyl-3-methyl-5-pyrazolone (PMP) pre-column derivatization. Polysaccharide samples were hydrolyzed with 2 mol/L trifluoroacetic acid (TFA) at 100 °C for 2 h, derivatized with PMP under alkaline conditions at 70 °C for 2 h, neutralized, and extracted repeatedly with chloroform to remove excess derivatization reagent. The aqueous phase was analyzed using an Agilent 1260 HPLC system equipped with a Zorbax SB-C18 column (Agilent Technologies, Santa Clara, CA, USA), employing an acetonitrile/phosphate buffer (pH 6.9) mobile phase. The purified polysaccharide fraction (TAPs-0.1M) was further characterized by Fourier-transform infrared (FTIR) spectroscopy (FTIR-8400S, Shimadzu Corporation, Kyoto, Japan). Freeze-dried samples were mixed with spectroscopic-grade KBr, compressed into transparent pellets, and scanned over the range of 4000–400 cm^{-1} to identify characteristic functional groups.

2.10. In Vitro Antioxidant Activity Evaluation

The antioxidant activities of crude and purified polysaccharides were evaluated using three commonly employed in vitro assays, including DPPH radical scavenging activity, hydroxyl radical scavenging activity, and ferric reducing antioxidant power (FRAP). Polysaccharide solutions were prepared at concentrations ranging from 0.2 to 1.0 mg/mL, and L-ascorbic acid (vitamin C, Vc) was used as the positive control in all assays.

2.10.1. DPPH Radical Scavenging Assay

This assay evaluates the ability of polysaccharides to donate hydrogen atoms to neutralize stable DPPH radicals. Briefly, 100 μ L of polysaccharide solution at different concentrations was mixed with an equal volume of 0.2 mmol/L DPPH solution (dissolved in 50% ethanol) in a 96-well plate. The mixture was incubated in the dark at room temperature for 30 min, and absorbance was measured at 517 nm. The scavenging activity was calculated as:

$$\text{Scavenging Rate (\%)} = [1 - (A_s - A_r)/A_0] \times 100 \quad (2)$$

where A_s is the absorbance of the sample mixed with DPPH solution, A_r is the absorbance of the sample blank (sample without DPPH), and A_0 is the absorbance of the control containing DPPH solution without sample.

2.10.2. Hydroxyl Radical (OH) Scavenging Assay

Hydroxyl radicals were generated via a classical Fenton reaction system to evaluate the ability of polysaccharides to scavenge highly reactive oxygen species (ROS). The reaction mixture contained 50 μ L sample solution, 50 μ L 0.1 mol/L FeSO_4 , 50 μ L 9 mmol/L salicylic acid–ethanol solution, and 50 μ L 0.01% H_2O_2 .

After incubation at 37 $^\circ\text{C}$ for 30 min in the dark, absorbance was measured at 520 nm. The scavenging rate was calculated using Equation (2), where A_r represents the background control prepared by replacing H_2O_2 with deionized water, and A_0 represents the control in which the polysaccharide solution was replaced with deionized water.

2.10.3. Ferric Reducing Power Assay

The reducing power assay reflects the electron-donating ability of polysaccharides by reducing Fe^{3+} to Fe^{2+} . In brief, 1 mL sample solution was mixed with 1 mL phosphate buffer (0.2 mol/L, pH 6.6) and 1 mL 1% (*w/v*) potassium ferricyanide, followed by incubation at 50 $^\circ\text{C}$ for 20 min.

The reaction was terminated by adding 1 mL 10% (*w/v*) trichloroacetic acid, followed by centrifugation at 2000 rpm for 10 min. Then, 2.5 mL supernatant was mixed with 2.5 mL deionized water and 0.5 mL 0.1% (*w/v*) FeCl_3 solution. After 10 min of reaction at room temperature, absorbance was measured at 700 nm. Higher absorbance indicates stronger reducing power.

2.11. Statistical Analysis

All experiments were performed using at least three independent biological replicates, with each biological replicate analyzed in triplicate unless otherwise specified. Data are presented as the mean $\pm$ standard deviation (SD). Response surface methodology (RSM) analyses, including regression model fitting and analysis of variance, were performed using Design-Expert software (Version 13.0, Stat-Ease Inc., Minneapolis, MN, USA). Statistical analyses for all other experiments were conducted using GraphPad Prism (Version 10.4.1, GraphPad Software, San Diego, CA, USA). Differences among multiple groups were evaluated using ANOVA followed by Tukey's multiple comparison test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Identification and Biological Characterization of the Fungal Strain

To ensure the authenticity and purity of the fungal material used in this study, the isolate was identified using both morphological and molecular approaches. Through tissue isolation from fresh specimens (Figure 1A) followed by successive purification, a genetically stable and homogeneous pure culture of the fungal strain was successfully

obtained. In macroscopic morphological evaluation, the strain exhibited robust growth on PDA medium. As shown in Figure 1B, after 14 days of incubation, the colony surface appeared dry, initially white and gradually turning pale yellow with prolonged cultivation. The mycelium was dense with abundant aerial hyphae, a slightly raised central region, and a regular, well-defined margin. The reverse side of the colony displayed a typical earthy-yellow pigmentation (Figure 1C), consistent with previously reported characteristics of *Termitomyces* species. Microscopically, observation at 1000 $\times$ magnification (Figure 1D) revealed transparent hyphae with regular branching patterns and distinct septa, consistent with the morphological characteristics of higher basidiomycetes [23]. In addition, sporulation structures were clearly observed, and their morphological features were highly consistent with those reported for *Termitomyces* spp. [24].

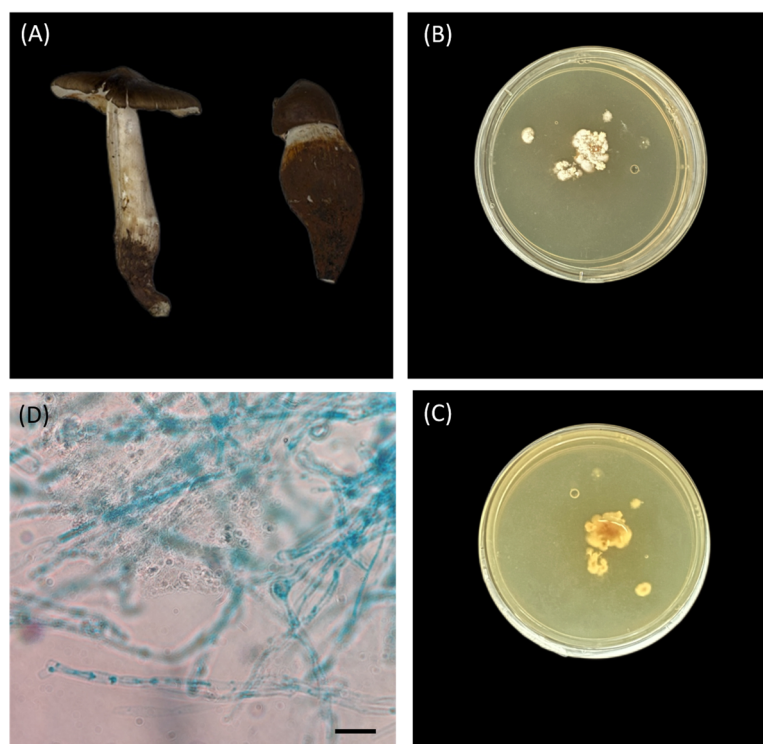


Figure 1. Morphological characterization of *T. albuminosus*. (A) Fruiting bodies of *T. albuminosus* collected from a natural habitat in July 2025. (B) Colony morphology after 14 days of cultivation in a 90-mm Petri dish, showing a dry surface with a color transition from white to pale yellow over time. The colony exhibited dense mycelial growth with abundant aerial hyphae, a slightly raised center, and well-defined, regular margins. (C) Reverse side of the colony displaying a characteristic earthy yellow pigmentation, consistent with previously reported features of *Termitomyces* species. (D) Microscopic morphology observed under a light microscope at 1000 $\times$ magnification, revealing transparent hyphae with typical branching patterns and distinct septate structures, characteristic of higher basidiomycetes. Bar, 20 μ m.

These macroscopic and microscopic characteristics collectively provided strong morphological evidence for preliminary taxonomic classification. However, considering the limitations of morphology-based identification, molecular validation was further conducted. The internal transcribed spacer (ITS) regions were amplified using universal primers ITS1/ITS4, and electrophoretic analysis of the PCR products (Figure 2A) showed a single, clear band at approximately 500–700 bp, confirming amplification specificity. The sequencing results were analyzed using BLAST (version 2.17.0) against the NCBI database, revealing up to 99% sequence similarity with multiple *Termitomyces* entries. To further clarify phylogenetic relationships, a neighbor-joining phylogenetic tree was constructed

(Figure 2B). The ITS sequence generated in this study has been deposited in the GenBank database under a pending accession number. The strain clustered within the *Termitomyces* clade with high bootstrap support, further confirming its taxonomic placement. The combined morphological observations and molecular analyses confirmed that the isolate used throughout this study was *T. albuminosus*, providing a reliable biological material for the subsequent extraction, purification, structural characterization, and antioxidant activity analyses.

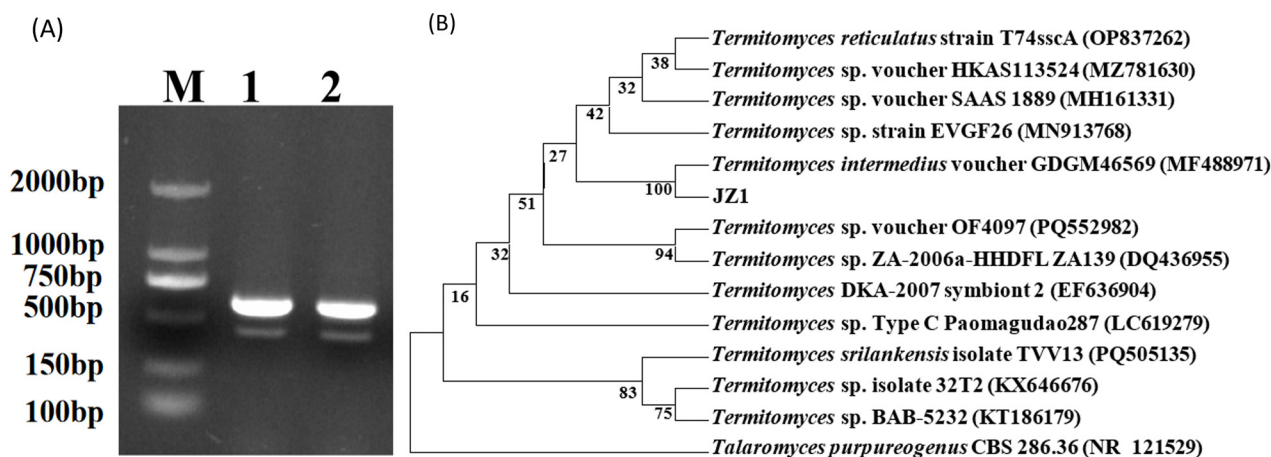


Figure 2. Molecular identification and phylogenetic analysis of the isolated strain. (A) PCR amplification of the ITS region using universal fungal primers ITS1/ITS4. Agarose gel electrophoresis (1%) revealed a single, clear band within the expected size range of approximately 500–700 bp, indicating high specificity and reliability of the amplification. Note: M, DL2000Ladder; 1, 2: amplified products. (B) Neighbor-joining phylogenetic tree based on ITS sequences showing the phylogenetic relationship between the isolated strain and representative *Termitomyces* species. Bootstrap values (%) based on 1000 replicates are indicated at the nodes.

3.2. Polysaccharide Extraction by HWE

Following taxonomic identification and characterization of the isolated *T. albuminosus* strain, attention was subsequently directed toward the efficient recovery of its bioactive polysaccharide components. As polysaccharides constitute the major antioxidant-related macromolecules in *T. albuminosus*, the development of an effective extraction strategy is a prerequisite for both structural analysis and bioactivity evaluation. However, the rigid chitin–glucan architecture of the fungal cell wall presents a substantial barrier to polysaccharide release, thereby necessitating careful selection and optimization of extraction approaches [25]. In this context, conventional hot water extraction (HWE) was first employed as a baseline reference method for subsequent comparisons [26]. It should be emphasized that all reported yields correspond to crude polysaccharide fractions, which contain target polysaccharides together with minor amounts of proteins and other water-soluble co-extracted constituents. These crude extracts were used for yield assessment and structural comparison, thereby reflecting the practical extraction efficiency of each process.

3.2.1. Single-Factor Analysis and Model Establishment

To define the operational space for multivariate optimization, the effects of extraction temperature, time, and solid–liquid ratio on polysaccharide yield were first evaluated individually (Figure 3A). All three variables exhibited a characteristic increase–decrease trend, indicating the existence of optimal extraction regions. Yield increased with temperature up to 50 °C and declined thereafter, which may be associated with partial thermal degradation or altered solubilization equilibrium. A similar pattern was observed for extraction time, with a maximum at 90 min, beyond which prolonged exposure led to

reduced yield, possibly due to structural degradation or co-extraction of impurities. The solid–liquid ratio showed an optimum at 30 mL/g. Based on these results, the parameter ranges for response surface methodology (RSM) were defined as 40–60 °C (temperature), 60–120 min (time), and 20–40 mL/g (solid–liquid ratio).

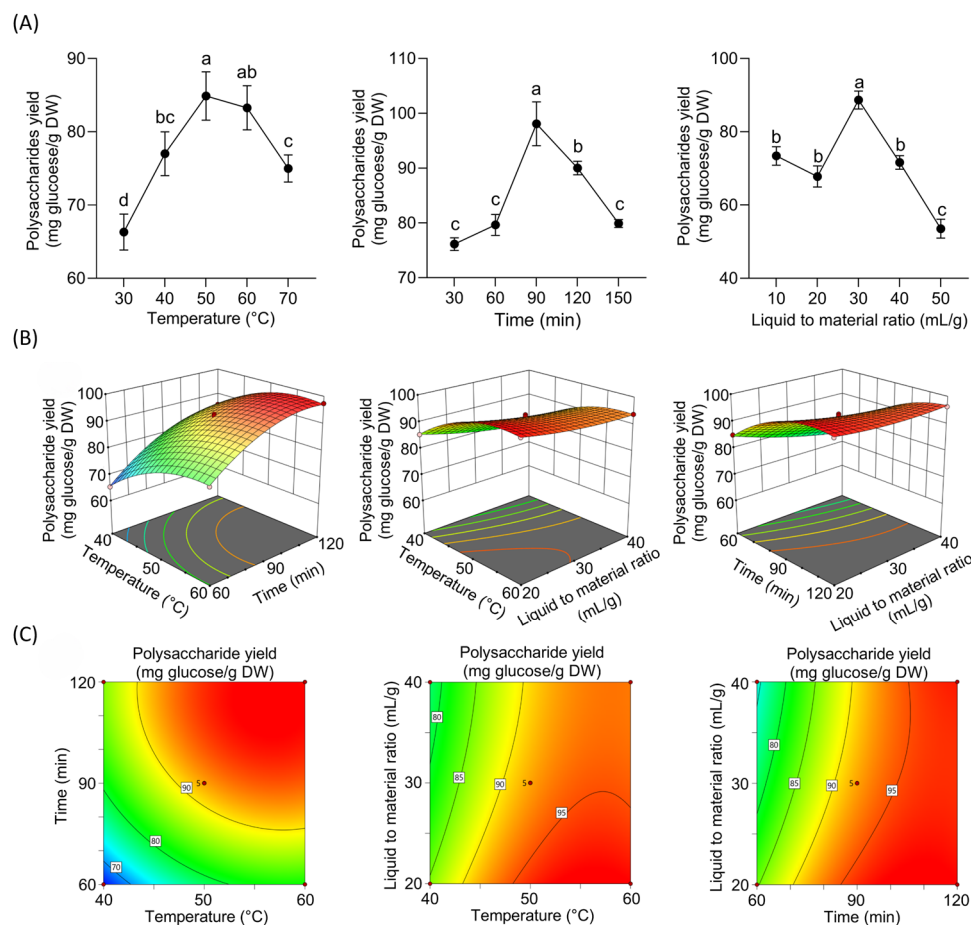


Figure 3. Optimization of polysaccharide extraction by HWE. (A) Effects of extraction temperature, time, and liquid-to-solid ratio on polysaccharide yield. All factors showed an initial increase followed by a decrease, indicating optimal extraction conditions. Different lowercase letters (a, ab, b, bc, c, d) above the bars indicate significant differences among groups according to Tukey's multiple comparison test ($p < 0.05$). Groups sharing one or more letters are not significantly different. (B,C) Response surface (B) and contour plots (C) illustrating interactions among variables, with significant interactions observed between time and temperature as well as time and liquid-to-solid ratio, while the interaction between temperature and liquid-to-solid ratio was relatively weak.

3.2.2. Model Fitting, Interaction Analysis, and Optimization

A Box–Behnken Design (BBD) with 17 experimental runs was employed to model the combined effects of the selected variables (Table 1). The quadratic regression model was highly significant ($p < 0.0001$), with a non-significant lack-of-fit ($p = 0.5565$), indicating strong model adequacy. The high coefficients of determination ($R^2 = 0.9981$, $R_{adj}^2 = 0.9957$) confirmed excellent agreement between predicted and experimental values. The fitted model was expressed as:

$$\text{Yield} = 95.269 + 5.457A + 1.257B - 2.116C - 0.004AB + 0.009AC + 0.008BC - 0.047A^2 - 0.006B^2 + 0.011C^2 \quad (3)$$

where A, B, and C denote the coded variables corresponding to extraction temperature (°C), extraction time (min), and liquid-to-solid ratio (mL/g), respectively. AB, AC, and BC represent interaction terms, whereas A^2 , B^2 , and C^2 denote quadratic effects.

Table 1. Response surface experimental design and ANOVA results for HWE.

Run	A *	B *	C *	Polysaccharide Yield (mg/g)	Source	Sum of Squares	df	Mean Square	F Value	p Value
1	40	120	30	85.76	Model	1316.24	9	146.25	409.72	<0.0001
2	50	90	30	93.18	A	381.22	1	381.22	1068.00	<0.0001
3	60	90	40	93.75	B	623.41	1	623.41	1746.52	<0.0001
4	50	60	20	85.04	C	62.96	1	62.96	176.39	<0.0001
5	50	120	20	97.20	AB	4.49	1	4.49	12.59	0.0094
6	40	90	40	78.10	AC	3.30	1	3.30	9.25	0.0188
7	50	90	30	91.67	BC	23.24	1	23.24	65.11	<0.0001
8	50	90	30	92.27	A ²	93.85	1	93.85	262.92	<0.0001
9	60	90	20	97.41	B ²	111.13	1	111.13	311.35	<0.0001
10	60	60	30	81.22	C ²	5.06	1	5.06	14.19	0.0070
11	50	90	30	91.74	Residual	2.50	7	0.36		
12	50	120	40	96.27	Lack of Fit	0.93	3	0.31	0.80	0.5565
13	40	90	20	85.39	Pure Error	1.56	4	0.39		
14	40	60	30	65.32	Cor Total	1318.74	16			
15	50	90	30	92.57	R ²	0.99				
16	60	120	30	97.42	R _{adj} ²	0.99				
17	50	60	40	74.47						

* A: Temperature (°C); B: Time (min); C: Liquid-to-solid ratio (mL/g).

Analysis of variance and response surface visualization (Figure 3B,C) consistently indicated that extraction time exerted the greatest influence on yield, followed by temperature, while the solid–liquid ratio had a comparatively minor effect. Significant interaction effects were observed between time and temperature, as well as time and solid–liquid ratio, whereas the interaction between temperature and solid–liquid ratio was weak. These observations suggest that the extraction efficiency of HWE is primarily influenced by extraction time and temperature, reflecting the combined effects of extraction kinetics and thermal diffusion.

3.2.3. Model Validation and Practical Implications

The model predicted optimal extraction conditions of 55.30 °C, 108.40 min, and 34.61 mL/g, with a corresponding yield of 97.60 mg/g. For practical implementation, these parameters were adjusted to 55 °C, 110 min, and 35 mL/g. The experimentally implemented conditions were obtained by rounding the model-predicted optimum to practically achievable operating parameters, without materially affecting the predicted extraction yield. Under these conditions, experimental validation ($n = 3$) yielded 97.15 ± 1.28 mg/g, closely matching the predicted value and confirming the robustness and predictive reliability of the model. Collectively, these results demonstrate that RSM provides an effective framework for optimizing HWE conditions. Although HWE achieved satisfactory extraction efficiency, the relatively long extraction duration and moderate polysaccharide yield highlight the inherent limitations of conventional thermal extraction, thereby justifying the exploration of more efficient extraction strategies in the subsequent sections.

3.3. Polysaccharides Extraction by the UAE

3.3.1. Single-Factor Analysis and Model Establishment

To define the operational domain for UAE optimization, the effects of extraction temperature, time, solid–liquid ratio, and ultrasonic power on polysaccharide yield were evaluated (Figure 4A). Temperature and time both exhibited a typical bell-shaped trend, with maximum yields at 60 °C and 120 min, respectively, indicating optimal conditions for polysaccharide release and stability. The solid–liquid ratio similarly showed an optimum at 30 mL/g, reflecting a balance between mass transfer efficiency and dilution effects. In

contrast, ultrasonic power (240–400 W) showed no statistically significant effect on yield ($p > 0.05$), suggesting that within this range, cavitation intensity was not the limiting factor. While acoustic cavitation is the primary mechanism for cell wall disruption, it is likely to reach a saturation point at or below 240 W in this specific system; beyond this threshold, increased power may primarily contribute to thermal effects rather than further mechanical lysis [27]. Therefore, ultrasonic power was excluded from subsequent response surface modeling. Based on these findings, the selected parameter ranges were 50–70 °C (temperature), 90–150 min (time), and 20–40 mL/g (solid–liquid ratio).

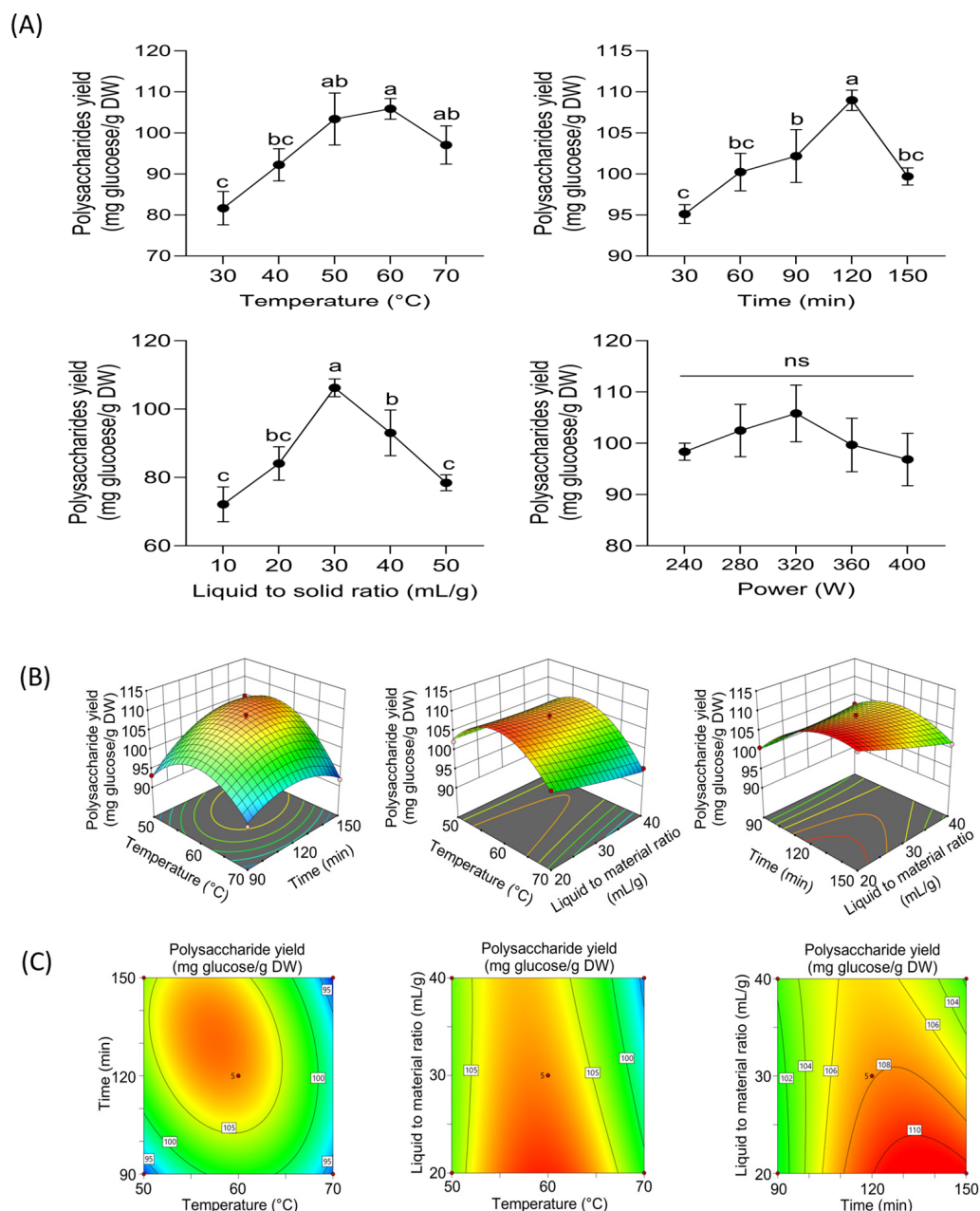


Figure 4. Optimization of polysaccharide extraction by UAE. **(A)** Effects of extraction temperature, time, liquid-to-solid ratio, and ultrasonic power on polysaccharide yield. Temperature, time, and liquid-to-solid ratio showed a typical increase–decrease trend with optimal values at 60 °C, 120 min, and 30 mL/g, respectively, while ultrasonic power had no significant effect within the tested range. Different lowercase letters above the bars indicate significant differences among groups according to Tukey’s multiple comparison test ($p < 0.05$). Groups sharing one or more letters are not significantly different. ns: not significant ($p > 0.05$). **(B,C)** Response surface **(B)** and contour plots **(C)** showing

interactions among variables. The interaction between temperature and time was the most significant, with temperature exerting a greater influence on yield, followed by time, whereas the effect of liquid-to-solid ratio was comparatively weaker.

3.3.2. Model Fitting, Interaction Analysis, and Optimization

The BBD was applied to model the combined effects of the selected variables (Table 2). The regression model was highly significant ($p < 0.0001$) with a non-significant lack-of-fit ($p = 0.3024$), confirming good model adequacy. The high coefficient of determination ($R^2 = 0.9883$) indicated strong predictive capability. The fitted quadratic model was:

$$\text{Yield} = -371.379 + 10.973A + 2.156B + 1.780C - 0.010AB - 0.017AC - 0.009BC - 0.079A^2 - 0.005B^2 + 0.003C^2 \quad (4)$$

where A, B, and C represent the coded variables corresponding to ultrasonic power (W), extraction time (min), and liquid-to-solid ratio (mL/g), respectively. The interaction terms (AB, AC, and BC) describe the combined effects of two variables, whereas A^2 , B^2 , and C^2 represent their quadratic effects.

Table 2. Response surface experimental design and ANOVA for UAE.

Run	A *	B *	C *	Polysaccharide Yield (mg/g)	Source	Sum of Squares	df	Mean Square	F Value	p Value
1	70	150	30	91.74	Model	573.26	9	63.7	65.93	<0.0001
2	60	120	30	106.96	A	58.76	1	58.76	60.82	0.0001
3	50	120	20	102.25	B	46.36	1	46.36	47.98	0.0002
4	70	90	30	92.65	C	24.37	1	24.37	25.22	0.0015
5	60	120	30	107.49	AB	38.08	1	38.08	39.42	0.0004
6	60	150	40	101.32	AC	11.78	1	11.78	12.2	0.0101
7	50	150	30	104.61	BC	30.00	1	30.00	31.05	0.0008
8	60	90	20	100.69	A^2	263.58	1	263.58	272.81	<0.0001
9	60	120	30	108.78	B^2	83.06	1	83.06	85.97	<0.0001
10	60	150	20	110.53	C^2	0.341	1	0.34	0.35	0.5712
11	60	90	40	102.43	Residual	6.76	7	0.97		
12	60	120	30	108.85	Lack of Fit	3.8	3	1.27	1.71	0.3024
13	60	120	30	107.42	Pure Error	2.97	4	0.74		
14	70	120	20	101.54	Cor Total	580.02	16			
15	70	120	40	94.86	R^2	0.99				
16	50	120	40	102.43	R_{adj}^2	0.97				
17	50	90	30	93.18						

* A: Temperature (°C); B: Time (min); C: Liquid-to-solid ratio (mL/g).

Analysis of variance and response surface visualization (Figure 4B,C) consistently showed that extraction temperature exerted the strongest influence on yield, followed by extraction time, while the solid–liquid ratio had a comparatively minor effect. Among interaction terms, the temperature–time interaction was the most pronounced, indicating that thermal effects and ultrasonic cavitation act synergistically to enhance polysaccharide release. These results suggest that, under UAE conditions, extraction efficiency is primarily governed by temperature-dependent cavitation dynamics rather than diffusion-limited processes.

3.3.3. Model Validation and Practical Implications

The model predicted optimal conditions of 61.28 °C, 132.25 min, and 20.53 mL/g, with a corresponding yield of 110.68 mg/g. For practical implementation, these were adjusted to 60 °C, 130 min, and 20 mL/g. The experimentally implemented conditions were obtained by rounding the model-predicted optimum to practically achievable operating parameters without materially affecting the predicted extraction yield. Under the optimized conditions,

the experimentally determined polysaccharide yield reached 110.32 ± 3.68 mg/g ($n = 3$), which closely agreed with the predicted value, confirming the robustness and predictive capability of the regression model. Compared with HWE, UAE produced a substantially higher polysaccharide yield while requiring a shorter extraction time. This improvement is likely attributable to ultrasonic cavitation, which enhances solvent penetration and facilitates the disruption of fungal cell walls [25], thereby promoting polysaccharide release. Collectively, these results demonstrate that UAE is a more efficient extraction strategy than conventional HWE for recovering crude polysaccharides from *T. albuminosus* under the conditions investigated [26,27].

3.4. Polysaccharides Extraction by the UA-ATPE

3.4.1. System Selection and Factor Screening

To further explore an alternative extraction strategy capable of integrating extraction with preliminary purification, ultrasound-assisted aqueous two-phase extraction (UA-ATPE) was subsequently investigated. To establish an efficient aqueous two-phase system (ATPS) for polysaccharide extraction, several ethanol/salt systems were initially screened, including ZnSO_4 , Na_2CO_3 , K_2CO_3 , $(\text{NH}_4)_2\text{SO}_4$, NaH_2PO_4 , K_2HPO_4 , and $\text{Na}_2\text{S}_2\text{O}_3$. Among the tested salt systems, $\text{Na}_2\text{S}_2\text{O}_3$ was excluded due to its interference with the phenol-sulfuric acid assay, where it reacts with sulfuric acid to generate elemental sulfur, thereby interfering with accurate polysaccharide quantification [28,29]. Among the remaining systems, the ethanol/ $(\text{NH}_4)_2\text{SO}_4$ system produced the highest polysaccharide yield (Figure 5A). Further optimization of phase composition identified 30% (*w/w*) ethanol and 16% (*w/w*) ammonium sulfate as optimal. Single-factor experiments (Figure 5B) revealed typical optimum ranges for temperature, time, and solid-liquid ratio, with the highest polysaccharide yield observed at 60 °C, 150 min, and 30 mL/g, respectively. In contrast, ultrasonic power showed no significant effect within the tested range ($p > 0.05$) and was therefore excluded from subsequent modeling. Based on these results, the factor ranges for response surface optimization were set as: temperature 50–70 °C, time 120–180 min, and solid-liquid ratio 20–40 mL/g.

3.4.2. Model Construction and Interaction Analysis

The BBD was employed again to model the extraction process, and the results are summarized in Table 3. The quadratic model was highly significant ($p < 0.0001$) with a non-significant lack of fit ($p = 0.1312$), indicating good model adequacy. The high coefficient of determination ($R^2 = 0.9859$) further confirmed strong predictive capability. ANOVA results demonstrated that temperature (A) was the dominant factor affecting polysaccharide yield, whereas time (B) and solid-liquid ratio (C) exerted comparatively minor effects. The fitted quadratic regression model was expressed as:

$$\text{Yield} = -10.636 + 4.816A - 0.772B + 0.044C + 0.003AB + 0.005AC + 0.002BC - 0.041A^2 + 0.002B^2 - 0.012C^2 \quad (5)$$

where A, B, and C represent the coded variables corresponding to ultrasonic power (W), extraction time (min), and liquid-to-solid ratio (mL/g), respectively. The interaction terms (AB, AC, and BC) describe the combined effects of two variables, whereas A^2 , B^2 , and C^2 represent their quadratic effects.

Response surface and contour analyses (Figure 5C,D) showed a moderate interaction between extraction temperature and extraction time, whereas the remaining interaction effects were comparatively weak. These observations were consistent with the ANOVA results, indicating that extraction temperature had the greatest influence on polysaccharide yield, followed by extraction time and liquid-to-solid ratio.

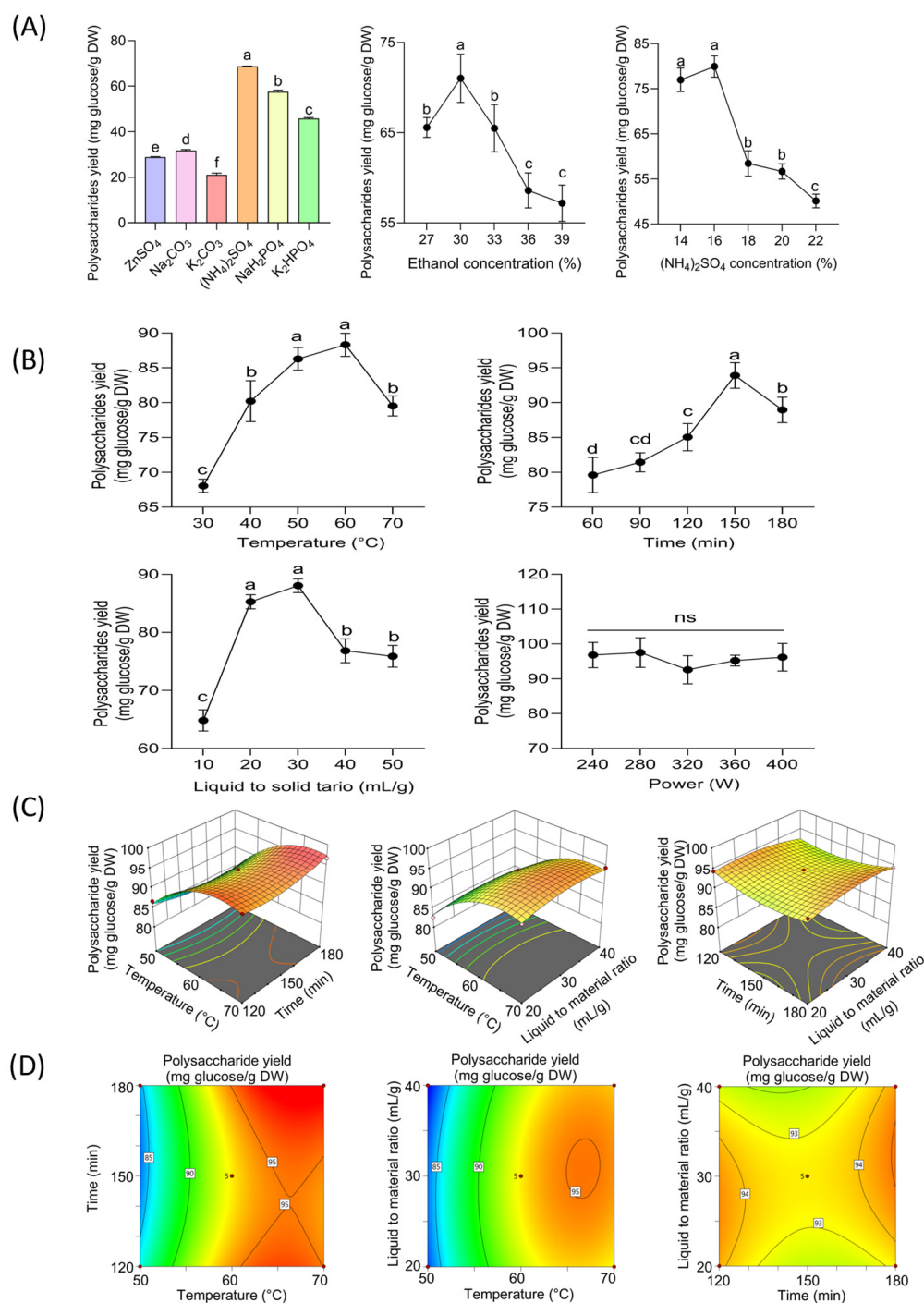


Figure 5. Optimization of polysaccharide extraction using UA-ATPE. **(A)** Selection and optimization of the aqueous two-phase extraction system. Among the tested systems, ammonium sulfate/ethanol showed the highest extraction efficiency. Optimization of system composition indicated that 30% ethanol and 16% ammonium sulfate yielded the maximum polysaccharide recovery. Different lowercase letters above the bars indicate significant differences among groups according to Tukey's multiple comparison test ($p < 0.05$). Groups sharing one or more letters are not significantly different. ns: not significant ($p > 0.05$). **(B)** Effects of key extraction parameters on polysaccharide yield. Temperature, time, and liquid-to-solid ratio exhibited optimal ranges with maximum yields at 60 $^{\circ}C$, 150 min, and 30 mL/g, respectively, while ultrasonic power showed no significant effect. **(C,D)** Response surface and contour plots illustrating interactions among variables. A moderate interaction was observed between temperature and time, whereas other interactions were relatively weak. Temperature exerted the strongest influence on yield, followed by time, while liquid-to-solid ratio showed the least effect.

Table 3. Response surface experimental design and ANOVA for UA-ATPE.

Run	A *	B *	C *	Polysaccharide Yield (mg/g)	Source	Sum of Squares	df	Mean Square	F Value	p Value
1	60	150	30	93.07	Model	329.45	9	36.61	54.55	<0.0001
2	70	120	30	95.12	A	235.82	1	235.82	351.43	<0.0001
3	50	150	20	82.53	B	0.47	1	0.47	0.71	0.4283
4	60	150	30	92.97	C	0.15	1	0.15	0.22	0.6516
5	60	120	40	91.65	AB	3.89	1	3.89	5.80	0.0469
6	60	180	40	94.45	AC	1.03	1	1.03	1.54	0.2549
7	70	150	20	92.87	BC	2.20	1	2.20	3.28	0.1128
8	50	150	40	82.12	A ²	70.74	1	70.74	105.41	<0.0001
9	60	120	20	94.29	B ²	9.79	1	9.79	14.59	0.0065
10	50	180	30	84.41	C ²	6.30	1	6.30	9.39	0.0182
11	60	150	30	93.29	Residual	4.70	7	0.67		
12	70	150	40	94.49	Lack of Fit	3.39	3	1.13	3.45	0.1312
13	60	180	20	94.12	Pure Error	1.31	4	0.33		
14	60	150	30	92.97	Cor Total	334.15	16			
15	60	150	30	94.32	R ²	0.99				
16	70	180	30	96.75	R _{adj} ²	0.97				
17	50	120	30	86.72						

* A: Temperature (°C); B: Time (min); C: Liquid-to-solid ratio (mL/g).

3.4.3. Model Validation and Process Implications

The model predicted optimal extraction conditions of 66.03 °C, 178.55 min, and 29.44 mL/g, corresponding to a theoretical yield of 97.23 mg/g. For practical implementation, these parameters were adjusted to 65 °C, 180 min, and 30 mL/g. The experimentally implemented conditions were obtained by rounding the model-predicted optimum to practically achievable operating parameters without materially affecting the predicted extraction yield. Under these conditions, the experimental yield reached 96.71 ± 2.15 mg/g, closely matching the predicted value. This agreement confirms the robustness and reliability of the model. Although the optimized UA-ATPE process produced a slightly lower polysaccharide yield than UAE, it integrates extraction and preliminary purification into a single process, thereby reducing subsequent purification requirements. Consequently, UA-ATPE may represent an attractive alternative when downstream processing efficiency and product enrichment are prioritized over maximum extraction yield. Therefore, this method provides a viable alternative for polysaccharide production when process integration and product purity are prioritized over maximum yield.

3.5. Chemical Composition and Structural Characterization of Crude Polysaccharides

To systematically evaluate the effects of different extraction strategies on the physicochemical properties of *T. albuminosus* polysaccharides, the crude polysaccharides obtained by hot water extraction (H-TAPs), ultrasound-assisted extraction (UH-TAPs), and ultrasound-assisted aqueous two-phase extraction (UT-TAPs) were comparatively characterized with respect to their chemical composition, molecular weight distribution, and monosaccharide composition.

As summarized in Table 4, the extraction method markedly influenced the composition of the crude polysaccharide preparations. Among the three extracts, UH-TAPs exhibited the highest carbohydrate content ($55.58 \pm 0.19\%$), followed by H-TAPs ($49.16 \pm 0.15\%$), whereas UT-TAPs contained substantially lower carbohydrate content ($25.35 \pm 0.25\%$). Conversely, H-TAPs contained the highest residual protein content ($6.24 \pm 0.49\%$), while UH-TAPs exhibited the lowest protein level ($3.17 \pm 0.64\%$). These observations indicate that the extraction strategy substantially affects the composition of the crude polysaccharide fractions, and that UAE produced crude extracts with a relatively higher carbohydrate

content and lower residual protein under the conditions investigated [30,31]. The lower carbohydrate content observed for UT-TAPs may be associated with the selective partitioning behavior of the aqueous two-phase system, which simultaneously extracts polysaccharides and other water-soluble constituents.

Table 4. Chemical composition of *T. albuminosus* crude polysaccharides from different extraction methods.

Component	H-TAPs	UH-TAPs	UT-TAPs
Carbohydrate (%)	49.16 ± 0.15	55.58 ± 0.19	25.35 ± 0.25
Protein (%)	6.24 ± 0.49	3.17 ± 0.64	3.87 ± 0.84
Man (mol%)	18.87	34.66	32.02
Rha (mol%)	15.71	25.14	ND
Glc (mol%)	13.53	13.74	1.71
Gal (mol%)	43.99	24.36	53.56
Xyl (mol%)	0.60	1.92	1.55
Fuc (mol%)	7.31	0.18	11.16
High-molecular-weight fraction (>2000 kDa), peak area (%)	20.65	15.78	31.57
Low-molecular-weight fraction (<50 kDa), peak area (%)	79.35	84.22	68.43

Note: Man, mannose; Rha, rhamnose; Glc, glucose; Gal, galactose; Xyl, xylose; Fuc, fucose; H-TAPs, crude polysaccharides extracted by hot water extraction (HWE); UH-TAPs, crude polysaccharides extracted by ultrasound-assisted extraction (UAE); UT-TAPs, crude polysaccharides extracted by ultrasound-assisted aqueous two-phase extraction (UA-ATPE); ND, not detected. Carbohydrate and protein contents are presented as mean ± SD ($n = 3$). Monosaccharide composition and molecular-weight distribution are expressed as relative molar percentages and chromatographic peak area percentages, respectively.

Molecular weight distributions were further analyzed by high-performance gel permeation chromatography (HPGPC) (Figure 6A). All three samples exhibited bimodal molecular weight distributions consisting of a high-molecular-weight fraction (>2000 kDa) and a low-molecular-weight fraction (<50 kDa), indicating that the extracted polysaccharides were heterogeneous. However, the relative proportions of these fractions differed considerably among the extraction methods. UH-TAPs contained the highest proportion of low-molecular-weight components (84.22%), whereas UT-TAPs exhibited the largest high-molecular-weight fraction (31.57%). These differences suggest that the extraction strategy is associated with changes in the molecular weight distribution of the recovered polysaccharides. In particular, ultrasonic treatment may facilitate partial fragmentation of larger polysaccharide molecules through cavitation-induced mechanical effects, whereas the aqueous two-phase system may preferentially partition polysaccharide fractions according to differences in molecular size and physicochemical properties [32,33].

The monosaccharide compositions of the crude polysaccharides were subsequently determined using PMP pre-column derivatization (Figure 6B). H-TAPs and UH-TAPs exhibited similar monosaccharide profiles, consisting primarily of mannose (Man), rhamnose (Rha), glucose (Glc), and galactose (Gal), together with smaller amounts of xylose (Xyl) and fucose (Fuc). Compared with H-TAPs, UH-TAPs contained higher proportions of mannose (34.66 mol%) and rhamnose (25.14 mol%). In contrast, UT-TAPs displayed a distinctly different monosaccharide composition, being predominantly composed of galactose (53.56 mol%), mannose (32.02 mol%), and fucose (11.16 mol%), while rhamnose was not detected and glucose was present only at a trace level (1.71 mol%). The marked differences in monosaccharide composition among the three crude polysaccharide preparations indicate that extraction strategy substantially influences the populations of polysaccharides recovered from *T. albuminosus*. In particular, the distinctive composition of UT-TAPs is likely attributable to the selective partitioning characteristics of the ethanol/ammonium sulfate aqueous two-phase system, which preferentially enriches polysaccharide fractions

according to differences in polarity, solubility, and molecular size [34,35]. Further purification and characterization of the major antioxidant-active fraction were therefore performed to investigate these structural differences in greater detail.

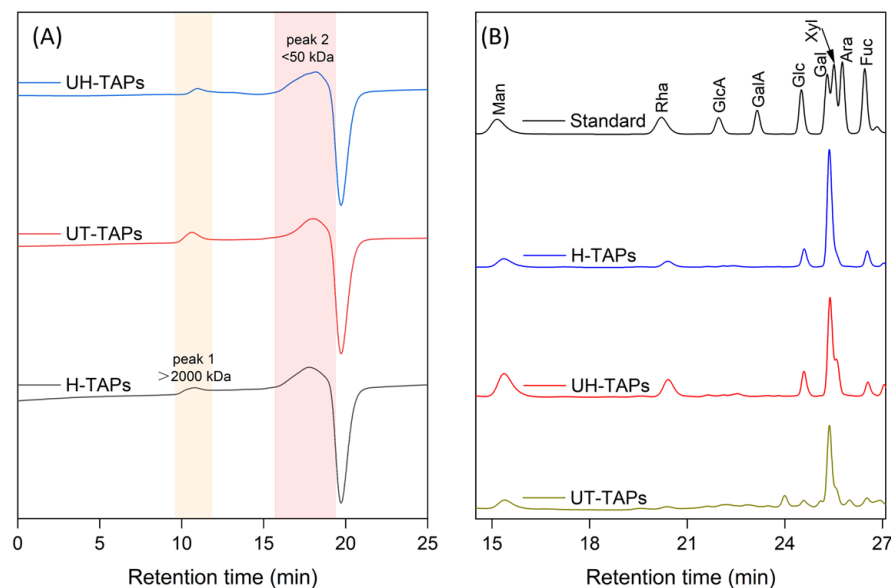


Figure 6. Chemical composition and structural characteristics of crude polysaccharides from different extraction methods. **(A)** Molecular weight distribution of H-TAPs, UH-TAPs, and UT-TAPs analyzed by HPGPC, showing distinct distribution profiles and varying proportions of high- and low-molecular-weight fractions among the three samples. **(B)** Monosaccharide composition and molar ratios determined by PMP pre-column derivatization, indicating significant differences in monosaccharide profiles depending on the extraction method, with UH-TAPs enriched in mannose and rhamnose, and UT-TAPs dominated by galactose, mannose, and fucose.

3.6. In Vitro Antioxidant Activity of Crude Polysaccharides

To compare the antioxidant properties of crude polysaccharides obtained using different extraction strategies, the free radical scavenging activities against DPPH and hydroxyl radicals, together with ferric reducing antioxidant power, were evaluated in vitro. Antioxidant activities increased with increasing polysaccharide concentration for all three crude extracts, demonstrating a concentration-dependent response (Figure 7).

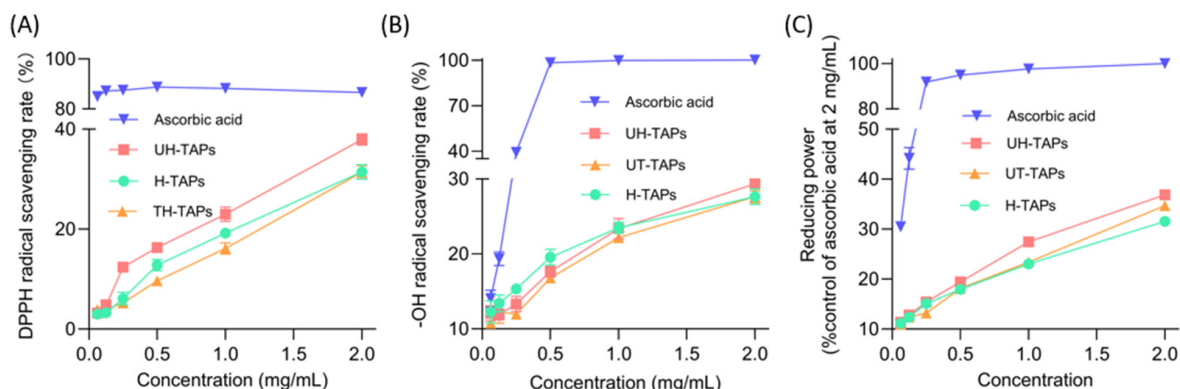


Figure 7. In vitro antioxidant activities of crude polysaccharides extracted by different methods. **(A)** DPPH radical scavenging activity. **(B)** Hydroxyl radical scavenging activity. **(C)** Ferric reducing antioxidant power. Data are presented as mean $\pm$ SD ($n = 3$).

As shown in Figure 7A, all crude polysaccharides exhibited progressively stronger DPPH radical scavenging activity as the concentration increased. Among the three samples,

UH-TAPs consistently showed the highest scavenging activity over the tested concentration range, followed by H-TAPs, whereas UT-TAPs exhibited comparatively lower activity. At the highest tested concentration, UH-TAPs achieved the greatest DPPH radical scavenging capacity, indicating that the extraction method influenced the antioxidant properties of the resulting crude polysaccharide preparations. A similar concentration-dependent trend was observed for hydroxyl radical scavenging activity (Figure 7B). UH-TAPs again exhibited the highest scavenging activity across the experimental concentration range, whereas H-TAPs and UT-TAPs showed comparatively lower activities. Although all three extracts effectively scavenged hydroxyl radicals, the magnitude of the response differed depending on the extraction strategy, suggesting that the physicochemical characteristics of the extracted polysaccharides varied among the three methods. The ferric reducing antioxidant power of the crude polysaccharides also increased with increasing concentration (Figure 7C). Consistent with the radical scavenging assays, UH-TAPs exhibited the strongest reducing capacity, followed by H-TAPs and UT-TAPs. The reducing power of all samples remained lower than that of the positive control, indicating that although the crude polysaccharides possessed measurable electron-donating ability, their reducing capacity was weaker than that of the standard antioxidant under identical experimental conditions.

Collectively, the three antioxidant assays consistently demonstrated that the extraction strategy was associated with differences in the antioxidant activities of the crude polysaccharide preparations. Under the experimental conditions evaluated, crude polysaccharides obtained by UAE exhibited higher antioxidant activities than those extracted using HWE or UA-ATPE. Because these crude extracts contain polysaccharides together with minor amounts of co-extracted components, further purification was performed to identify the major antioxidant-active polysaccharide fraction and to facilitate subsequent structural characterization.

3.7. Screening of Antioxidant-Active Polysaccharide Fractions from *T. albuminosus*

To identify the polysaccharide fraction primarily responsible for the antioxidant activity observed in the crude extracts, the crude polysaccharide preparations obtained from HWE, UAE, and UA-ATPE were further fractionated by DEAE-52 anion-exchange chromatography. As shown in Figure 8A, each crude polysaccharide preparation was separated into several fractions by sequential elution with distilled water and increasing concentrations of NaCl solution.

The antioxidant activities of the collected fractions were subsequently evaluated using the same *in vitro* antioxidant assays described above. As shown in Figure 8B,D, marked differences in antioxidant activity were observed among the purified fractions. Among all fractions, TAPs-0.1M, eluted with 0.1 M NaCl, consistently exhibited the strongest DPPH radical scavenging activity, hydroxyl radical scavenging activity, and ferric reducing antioxidant power throughout the tested concentration range. The remaining fractions displayed comparatively lower antioxidant activities under identical experimental conditions. The consistent performance of TAPs-0.1M across the three independent antioxidant assays suggests that this fraction contains the major antioxidant-active polysaccharide population recovered from *T. albuminosus*. Consequently, TAPs-0.1M was selected for subsequent physicochemical characterization and detailed structural analysis.

It should be noted that the antioxidant activities reported here represent comparative evaluations of purified polysaccharide fractions under identical *in vitro* experimental conditions. The present results identify TAPs-0.1M as the major antioxidant-active fraction within the extracted polysaccharides but do not establish the structural determinants responsible for its antioxidant activity. Further structural characterization was therefore performed to investigate the physicochemical properties of this fraction.

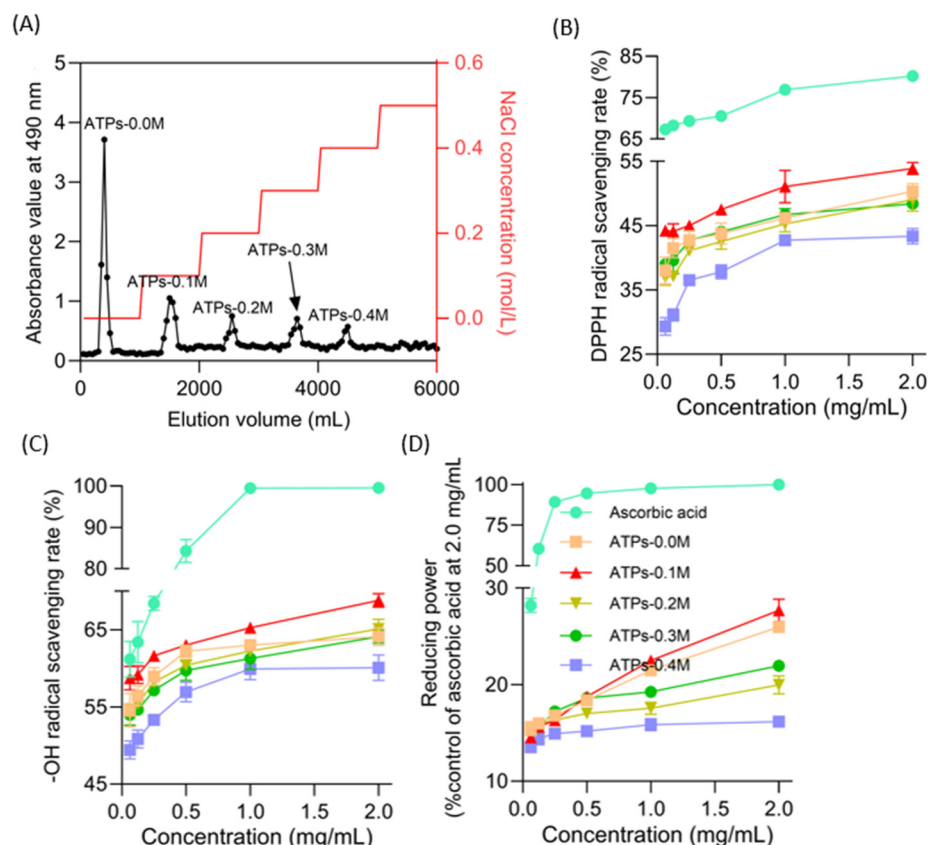


Figure 8. Screening of antioxidant-active polysaccharide fractions obtained by DEAE-52 anion-exchange chromatography. (A) Elution profile of crude *T. albuminosus* polysaccharides on a DEAE-52 cellulose column using distilled water followed by stepwise NaCl elution. (B) DPPH radical scavenging activity of the purified fractions. (C) Hydroxyl radical scavenging activity of purified fractions. (D) Ferric reducing antioxidant power of the purified fractions. Data are presented as mean $\pm$ SD ($n = 3$).

3.8. Structural Characterization of TAPs-0.1M

Based on the antioxidant screening described above, TAPs-0.1M was identified as the major antioxidant-active polysaccharide fraction and was therefore selected for further physicochemical characterization. The purified fraction was analyzed with respect to molecular weight distribution, monosaccharide composition, and functional group characteristics to obtain preliminary structural information.

The molecular weight distribution of TAPs-0.1M was determined by high-performance gel permeation chromatography (HPGPC). As shown in Figure 9A, TAPs-0.1M exhibited a single symmetrical chromatographic peak, indicating that the purification procedure effectively enriched a relatively homogeneous polysaccharide fraction. Based on calibration with dextran standards, the average molecular weight of TAPs-0.1M was estimated to be 6.37 kDa, which was substantially lower than the predominant high-molecular-weight fractions observed in the crude polysaccharide preparations. The monosaccharide composition of TAPs-0.1M was subsequently analyzed using PMP pre-column derivatization (Figure 9B). The purified polysaccharide consisted primarily of glucose, mannose, galactose, xylose, glucuronic acid, rhamnose, and fucose, demonstrating that TAPs-0.1M is an acidic heteropolysaccharide. Compared with the corresponding crude polysaccharide preparation, the relative proportions of individual monosaccharides changed after purification, indicating that DEAE-52 anion-exchange chromatography selectively enriched a specific polysaccharide population. The monosaccharide composition reported here corresponds to the purified TAPs-0.1M fraction and therefore differs from that of the crude polysaccha-

ride preparations shown in Table 4, reflecting the selective enrichment of a specific acidic polysaccharide fraction during DEAE-52 anion-exchange chromatography.

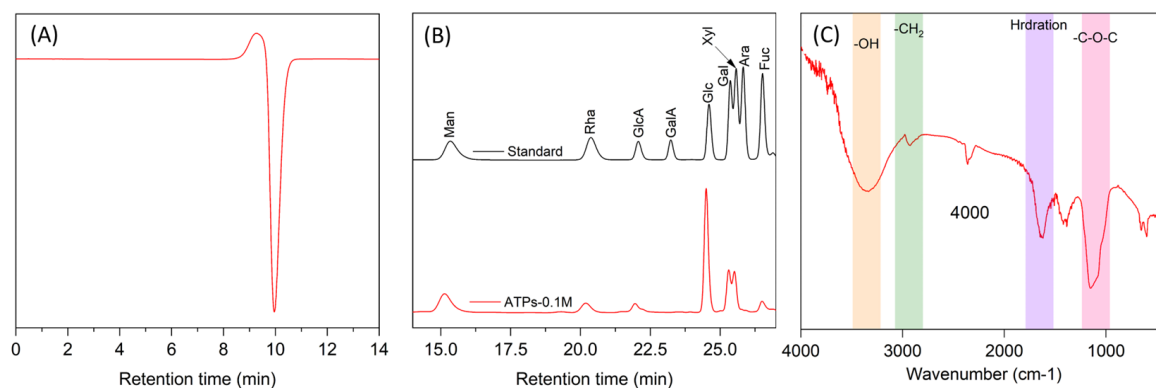


Figure 9. Structural characterization of TAPs-0.1M. (A) High-performance gel permeation chromatogram showing the molecular weight distribution of TAPs-0.1M. (B) Monosaccharide composition of TAPs-0.1M determined by PMP pre-column derivatization. (C) FTIR spectrum of TAPs-0.1M showing the characteristic absorption bands of polysaccharides.

The structural features of TAPs-0.1M were further examined by Fourier-transform infrared (FTIR) spectroscopy (Figure 9C). A broad absorption band around 3400 cm⁻¹ was assigned to O–H stretching vibrations, while the weak absorption near 2930 cm⁻¹ corresponded to C–H stretching vibrations. Characteristic absorption bands observed in the 1600–1400 cm⁻¹ region were consistent with the presence of carboxyl groups, supporting the existence of uronic acid residues within the polysaccharide. Strong absorption bands between 1200 and 1000 cm⁻¹ were attributed to C–O–C and C–O stretching vibrations characteristic of polysaccharide glycosidic structures. In addition, the absorption signal observed in the 800–900 cm⁻¹ region suggested the presence of glycosidic linkages typical of polysaccharides.

Collectively, these results demonstrate that TAPs-0.1M is a relatively homogeneous acidic heteropolysaccharide with a moderate molecular weight and a complex monosaccharide composition. The structural characteristics identified here provide a physicochemical basis for subsequent discussion of the antioxidant properties of this fraction. However, the present analyses do not establish direct relationships between individual structural features and antioxidant activity. More detailed structural elucidation, including glycosidic linkage analysis and nuclear magnetic resonance (NMR) spectroscopy, will be required to further define the structure of TAPs-0.1M.

4. Discussion

4.1. Comparison of Extraction Strategies and Process Optimization

Efficient extraction is fundamental to the structural characterization and functional evaluation of mushroom polysaccharides. Because fungal cell walls possess a rigid chitin–β-glucan matrix that restricts polysaccharide release, the extraction strategy can markedly influence not only extraction yield but also the physicochemical characteristics of the recovered polysaccharides. In this study, three representative extraction methods—HWE, UAE, and UA-ATPE—were systematically optimized and comparatively evaluated under their respective optimal conditions. Among the three methods, UAE achieved the highest crude polysaccharide yield, followed by HWE and UA-ATPE. This finding agrees with recent studies demonstrating that ultrasonic cavitation enhances solvent penetration, disrupts fungal cell walls, and accelerates mass transfer, thereby improving polysaccharide recovery while reducing extraction time and energy consumption [36–41]. When ultrasonic param-

ters are appropriately controlled, UAE can improve extraction efficiency without causing substantial degradation of polysaccharide structures, making it an effective approach for extracting bioactive polysaccharides from edible and medicinal fungi [40,41]. The present results further support the applicability of UAE for recovering crude polysaccharides from *T. albuminosus* under the optimized conditions employed in this study. Although UA-ATPE produced a slightly lower extraction yield than UAE, it exhibited distinct advantages in process integration. Unlike conventional extraction methods, UA-ATPE combines extraction with preliminary fractionation by exploiting the differential partitioning of biomolecules between two immiscible aqueous phases. Recent studies have shown that aqueous two-phase systems can selectively enrich polysaccharides according to differences in molecular size, charge, hydrophilicity, and solubility while simultaneously simplifying downstream purification [42–44]. Consistent with these reports, UA-ATPE produced crude polysaccharides with physicochemical characteristics distinct from those obtained by HWE and UAE, indicating that extraction strategy influences not only extraction efficiency but also the composition of the recovered polysaccharide populations.

Response surface methodology (RSM) proved to be an effective optimization tool for all three extraction strategies. The regression models exhibited high statistical significance, non-significant lack-of-fit values, and excellent agreement between predicted and experimentally validated yields, demonstrating their robustness and predictive capability. Similar optimization strategies have been widely applied in recent studies because they efficiently evaluate interactions among multiple processing variables while substantially reducing experimental workload [19,45,46].

Collectively, the present results demonstrate that each extraction strategy possesses distinct advantages. UAE is advantageous when maximizing crude polysaccharide recovery is the primary objective, whereas UA-ATPE offers additional benefits by integrating extraction with preliminary purification. Therefore, selection of an extraction method should be guided by the intended application rather than extraction yield alone. More importantly, unlike most previous studies that optimized a single extraction procedure, the present work systematically compared three independently optimized extraction strategies using the same biological material. This experimental design enabled a direct evaluation of how different extraction approaches are associated with variations in polysaccharide yield, physicochemical characteristics, and antioxidant activity, thereby providing a more comprehensive assessment of extraction strategy than has generally been reported. Since extraction conditions can influence both polysaccharide recovery and structural characteristics, comparative structural characterization is essential for understanding the biological properties of the extracted polysaccharides and is discussed in the following section.

4.2. Structural Characteristics of Crude and Purified Polysaccharides

The physicochemical characteristics of polysaccharides, including chemical composition, molecular weight distribution, and monosaccharide composition, are widely recognized as important factors influencing their biological properties. Recent studies have demonstrated that these characteristics are strongly affected by extraction and purification procedures, which can selectively recover different polysaccharide populations even from the same fungal species [13]. Consequently, understanding how extraction strategies influence polysaccharide characteristics has become an important focus in contemporary fungal polysaccharide research. In the present study, the three extraction methods produced crude polysaccharides with distinct physicochemical characteristics. UH-TAPs exhibited the highest carbohydrate content and the lowest residual protein content, whereas UT-TAPs contained comparatively lower carbohydrate levels. Similar observations have been reported for mushroom polysaccharides extracted using different techniques, where ex-

traction conditions affected not only polysaccharide recovery but also the co-extraction of proteins and other water-soluble constituents [19]. These findings suggest that different extraction strategies recover chemically distinct polysaccharide populations rather than simply altering extraction efficiency.

The molecular weight distributions of the crude polysaccharides also differed markedly among the three extraction methods. UH-TAPs contained a greater proportion of low-molecular-weight components, whereas UT-TAPs were enriched in relatively high-molecular-weight fractions. Comparable results have been reported in recent studies of ultrasound-assisted extraction, in which controlled ultrasonic treatment was associated with shifts in molecular weight distribution, likely because acoustic cavitation enhances solvent penetration and mass transfer while promoting limited fragmentation of larger polysaccharide molecules [18]. In contrast, aqueous two-phase extraction primarily separates biomolecules according to differences in molecular size, charge, polarity, and hydrophilicity, thereby selectively enriching specific polysaccharide fractions rather than inducing structural degradation [33]. Therefore, the molecular weight profiles observed in the present study are more likely to reflect differences in extraction selectivity than irreversible structural modification. Distinct differences were likewise observed in monosaccharide composition. UH-TAPs contained relatively higher proportions of mannose and rhamnose, whereas UT-TAPs were enriched in galactose and fucose, with no detectable rhamnose. Similar extraction-dependent variations have been reported for polysaccharides isolated from edible fungi and medicinal plants, suggesting that different extraction procedures preferentially recover chemically distinct polysaccharide populations [19,43]. Because fungal polysaccharides naturally exist as heterogeneous mixtures differing in molecular architecture and charge properties, these compositional differences are more likely to result from selective extraction than from changes in the intrinsic chemical structures of individual polysaccharides.

Purification by DEAE-52 anion-exchange chromatography further altered the composition of the recovered polysaccharides. Compared with the corresponding crude extract, TAPs-0.1M exhibited a markedly different monosaccharide profile, including the appearance of glucuronic acid and an increased proportion of glucose. This finding is consistent with the separation mechanism of anion-exchange chromatography, which enriches acidic polysaccharide fractions according to differences in charge density [33]. Similar compositional shifts after DEAE-cellulose purification have been widely reported for fungal polysaccharides and are generally interpreted as the selective enrichment of chemically distinct polysaccharide populations rather than chemical transformation during purification. This mechanism also explains the differences between the monosaccharide compositions of the crude polysaccharides (Table 4) and the purified TAPs-0.1M fraction observed in the present study. The purified TAPs-0.1M fraction also exhibited a relatively homogeneous molecular weight (6.37 kDa) and characteristic FTIR absorption bands typical of acidic heteropolysaccharides. Together, these results provide preliminary evidence for the structural characteristics of the purified polysaccharide. However, molecular weight, monosaccharide composition, and FTIR spectroscopy alone are insufficient to establish detailed polysaccharide structures. Recent studies consistently emphasize that comprehensive structural elucidation requires complementary techniques such as methylation analysis, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry to determine glycosidic linkage patterns, branching structures, and higher-order conformations [17]. Therefore, while the present study demonstrates that different extraction and purification strategies are associated with distinct physicochemical characteristics of *T. albuminosus* polysaccharides, further structural investigations are required to establish definitive structure–function relationships.

4.3. Antioxidant Activity and Potential Structure–Activity Relationships

Fungal polysaccharides have attracted considerable attention because of their antioxidant properties and their potential applications as functional food ingredients and natural antioxidants. Consistent with previous studies, all crude polysaccharides isolated in the present study exhibited concentration-dependent DPPH radical scavenging activity, hydroxyl radical scavenging activity, and ferric reducing antioxidant power, with UH-TAPs showing the highest antioxidant activity among the three crude extracts under the experimental conditions investigated [4,8]. These findings indicate that different extraction strategies are associated with differences in the antioxidant properties of the recovered polysaccharides.

Current evidence suggests that the antioxidant activity of polysaccharides is determined by the combined effects of multiple structural characteristics rather than by a single physicochemical parameter. Molecular weight, monosaccharide composition, uronic acid content, glycosidic linkage patterns, branching degree, molecular conformation, and residual non-polysaccharide components have all been proposed to contribute to antioxidant activity [10,13,35]. However, these structural features are often interdependent, and their relative contributions remain controversial because naturally occurring polysaccharides are highly heterogeneous. Consequently, definitive structure–activity relationships have not yet been established for most fungal polysaccharides.

In the present study, UH-TAPs contained a greater proportion of low-molecular-weight polysaccharides and exhibited relatively stronger antioxidant activities than H-TAPs and UT-TAPs. Similar associations have been reported in recent studies of mushroom polysaccharides, where fractions with relatively lower molecular weights frequently displayed stronger radical-scavenging activities [19,22]. Lower-molecular-weight polysaccharides are generally considered to possess improved aqueous solubility and greater accessibility of reactive hydroxyl groups, which may facilitate interactions with free radicals. Nevertheless, opposite trends have also been reported, indicating that molecular weight alone cannot adequately explain antioxidant activity [10,15]. Therefore, the present findings suggest an association between molecular-weight distribution and antioxidant activity but do not establish a causal relationship.

The purified fraction TAPs-0.1M exhibited the strongest antioxidant activity among the chromatographically separated fractions and was identified as an acidic heteropolysaccharide containing glucuronic acid. Previous studies have suggested that acidic polysaccharides may exhibit relatively strong antioxidant activities because carboxyl groups can increase molecular hydrophilicity and facilitate interactions with reactive oxygen species or transition metal ions [8,35]. However, antioxidant activity is also influenced by glycosidic linkage patterns, branching structures, molecular conformation, and other structural features [10,13]. Consequently, the presence of glucuronic acid in TAPs-0.1M should not be interpreted as direct evidence that uronic acid is responsible for the enhanced antioxidant activity observed in the present study.

Overall, the results demonstrate that different extraction and purification strategies are associated with differences in the antioxidant activities of *T. albuminosus* polysaccharides. However, the present study establishes only preliminary associations between physicochemical characteristics and antioxidant activity. In addition, the antioxidant activities were evaluated exclusively using in vitro chemical assays, which primarily reflect radical-scavenging and reducing capacities under simplified experimental conditions and do not fully represent biological antioxidant efficacy under physiological conditions. Comprehensive structural characterization, including methylation analysis, nuclear magnetic resonance (NMR) spectroscopy, and three-dimensional conformational analysis, together with mechanistic investigations in cell-based and animal models, will be required to fur-

ther clarify how individual structural features contribute to the biological activities of *T. albuminosus* polysaccharides [17,18].

5. Conclusions

This study systematically compared three extraction strategies—HWE, UAE, and UA-ATPE—for the recovery of polysaccharides from *T. albuminosus*. Extraction conditions for each method were successfully optimized using Box–Behnken response surface methodology, enabling a direct comparison of extraction efficiency, physicochemical characteristics, and antioxidant activities under independently optimized conditions. Among the three methods, UAE achieved the highest crude polysaccharide yield, whereas UA-ATPE provided the additional advantage of integrating extraction with preliminary purification. Comparative characterization demonstrated that different extraction strategies were associated with distinct carbohydrate contents, molecular weight distributions, monosaccharide compositions, and antioxidant activities of the recovered polysaccharides. The purified fraction, TAPs-0.1M, exhibited the strongest antioxidant activity among the isolated fractions and was characterized as an acidic heteropolysaccharide with an average molecular weight of 6.37 kDa. However, the present findings establish associations between extraction strategy, physicochemical characteristics, and antioxidant activity rather than definitive structure–activity relationships. Overall, this work provides a systematic evaluation of three commonly used extraction strategies for *T. albuminosus* polysaccharides and demonstrates that extraction methodology influences not only polysaccharide recovery but also the physicochemical characteristics of the extracted fractions. These findings provide a useful reference for selecting appropriate extraction methods according to different production objectives and lay a foundation for the future development and utilization of *T. albuminosus* polysaccharides as functional food ingredients and natural antioxidant resources. Further studies employing comprehensive structural characterization and biological validation will be necessary to elucidate the mechanisms underlying their antioxidant activities.

Author Contributions: Conceptualization, Z.L. and C.Q.; methodology, Z.L. and Y.T.; software, Z.L. and X.T.; validation, L.Y., J.C. and L.C.; formal analysis, Z.L. and F.Z.; investigation, Z.L., Y.T. and X.T.; resources, C.Q., L.Y. and J.C.; data curation, Z.L. and F.Z.; writing—original draft preparation, Z.L.; writing—review and editing, C.Q. and Y.T.; visualization, Z.L. and X.T.; supervision, C.Q.; project administration, C.Q. and L.Y.; funding acquisition, C.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Sichuan Provincial Science and Technology Department, grant number 24ZHSF0118 (Sichuan Provincial Demonstration Project for the Transfer and Transformation of Scientific and Technological Achievements).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors would like to thank the technical staff at the Sichuan Food and Fermentation Industry Research & Design Institute and the College of Bioengineering, Tianjin University of Science and Technology, for their assistance with structural characterization and chemical analysis.

Conflicts of Interest: Zhenjiang Li, Jing Chen, Lan Chen, Fangyuan Zeng are employees of Chengdu Jinkai Biology Engineering Co., Ltd. Youpeng Tuo, Xiaofang Tang, Li Ye are employees of Sichuan Food and Fermentation Industry Research & Design Institute Co., Ltd. The other author declares no conflicts of interest. Chengdu Jinkai Biology Engineering Co., Ltd. and Sichuan Food and Fermentation Industry Research & Design Institute Co., Ltd. had no role in the design of the study; in

the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

HWE	Hot water extraction
UAE	Ultrasound-assisted extraction
ATPE	Aqueous two-phase extraction
UA-ATPE	Ultrasound-assisted aqueous two-phase extraction
RSM	Response surface methodology
BBD	Box–Behnken design
DPPH	1,1-diphenyl-2-picrylhydrazyl
ATPs-0.1M	Major active polysaccharide fraction eluted with 0.1M NaCl
kDa	Kilodaltons
ROS	Reactive oxygen species
SOD	Superoxide dismutase
MDA	Malondialdehyde
ATPS	Aqueous two-phase system
PDA	Potato Dextrose Agar
ITS	Internal Transcribed Spacer
PCR	Polymerase Chain Reaction
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
ANOVA	Analysis of variance
H-TAPs	<i>Termitomyces albuminosus</i> polysaccharides obtained by HWE
UH-TAPs	<i>Termitomyces albuminosus</i> polysaccharides obtained by UAE
UT-TAPs	<i>Termitomyces albuminosus</i> polysaccharides obtained by UA-ATPE

References

1. Yu, Y.; Shen, M.; Song, Q.; Xie, J. Biological activities and pharmaceutical applications of polysaccharide from natural resources: A review. *Carbohydr. Polym.* **2018**, *183*, 91–101. [[CrossRef](#)] [[PubMed](#)]
2. Liu, J.; Wang, D.; Ren, N.; Zhang, L.; Wang, T. Metabolites of *Astragalus membranaceus* and Their Pro-Apoptotic and Cytotoxic Activities: Insights into Targeted Metabolic Pathways. *Front. Pharmacol.* **2025**, *16*, 1647958. [[CrossRef](#)] [[PubMed](#)]
3. Ji, X.; Yu, Y.; Zhang, Y.; Sun, X.; Huang, S.; Li, Z.; Yang, S.; Shi, Y. Extraction, purification, structural characteristics, modifications, biological activities, and applications of jujube polysaccharides: A review. *J. Future Foods*, 2025; *in press*. [[CrossRef](#)]
4. Ali, S.S.; Alsharbaty, M.H.M.; Al-Tohamy, R.; Naji, G.A.; Elsamahy, T.; Mahmoud, Y.A.G.; Kornaros, M.; Sun, J. A review of the fungal polysaccharides as natural biopolymers: Current applications and future perspective. *Int. J. Biol. Macromol.* **2023**, *273*, 132986.
5. He, W.; Jiang, H.; Rong, M.; Li, X.; Xu, J.; Guo, Y. A fungal polysaccharide from *Trametes orientalis*: Structural elucidation and multifaceted antitumor mechanisms via metastasis, angiogenesis, and immunomodulation. *Carbohydr. Polym.* **2025**, *368*, 124151. [[CrossRef](#)] [[PubMed](#)]
6. Li, S.; Zhao, Z.; He, Z.; Yang, J.; Feng, Y.; Xu, Y.; Wang, Y.; He, B.; Ma, K.; Zheng, Y.; et al. Effect of structural features on the antitumor activity of plant and microbial polysaccharides: A review. *Food Biosci.* **2024**, *61*, 104648. [[CrossRef](#)]
7. Zhang, M.; Zhang, L. Lentinan as an immunotherapeutic for treating lung cancer: A review of 12 years of clinical studies in China. *J. Cancer Res. Clin. Oncol.* **2023**, *149*, 1689–1702.
8. Ding, L.; Shangguan, H.; Wang, X.; Liu, J.; Shi, Y.; Xu, X.; Xie, Y. Extraction, purification, structural characterization, biological activity, mechanism of action and application of polysaccharides from *Ganoderma lucidum*: A review. *Int. J. Biol. Macromol.* **2025**, *288*, 138575. [[CrossRef](#)] [[PubMed](#)]
9. Hong, Y.; Ying, T. Isolation, molecular characterization and antioxidant activity of a water-soluble polysaccharide extracted from the fruiting body of *Termitomyces albuminosus* (Berk.) Heim. *Int. J. Biol. Macromol.* **2019**, *122*, 115–126. [[CrossRef](#)] [[PubMed](#)]
10. Chen, N.; Jiang, T.; Xu, J.; Xi, W.; Shang, E.; Xiao, P.; Duan, J.A. The relationship between polysaccharide structure and its antioxidant activity needs to be systematically elucidated. *Int. J. Biol. Macromol.* **2024**, *270*, 132391. [[CrossRef](#)] [[PubMed](#)]

11. Zhao, H.; Li, H.; Feng, Y.; Zhang, Y.; Yuan, F.; Zhang, J.; Ren, H.; Jia, L. Mycelium Polysaccharides from *Termitomyces albuminosus* Attenuate CCl₄-Induced Chronic Liver Injury Via Inhibiting TGF β 1/Smad3 and NF- κ B Signal Pathways. *Int. J. Mol. Sci.* **2019**, *20*, 4872. [[CrossRef](#)] [[PubMed](#)]
12. Pinho, S.S.; Alves, I.; Gaifem, J.; Rabinovich, G.A. Immune regulatory networks coordinated by glycans and glycan-binding proteins in autoimmunity and infection. *Cell. Mol. Immunol.* **2023**, *20*, 1101–1113. [[CrossRef](#)] [[PubMed](#)]
13. Guo, D.; Liu, C.; Zhu, H.; Cheng, Y.; Guo, Y.; Yao, W.; Jiang, J.; Qian, H. Advanced insights into mushroom polysaccharides: Extraction methods, structure-activity, prebiotic properties, and health-promoting effects. *Int. J. Biol. Macromol.* **2025**, *308*, 142319. [[CrossRef](#)] [[PubMed](#)]
14. Kadam, S.U.; Tiwari, B.K.; O'Donnell, C.P. Extraction, structure and biofunctional activities of laminarin from brown algae. *Int. J. Food Sci. Technol.* **2015**, *50*, 24–31.
15. Yang, M.; Ren, W.; Li, G.; Yang, P.; Chen, R.; He, H. The effect of structure and preparation method on the bioactivity of polysaccharides from plants and fungi. *Food Funct.* **2022**, *13*, 12541–12560. [[CrossRef](#)] [[PubMed](#)]
16. Wen, L.; Jiang, Y.; Yang, J.; Zhao, M. Recent advances in extraction and characterization of polysaccharides from natural resources. *Trends Food Sci. Technol.* **2024**, *143*, 104189.
17. Huang, X.; Ai, C.; Yao, H.; Zhao, C.; Xiang, C.; Hong, T.; Xiao, J. Guideline for the extraction, isolation, purification, and structural characterization of polysaccharides from natural resources. *eFood* **2022**, *3*, e37. [[CrossRef](#)]
18. Cui, F.J.; Qian, L.S.; Sun, W.J.; Zhang, J.S.; Yang, Y.; Li, N.; Zhuang, H.N.; Wu, D. Ultrasound-assisted extraction of polysaccharides from *Volvariella volvacea*: Process optimization and structural characterization. *Molecules* **2018**, *23*, 1706. [[CrossRef](#)] [[PubMed](#)]
19. Liu, X.Q.; Wang, N.; Liang, J.; Yu, L.S.; Zhou, F.Y.; Lei, Y.; Wang, Y.; Kuang, H.X.; Xia, Y.G. Effect of Different Extraction Methods on the Structures and Bioactivities of Polysaccharides from *Auricularia auricula-judae*. *ACS Food Sci. Technol.* **2025**, *5*, 2843–2857. [[CrossRef](#)]
20. Zheng, Y.; Cui, J.; Chen, A.H.; Zong, Z.M.; Wei, X.Y. Optimization of ultrasonic-microwave assisted extraction and hepatoprotective activities of polysaccharides from *Trametes orientalis*. *Molecules* **2019**, *24*, 147. [[CrossRef](#)] [[PubMed](#)]
21. Guo, Q.; Liang, S.; Xiao, Z.; Ge, C. Research progress on extraction technology and biological activity of polysaccharides from Edible Fungi: A review. *Food Rev. Int.* **2023**, *39*, 4909–4940.
22. Cao, H.; Wang, X.; Shi, M.; Guan, X.; Zhang, C.; Wang, Y.; Qiao, L.; Song, H.; Zhang, Y. Influence of physicochemical changes and aggregation behavior induced by ultrasound irradiation on the antioxidant effect of highland barley β -glucan. *Food Chem. X* **2023**, *19*, 100793. [[CrossRef](#)] [[PubMed](#)]
23. Paloi, S.; Kumla, J.; Phonrob, W.; Paloi, B.P.; Suwannarach, N. Unveiling the Hidden Diversity of *Termitomyces* (Lyophyllaceae, Agaricales) in Northern Thailand: Identification of Five New Species and the First Report of *Termitomyces acriumbonatus*. *J. Fungi* **2025**, *11*, 830. [[CrossRef](#)]
24. Wei, T.Z.; Tang, B.H.; Yao, Y.J. Revision of *Termitomyces* in China. *Mycotaxon* **2009**, *108*, 257–285. [[CrossRef](#)]
25. Kang, X.; Kirui, A.; Muszyński, A.; Widanage, M.C.D.; Chen, A.; Azadi, P.; Wang, P.; Mentink-Vigier, F.; Wang, T. Molecular architecture of fungal cell walls revealed by solid-state NMR. *Nat. Commun.* **2018**, *9*, 2747. [[CrossRef](#)] [[PubMed](#)]
26. Gong, P.; Wang, S.; Liu, M.; Chen, F.; Yang, W.; Chang, X.; Liu, N.; Zhao, Y.; Wang, J.; Chen, X. Extraction methods, chemical characterizations and biological activities of mushroom polysaccharides: A mini-review. *Carbohydr. Res.* **2020**, *494*, 108037. [[CrossRef](#)] [[PubMed](#)]
27. Chemat, F.; Rombaut, N.; Sicaire, A.G.; Meullemiestre, A.; Fabiano-Tixier, A.S.; Abert-Vian, M. Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrason. Sonochem.* **2017**, *34*, 540–560. [[CrossRef](#)] [[PubMed](#)]
28. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [[CrossRef](#)]
29. Albalasmeh, A.A.; Berhe, A.A.; Ghezzehei, T.A. A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry. *Carbohydr. Polym.* **2013**, *97*, 253–261. [[CrossRef](#)] [[PubMed](#)]
30. Tan, J.; Cui, P.; Ge, S.; Cai, X.; Li, Q.; Xue, H. Ultrasound assisted aqueous two-phase extraction of polysaccharides from *Cornus officinalis* fruit: Modeling, optimization, purification, and characterization. *Ultrason. Sonochem.* **2022**, *84*, 105966. [[CrossRef](#)] [[PubMed](#)]
31. Wu, J.Y. Ultrasound-assisted extraction of polysaccharides from edible and medicinal fungi: Major factors and process kinetics. *MOJ Food Process. Technol.* **2017**, *4*, 48–52. [[CrossRef](#)]
32. Grilo, A.L.; Aires-Barros, M.R.; Azevedo, A.M. Partitioning in aqueous two-phase systems: Fundamentals, applications and trends. *Sep. Purif. Rev.* **2016**, *45*, 68–80.
33. Mao, G.; Yu, P.; Zhao, T.; Chen, Y.; Feng, W.; Zhang, Q.; Yang, L.; Wu, X. Aqueous two-phase simultaneous extraction and purification of a polysaccharide from *Grifola frondosa*: Process optimization, structural characteristics and antioxidant activity. *Ind. Crops Prod.* **2022**, *184*, 114962. [[CrossRef](#)]

34. Iqbal, M.; Tao, Y.; Xie, S.; Zhu, Y.; Chen, D.; Wang, X.; Huang, L.; Peng, D.; Sattar, A.; Shabbir, M.A.; et al. Aqueous two-phase system (ATPS): An overview and advances in its applications. *Biol. Proced. Online* **2016**, *18*, 18. [[CrossRef](#)] [[PubMed](#)]
35. Wang, W.; Tan, J.; Nima, L.; Sang, Y.; Cai, X.; Xue, H. Polysaccharides from fungi: A review on their extraction, purification, structural features and biological activities. *Food Chem. X* **2022**, *15*, 100414. [[CrossRef](#)] [[PubMed](#)]
36. Sun, Y.; He, H.; Wang, Q.; Yang, X.; Jiang, S.; Wang, D. A review of development and utilization for edible fungal polysaccharides: Extraction, chemical characteristics, and bioactivities. *Polymers* **2022**, *14*, 4454. [[CrossRef](#)] [[PubMed](#)]
37. Kumla, J.; Thangrongthong, S.T.; Kaewnunta, A.K.; Suwannarach, N. Research advances in fungal polysaccharides: Production, extraction, characterization, properties, and their multifaceted applications. *Front. Cell. Infect. Microbiol.* **2025**, *15*, 1604184. [[CrossRef](#)] [[PubMed](#)]
38. Liu, Y.; Gao, L.; Yu, Z. Revealing the complexity of polysaccharides: Advances in NMR spectroscopy for structural elucidation and functional characterization. *Appl. Sci.* **2025**, *15*, 5246. [[CrossRef](#)]
39. Zhou, H.; Fan, Z.; Li, Y.; Liu, X.; Wang, B.; Xing, J.; He, J.; Zheng, R.; Li, J. Structure–Antioxidant Activity Relationship of Polysaccharides Isolated by Microwave/Ultrasonic-Assisted Extraction from *Pleurotus ferulae*. *Antioxidants* **2025**, *14*, 91. [[CrossRef](#)] [[PubMed](#)]
40. Gao, D.; Chen, H.; Liu, H.; Yang, X.; Guo, P.; Cao, X.; Cai, Y.; Xu, H.; Yang, J. Structure characterization and antioxidant activity analysis of polysaccharides from Lanzhou lily. *Front. Nutr.* **2022**, *9*, 976607. [[CrossRef](#)] [[PubMed](#)]
41. Yu, T.E.; Wu, Q.; Liang, B.; Wang, J.; Wu, D.; Shang, X. The current state and future prospects of *Auricularia auricula* polysaccharide processing technology portfolio. *Molecules* **2023**, *28*, 582. [[CrossRef](#)] [[PubMed](#)]
42. Zhao, J.; Hu, Y.; Qian, C.; Hussain, M.; Liu, S.; Zhang, A.; He, R.; Sun, P. The interaction between mushroom polysaccharides and gut microbiota and their effect on human health: A review. *Biology* **2023**, *12*, 122. [[CrossRef](#)] [[PubMed](#)]
43. He, G.; Yan, J.; Liang, L.; Liu, W.; Chen, S.; Chen, P. Characterization and biological activities of polysaccharides extracted from *Auricularia auricula* with different extraction methods. *Prep. Biochem. Biotechnol.* **2024**, *54*, 859–871. [[PubMed](#)]
44. de Carvalho, M.M.; Ellefsen, C.F.; Eltvik, A.A.; Hiorth, M.; Samuelsen, A.B.C. Chemical structure characterization of polysaccharides using diffusion ordered NMR spectroscopy (DOSY). *Carbohydr. Polym.* **2025**, *349*, 123021. [[CrossRef](#)] [[PubMed](#)]
45. Du, B.; Peng, F.; Xie, Y.; Wang, H.; Wu, J.; Liu, C.; Yang, Y. Optimization of extraction and antioxidant activity of crude polysaccharides from chestnut mushroom (*Agrocybe aegerita*) by accelerated solvent extraction combined with response surface methodology. *Molecules* **2022**, *27*, 2380. [[CrossRef](#)] [[PubMed](#)]
46. Khalil, A.S.E.; Łukasiewicz, M. The optimization of the hot water extraction of the polysaccharide-rich fraction from *Agaricus bisporus*. *Molecules* **2024**, *29*, 4783. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.