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Growth Factors Optimization and Antioxidant Evaluation of β-Glucan Extracted from *Lignosus rhinocerus* Mycelium using Microwave-Assisted Extraction

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Abstract

This study aimed to optimize the cultivation of *Lignosus rhinocerus* mycelium at a laboratory level and evaluate the antioxidant activity of β-glucan extracted from the mycelium. Using the Response Surface Methodology (RSM) and Central Composite Design (CCD), optimal conditions for mycelium growth were identified at a temperature of 31.5 °C, 15 % potato powder, and 3.5 % glucose concentration, yielding a maximum mycelial biomass of 44.7 g/L and β-glucan production of 15.08 g/L. The β-glucan was extracted using a household microwave, significantly improving the extraction efficiency. The extracted β-glucan demonstrated notable antioxidant activity, with DPPH radical scavenging activity at approximately 53.31 % and ABTS radical scavenging activity around 47.31 %, albeit slightly lower than the 71.94 % activity of ascorbic acid. Comparative analyses highlighted the consistency in antioxidant capacities across different morphological stages, with aqueous methanol extracts of mycelium (LR-MH, LR-MT) and culture broth (LR-BH, LR-BT) showing either higher or comparable antioxidant activities to sclerotium extracts (LR-SC). Field Emission Scanning Electron Microscopy (FESEM) and Fourier Transform Infrared (FTIR) spectroscopy confirmed the structural integrity of the extracted β-glucan. The findings support the feasibility of using mycelium for β-glucan extraction, offering a sustainable alternative to traditional methods.

Keywords: *Lignosus rhinocerus*, β-glucan, Mycelium cultivation, Antioxidant activity, Response surface methodology, Microwave-assisted extraction

Introduction

Prebiotics are dietary components that resist digestion in the small intestine, ensuring they arrive intact in the large intestine, where they nourish probiotic bacteria. These substances are typically found in complex carbohydrates such as inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), xylo-oligosaccharides (XOS), and notably β-glucan [1].

Among these prebiotics, β-glucan a crucial element found in the cell walls of of various organisms such as algae, plants, fungi, yeast cells, and edible mushrooms [2-4]. Mushrooms are a significant source of β-glucan because their cell walls are rich in β-glucans, glucose polymer subunits with β-1,3 and β-1,6 linkages. Additionally, β-glucans in some mushrooms are known for their unique benefits, such as medicinal and antioxidant properties [5]. The focus on β-glucan corresponds to using natural substances to enhance animal health and reduce reliance on antibiotics [6,7]. Furthermore, β-glucan is crucial in boosting and stimulating the innate immune system in humans and animals, significantly affecting the mucosal immune system [8-10].

Lignosus rhinocerus is an interesting wild edible mushroom widely distributed in Asian countries. This mushroom is unique because its mycelium can

proliferate rapidly. This makes it a promising candidate for consumption, as well as for functional and nutritional supplement purposes. Previous research has indicated that this mushroom species is a rich source of potent antioxidant substances when tested *in vitro* [11]. *L. rhinocerus* is a highly valued medicinal mushroom. Indigenous tribes in Southeast Asia have used it as a medicinal herb to treat many ailments and promote health. Mushrooms also have high antioxidant activity to reduce free radicals [12].

Free radicals occur in metabolic processes in the body. If there are too many, they will harm various cells, causing cell degeneration. As a result, cells age and can eventually cause cell death. It was found that β-glucan from many types of fungi and mushrooms has antioxidant properties and properties as a chelating agent, able to bind to heavy metal ions [13]. However, most of the test results were derived from extracts of substances from mushroom fruiting bodies. There were no findings related to extracts from the mycelium. Due to the prolonged cultivation period required for mushrooms, the process of deriving β-glucan from the fruiting body of the mushrooms is notably costly. Furthermore, the escalating consumption demand for mushrooms renders their availability as a source for β-glucan extraction inadequate. An alternative and feasible approach involves extracting β-glucan from mushroom mycelium grown in broth cultures. This method allows for rapid cultivation of mushroom mycelium in the laboratory. Moreover, maintaining a constant production standard is easier to manage, and the production time is shorter.

In mycelium cultivation, the composition of the growth medium is critical in determining both biomass production and the yield of bioactive compounds such as β-glucan. Previous research has shown that selecting and optimizing key media components, including carbon and nitrogen sources, greatly influences the growth and metabolic activity of the mycelium [14]. Common carbon sources include potato dextrose broth (PDB) and glucose, which are widely used to promote mycelial growth. Nitrogen, another essential component, can be provided in both organic and inorganic forms. Organic nitrogen sources, such as yeast extract, peptone, and urea, support robust mycelial development. Additionally, inorganic nitrogen sources like ammonia solution, ammonium phosphate dibasic, ammonium citrate, ammonium nitrate, and potassium nitrate are also effective in promoting mycelium growth [15]. For mushrooms such as *L. rhinocerus*, the use of starchy substrates like potato powder and glucose has been shown to significantly improve growth performance [16]. Moreover, temperature optimization is another key factor that affects mycelium metabolism and enzyme activity, thus influencing the overall yield of desired bioactive compounds [17].

Multiple techniques exist for extracting β-glucan, including hot water extraction, a conventional approach that typically results in a low yield of β-glucan [18]. β-glucan is extracted from more resilient cell wall sources like mushrooms or yeast through alkaline hydrolysis, although this method may lead to environmental concerns due to chemical waste [19]. An alternative eco-friendly technique involves using specific enzyme extraction to decompose the cell walls and liberate β-glucan without harming its integrity. Nevertheless, enzymatic extraction is expensive and not feasible for large-scale production [20]. Microwave-assisted extraction is a more efficient technique that uses microwave energy to rapidly heat the solvent and biological material, thereby enhancing extraction efficiency and facilitating the solubilization of β-glucan [20]. Studies have shown that microwave radiation improves heating efficiency and reduces reaction time compared to acid hydrolysis for extracting β-glucan from grey oyster mushrooms (*Pleurotus sajor-caju* (Fr.) Sing.) [21]. Another study examined the microwave extraction method at a frequency of 2450 MHz and found that the β-glucan yield was significantly higher than with traditional methods [22]. Additionally, research on microwave-assisted extraction of polysaccharides from the fruiting body of *Hericium erinaceum* demonstrated that a 5-min microwave treatment at 140 °C was almost as effective as conventional heating for 6 h at 100 °C, indicating that microwave irradiation is more efficient in terms of extraction time for polysaccharides from mushrooms [23].

This research aimed to extract β-glucan from cultivating *L. rhinocerus* mushroom mycelia. The optimization condition for growing mushroom mycelium was initially explored using the Response Surface Methodology (RSM) technique, followed by the extraction of β-glucan with a household microwave for

cost-effectiveness and ease of accessibility. The optimization condition for growing mushroom mycelium was initially explored using the RSM. This was followed by a household microwave to extract β-glucan from mycelium. Subsequently, the study will explore the antioxidant capabilities of β-glucan derived from the mycelium of both mushrooms.

Materials and methods

Mushroom samples and cultivation

The *L. rhinocerus* was purchasing the fruiting bodies samples from the mushroom's farmers. Preliminary culturing mushroom mycelium begins with using a sterile technique to clean the outer surface of the mushroom with 90 % ethanol. Use a surgical blade to cut and separate the mushrooms. Mushroom tissue was collected from the inside of the mushrooms. The inner tissue was cut into small pieces, placed in the middle of a PDA dish, and then incubated at 30 °C for 5 days. A Cork borer was then used to pierce the active mycelium around the outer ring of the colony. Active mycelium on PDA was continue to grow in PDB at shaking incubator at 30 °C with shaking at 120 rpm for seven days.

Classification of mushroom species PCR

Even though the type of mushroom is known, we still need to confirm its genus and species by amplifying the 18S and ITS genes. Details of the preparation of mushroom mycelium samples are as follows. Mushroom mycelium grown in PDB liquid medium was blended to fine mycelium with a laboratory blender (Waring commercial 7010S: USA). Then, 400 µL of mycelium was placed in a 1.5 mL microcentrifuge tube and subjected to sedimentation at 14,000 rpm for 10 min and discarded the supernatant. The mycelium precipitate was extracted for DNA according to the method specified in the extraction kit's manual (GF-1 Fungus DNA Extraction Kit; Vivantis: Malaysia). The 18S and ITS genes were amplified using the primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'TCCTCCGCTTA TTGATATGC-3'). The PCR reaction mixture (50 µL total volume) contained 1X buffer (100 mM KCl, 20 mM Tris), 2.5 mM MgCl2, 0.2 mM dNTPs, 0.2 pmol of each primer, 0.5 Units of Taq polymerase, 2 µL of the extracted DNA template, and deionized water to reach the final volume. The PCR program consisted of an initial denaturation step at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 50 s, annealing at 55 °C for 1 min and 30 s, and extension at 72 °C for 1 min. A final extension step was carried out at 72 °C for 5 min. The PCR program consisted of an initial denaturation step at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 50 s, annealing at 55 °C for 1 min and 30 s, and extension at 72 °C for 1 min. A final extension step was carried out at 72 °C for 5 min.

Optimal conditions for mycelium growth

The study focused on optimizing the ratios and components of the PDB liquid media to facilitate the best growth conditions for *L. rhinocerus* mycelium across 5 different formulations. Formula A was prepared using a basic PDB composition that included 20 % potato infusion and 2 % dextrose (Himedia). Formula B utilized 20% potato powder (Himedia) and 2 % food-grade glucose, while Formula C comprised the same percentage of potato powder with 2 % sucrose. Formulas D and E were adjusted to contain 10 % potato powder, with Formula D adding 2 % food-grade glucose and Formula E adding 2 % sucrose. For each formulation, 50 mL of media was prepared in a 250 mL flask, and the pH was adjusted to 5 ± 0.2. Each was inoculated with 5 equal pieces of active mycelium using Cork borer. The cultures were incubated at 30 °C with a shaking speed of 100 rpm. After ten days, the growth of the mushroom mycelium in each formula was assessed by measuring the biomass. The mycelium was then filtered through No. 1 filter paper with the aid of a vacuum pump for efficient filtration. The filtered mycelium was dried at 50 °C, and its dry weight was measured. Results were expressed in grams per 50 mL of media volume and statistically analyzed using a Two-Way ANOVA (GraphPad Prism V.5).

The optimal growth conditions for *L. rhinocerus* mycelium from the shake flask method will be further optimized to the most suitable condition for its growth. This analysis will use Response Surface Methodology (RSM) paired with a Central Composite Design (CCD). The study focuses on identifying the variables that affect the proliferation of mushroom mycelium and percentage of β-glucan by exploring the potato powder concentration (10 – 20 %), glucose levels (2 – 5 %), and temperature variations (28 - 35 °C) influence growth. The analysis of variance (ANOVA) and the creation of

three-dimensional (3D) graphs for the experimental data were performed using Design-Expert® Software version 13.0 (StatEase, Inc., Minneapolis, MN, USA, 2019) [20]. An empirical model was developed to investigate the effects and interactions of the variables, utilizing a second-order quadratic equation to describe the responses, as shown in Eq. (1).

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i=0}^{n} \sum_{j>0}^{n} b_{ij} X_i X_j \quad (1)$$

where Y is the predicted response, $b0$ is the constant coefficient, bi is the linear coefficient, bij is the interaction coefficient, bii is the quadratic coefficient, and Xi and Xj are the coded values.

Extraction of polysaccharides from mushroom mycelium using a microwave

A household microwave oven (ELECTROLUX model EMM30D510EB: Magnetron power 1,580 W, frequency 2,450 MHz) was chosen for extraction. The extraction method was adapted from the previous method [24]. The process involved cultivating the mycelium in 200 mL of PDB media within a 1,000 mL flask at a temperature of 30 °C and a speed of 100 rpm. After cultivation, the mushroom mycelium was finely blended using a laboratory blender (Waring 7011HS 2-Speed Heavy-Duty Lab Blender) for 5 min. The blended material was then washed with sterile distilled water and centrifuged at 5,000 rpm at a temperature of 4 °C for 10 min, repeated 4 - 5 times. The material was freeze-dried (Christ / Delta 2-24 LSCplus). The prepared fiber mass was weighed, and 0.5 g of mushroom mycelium was measured into a 50 mL centrifuge tube with 25 mL of distilled water (fiber-to-water ratio of 1:50 w/v) and microwaved. The microwave power settings started at 200 watts for 2 min, increased to 600 watts for 3 min, then to 800 watts for 3 min, and finally to 1,000 watts for 5 min. The microwave-extracted mushroom fiber solution was then blended to separate the supernatant and the residue. The residue was subjected to freeze-drying to prepare for residual polysaccharide analysis, and the supernatant was precipitated by adding 95 % ethanol in a 3:1 (V/V) ratio and left to precipitate at 4 °C for 24 h. The precipitate was centrifuged at 10,000 rpm at 4 °C for 10 min and freeze-dried. The resulting data were used to calculate the percentage of residual and precipitated fractions according to Eqs. (2) and (3):

$$\text{Yield of residue} = \frac{\text{weight of residue after extraction}}{\text{weight of mycelium powder}} \times 100 \quad (2)$$

$$\text{Yield of precipitate} = \frac{\text{weight of precipetate after extraction}}{\text{weight of mycelium powder}} \times 100 \quad (3)$$

Analysis of β-glucan content

The analysis of β-glucan content utilizes a total β-glucan assay kit for fungi and yeast (β-Glucan Assay Kit: Megazyme). The principle involves determining total glucan content (α-glucan + β-glucan) and then subtracting the amount of α-glucan to isolate the value of β-glucan. The total glucan is digested with HCL acid to convert it into D-glucose. The amount of D-glucose corresponds to the total amount of glucan present. The extraction process and calculation of β-glucan were conducted according to the instructions provided with the kit [25].

Freeze drying process

The extracted β-glucan samples are subjected to freeze drying for quantitative analysis and further analysis of other properties using a Christ brand Freeze Dryer, model Delta 2-24 LSCplus. The program settings are as follows: at 0 °C with a pressure of 1.000 mBar for 1 h, at 10 °C with a pressure of 1.000 mBar for 1 h, at 20 ºC with a pressure of 0.600 mBar for 30 min, and at 25 °C with a pressure of 0.600 mBar for 30 min.

Characterization of β-glucan

Morphological structure

The morphological features of the β-glucan were examined using a field emission scanning electron microscope (FEI-SEM), model Quanta 450. Before analysis, the β-glucan samples were coated with a mixture of gold (Au) and palladium (Pd) to a specific thickness of 520.20 Å.

Function group characterization by Fourier Transform Infrared (FTIR) spectroscopy

The characterization of the functional groups in the EPS was conducted using Fourier Transform Infrared (FTIR) spectroscopy. For this, purified β-glucan pellets were prepared by compressing freeze-dried β-glucan samples with potassium bromide (KBr) powder at a

5:100 w/w ratio. The FTIR spectra were recorded in transmittance mode over a spectral range of 4000 to 400 cm^{-1}. This process involved accumulating 15 scans at a resolution of 4 cm^{-1} using a Fourier Transform Infrared Spectrometer (VERTEX 70, Bruker, Germany). The peaks identified in the spectra were compared with known standards β-glucan. [26].

Nuclear Magnetic Resonance (NMR) spectroscopy

Spectral analysis of the structure of β-glucan extracted from *L. rhinocerus* mycelium was performed using Nuclear Magnetic Resonance (NMR) with a 500 MHz NMR spectrometer (BRUKER/AVANCE™ NEO, ASCEND™, Switzerland). Measurements were conducted using a CryoProbe™ Prodigy 5 mm BBO 500 MHz, optimized for broad bandwidth and enhanced sensitivity. The spectral data were processed and analyzed using TopSpin NMR software, also provided by the manufacturer in Switzerland.

Antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The extracted β-glucan solutions were prepared by dissolving 0.1 g of β-glucan in 2 mL of methanol and then agitating the mixture using a vortex mixer for approximately 10 min. The solution was then left at room temperature for 2 h, followed by filtration using Whatman No.4 filter paper to collect the clear part of the solution. Subsequently, the antioxidant activity against DPPH (DPPH solution at a concentration of 0.6 mM dissolved in methanol) was tested. This testing was conducted in a 96-well plate, where 100 µL of the β-glucan solution obtained earlier was added, followed by 100 µL of the DPPH solution to each well. The plate was then agitated to ensure thorough mixing. Ascorbic acid at concentrations of 10, 20, 40, 60, 100, and 200 mg/L was used as a standard antioxidant for comparative testing, and β-glucan from yeast cell walls (Innovacan; Thailand) was used as a comparative β-glucan sample. The samples were then incubated at room temperature in darkness for 30 min before measuring the absorbance using a microplate reader (EZread 2000: Biochrom; UK) at a wavelength of 517 nm. If there were antioxidants present, the color of the solution would change from purple to yellow. The scavenging ability was calculated using the Eq. (4) [27]:

$$\% \text{ DPPH activity} = \left[\left(A_{\text{Control}} - A_{\text{Sample}}\right)/A_{\text{Control}}\right] \times 100 \quad (4)$$

$A_{control}$ is the absorbance of the DPPH solution without the sample (control).

A_{sample} is the absorbance of the sample.

3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay

For the ABTS assay, a solution of 7 mM ABTS was prepared and mixed in a 1:1 (v/v) ratio with a 2.45 mM potassium persulfate solution. This mixture was allowed to react at room temperature for 24 h. Prior to use, the ABTS solution was diluted with water to adjust the absorbance to approximately 0.7 ± 0.005 at 734 nm. A total of 50 µL of the β-glucan was then added to 100 µL of the diluted ABTS solution. This mixture was thoroughly combined and left in the dark for 10 min. Absorbance was measured at 734 nm using a microplate reader. The presence of antioxidants in the samples resulted in a color change from green to clear, indicating the scavenging of ABTS radicals. The antioxidant capacity was quantified using the scavenging rate formula to calculate the percentage of antioxidant activity [28]:

$$\text{Scavenging activity (\%)} = [(A_{\text{Control}} - A_{\text{Sample}})/A_{\text{Control}}] \times 100 \quad (5)$$

Acontrol is the absorbance of the ABTS solution without the sample (control).

Asample is the absorbance of the sample.

Results and discussion

Identification

The classification of mushrooms was analyzed based on the morphology of the collected specimens and confirmed by nucleotide sequence analysis in the ITS region, covering ITS-1 and ITS-2 (**Figure 1**). The nucleotides were compared (Blast) with sequences in the ITS database UNITE [29], a database specifically for fungal and mushroom sequences. The study's results classified the mushrooms' mycelium with 99 % homology to *L. rhinocerus* (FJ380871).

The study's results revealed a 99 % homology of the mushroom's mycelium to *L. rhinocerus* (ITS database UNITE accession number FJ380871). A 99 % homology provides strong evidence for the accurate

classification of the species. The genetic analysis corroborates the initial morphological observations, thereby strengthening the confidence in the identification.

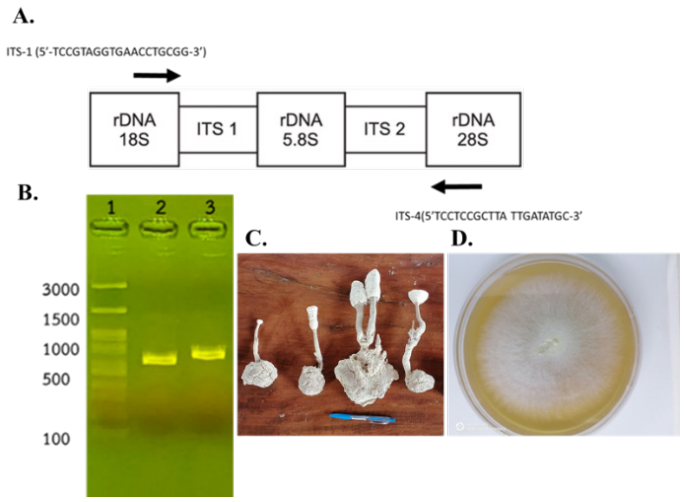

Figure 1 Morphological and molecular analysis of mushroom specimens. (A) Positions and primers for mushroom classification and PCR results in the ITS area. (B) PCR products, Lane 1 100 bp. DNA marker, lane 2 and 3 are PCR products of *L. rhinocerus*. (C) Photograph of collected mushroom specimens, illustrating their morphological features for preliminary identification. (D) Culture of the mushroom mycelium grown on an agar plate.

Optimal conditions for mycelium growth

The mushroom mycelium grew into a spherical ball shape when grown in a broth medium at 30 °C and shaken at 120 round per min (RPM) for 2 weeks. The study results showed that mycelium tended to grow best in formulas A and B, generating the most mycelium biomass (MB). Statistical tests comparing the growth of mycelium in medium A, with the growth of mycelium in other medium found no statistical difference in the growth of mycelium in medium A, and B. Mycelium grown in medium A and B was significantly better than those in formulas C, D, and E, ($p < 0.05$) (**Figure 2**).

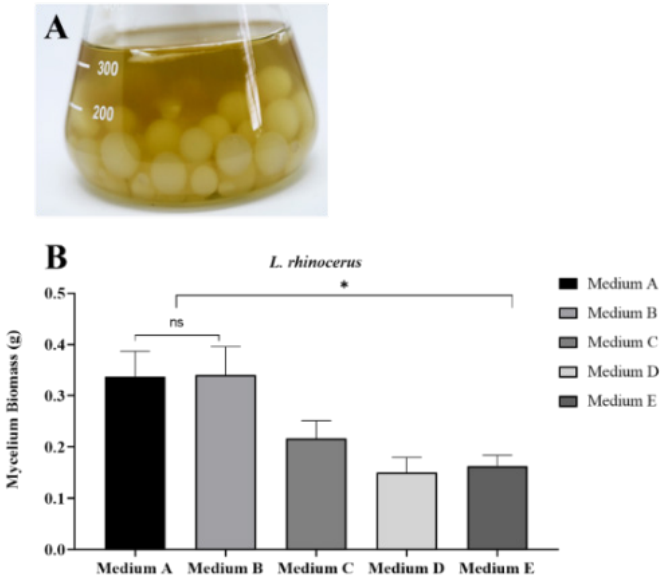

Figure 2 (A) Spherical mycelium growths observed in medium. (B) Bar graph displaying the biomass production of mushroom mycelium across 5 different media formulations (A, B, C, D, E). The statistical analysis that shows significant differences between the growth outcomes in media A and B versus C, D, and E (* $p < 0.05$).

Response surface method (RSM) optimization

β-glucan is a polysaccharide that contains a large amount of glucose. It can be found in many sources, including plants and grains, but the most important and common is fungi, including yeast, mold, and mushrooms. β-glucan is highly popular for its use as a functional food additive for Humans and livestock [30]. It is also essential in cosmetics [31] and topical medicines to treat infectious diseases [32]. Its properties stimulate the activity of white blood cells, causing white blood cells to respond to infection. It can be improved [33,34]. Therefore, the development of β-glucan to utilize it is an approach that is currently receiving significant interest, especially as a dietary supplement for livestock.

Based on preliminary experiments, medium B was the most effective for growing *L. rhinocerus* mycelium. Consequently, medium B was selected for further optimization using the response surface method (RSM) paired with a Central Composite Design (CCD). The experiment determined the optimal conditions for mycelium growth, investigating factors such as temperature (°C), potato powder (%), and glucose (%). The responses measured were mycelial biomass (MB; dry weight in g/L) and β-glucan (g/L). he actual and predicted values of MB and β-glucan yield were summarized in **Table 1**.

Table 1 Response surface method (RSM) results for *L. rhinocerus* mycelium growth and β-glucan production.

Run	Temperature (°C)	Potato powder (%)	Glucose (%)	Mycelium biomass g/L		β-glucan g/L	
				Actual value	Predicted value	Actual value	Predicted value
1	31.5	15	3.5	38.9	37.52	9.45	12.61
2	31.5	15	3.5	43.1	37.52	15.08	12.61
3	31.5	15	3.5	44.7	37.52	14.3	12.61
4	31.5	6.59	3.5	20.12	19.88	7.04	7.14
5	28	20	5	34.1	32.72	11.59	11.79
6	28	10	2	19.47	20.97	6.81	6.73
7	35	10	2	17.23	17.44	6.3	6.03
8	31.5	15	3.5	39.7	37.52	13.89	12.61
9	31.5	15	3.5	37.8	37.52	13.23	12.61
10	35	10	5	15.32	16.09	5.36	6.14
11	28	10	5	22.31	23.18	7.8	7.15
12	25.61	15	3.5	16.31	17.43	5.7	6.49
13	35	20	2	16.35	17.02	5.7	6.28
14	28	20	2	32.24	31.81	11.28	10.43
15	31.5	15	3.5	27.8	37.52	9.73	12.61
16	31.5	15	6.02	32.56	33.84	11.39	11.15
17	31.5	15	0.97	27.35	26.55	9.57	9.91
18	31.5	23.40	3.5	32.14	29.84	11.24	11.25
19	35	20	5	20.1	19.97	7.32	7.33
20	37.38	15	3.5	8.33	5.21	2.83	2.15

Mycelial biomass and β-glucan optimization

Mycelium biomass production

The quadratic model for MB was evaluated using ANOVA, and the results are presented in **Table 2**. The model was found to be significant (p = 0.0005), indicating a strong relationship between the independent variables (temperature, potato powder, and glucose) and the dependent variable mycelium biomass. The significant factors influencing MB (p = 0.0043), and glucose (p = 0.0298). The glucose factor was not significant as a linear term (p = 0.3815), nor were the interaction terms AB (temperature × potato powder), AC (temperature × glucose), and BC (potato powder × glucose), with p-values of 0.1374, 0.8276, and 0.7221, respectively. The lack of fit for the model was not significant (p = 0.9662), suggesting that the model fits the data well. The coefficient of determination (R^2) was high, reflecting the model's ability to explain the variability in mycelium biomass production. The quadratic regression model for mycelium biomass is given by Eq. (6). Significant linear terms were temperature and potato powder, with p-values of 0.0105 and 0.0155, respectively. The quadratic terms for temperature, potato powder, and glucose were also significant (p < 0.0001, p = 0.0043, and p = 0.0298, respectively). This suggests that while the main effects of temperature and potato powder are crucial, their squared terms and the squared term of glucose also have significant impacts, highlighting non-linear effects on mycelium biomass production.

$$\text{Mycelium biomass} = -818.28030 + (48.93183\times\text{Temperature}) + (10.33104\times\text{Potato powder}) + (11.10691\times\text{Glucose}) - (0.147571\times\text{Temperature}\times\text{Potato powder}) - (0.068095\times\text{Temperature}\times\text{Glucose}) + (0.078000\times\text{Potato powder}\times\text{Glucose}) - (0.755224\times\text{Temperature}^2) - (0.174757\times\text{Potato powder}^2) - (1.34070\times\text{Glucose}^2) \quad (6)$$

β-glucan production

The quadratic model for β-glucan production was also assessed using ANOVA, with results summarized in **Table 3**. The model was significant (p = 0.0038), indicating a robust relationship between the independent variables and β-glucan production. Temperature (p = 0.0249), potato powder (p = 0.0317), and the quadratic terms for temperature (p = 0.0001) and potato powder (p = 0.0295) were significant factors affecting β-glucan production. The glucose factor was not significant as a linear term (p = 0.4683), and the interaction terms AB, AC, and BC were also not significant, with p-values of 0.2073, 0.9060, and 0.7210, respectively. The lack of fit for the model was not significant (p = 0.9822), indicating that the model fits the data well. The high R^2 value demonstrated the model's effectiveness in explaining the variability in β-glucan production. The quadratic regression model for β-glucan production is given by Eq. (7). Significant linear terms were temperature and potato powder, with p-values of 0.0249 and 0.0317, respectively. The quadratic terms for temperature and potato powder were significant (p = 0.0001 and p = 0.0295, respectively), indicating that these variables have a non-linear influence on β-glucan production. The glucose factor was not significant as a linear term (p = 0.4683) and the interaction terms were not significant, suggesting that the individual effects of temperature and potato powder are more critical than their interactions with glucose in determining β-glucan production.

$$\beta\text{-glucan} = -255.98330 + (15.50353\times\text{Temperature}) + (3.13785\times\text{Potato powder}) + (2.52820\times\text{Glucose}) - (0.049286\times\text{Temperature}\times\text{Potato powder}) - (0.014762\times\text{Temperature}\times\text{Glucose}) + (0.031333\times\text{Potato powder}\times\text{Glucose}) - (0.239386\times\text{Temperature}^2) - (0.048356\times\text{Potato powder}^2) - (0.326733\times\text{Glucose}^2) \quad (7)$$

Table 2 ANOVA for Quadratic model for mycelium biomass.

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	1903.44	9	211.49	10.34	0.0005	significant
A-Temperature	202.13	1	202.13	9.88	0.0105	
B-Potatose powder	173.49	1	173.49	8.48	0.0155	
C-glucose	17.15	1	17.15	0.8381	0.3815	
AB	53.35	1	53.35	2.61	0.1374	

Source	Sum of squares	df	Mean square	F-value	*p*-value	
AC	1.02	1	1.02	0.0500	0.8276	
BC	2.74	1	2.74	0.1338	0.7221	
A^2	1233.47	1	1233.47	60.29	< 0.0001	
B^2	275.08	1	275.08	13.45	0.0043	
C^2	131.14	1	131.14	6.41	0.0298	
Residual	204.59	10	20.46			
Lack of Fit	28.58	5	5.72	0.1624	0.9662	not significant
Pure Error	176.01	5	35.20			
Cor Total	2108.03	19				

Table 3 ANOVA for Quadratic model for β-glucan production.

Source	Sum of squares	df	Mean square	F-value	*p*-value	
Model	189.53	9	21.06	6.43	0.0038	significant
A-Temperature	22.75	1	22.75	6.95	0.0249	
B-Potatose powder	20.38	1	20.38	6.23	0.0317	
C-glucose	1.86	1	1.86	0.5683	0.4683	
AB	5.95	1	5.95	1.82	0.2073	
AC	0.0480	1	0.0480	0.0147	0.9060	
BC	0.4418	1	0.4418	0.1349	0.7210	
A^2	123.93	1	123.93	37.85	0.0001	
B^2	21.06	1	21.06	6.43	0.0295	
C^2	7.79	1	7.79	2.38	0.1540	
Residual	32.74	10	3.27			
Lack of Fit	3.48	5	0.6958	0.1189	0.9822	not significant
Pure Error	29.26	5	5.85			
Cor Total	222.27	19				

3D surface plot analysis

The 3D surface plots analysis revealed that the mycelium biomass reached its peak at approximately 44.7 g/L. This maximum MB was observed under optimal conditions (temperature 31.5 °C, potatoes powder 15 % and glucose 3.5 %). The data demonstrates a quadratic relationship between temperature and mycelium biomass, where an increase in temperature results in increased biomass up to an optimal point, beyond which further temperature increases lead to a decline in MB. Similarly, the concentration of potato powder exhibited a quadratic effect; initial increases in potato powder concentration led to higher MB, but beyond a certain threshold, further increases caused a decrease in MB. The curved nature of the surface plot underscores the significant interaction between temperature and potato powder concentration, indicating that optimal MB is contingent on a precise combination of these variables. The contour plot at the base of the 3D graph provides a 2D perspective of

biomass distribution, where concentric lines demarcate regions of equal biomass, with the innermost contours indicating the highest biomass levels (**Figure 3(A)**).

For β-glucan yield, the highest production was recorded at 15.08 g/L within a specific range of temperature and potato powder concentration, as indicated by the red area on the plot. Conversely, the lowest production, at 2.83 g/L, is represented by the blue area. The surface plot illustrates a quadratic relationship between the variables and β-glucan production, with both temperature and potato powder exerting significant non-linear effects. The interaction between these factors is evident from the plot's curvature, highlighting the non-linear combined effect on β-glucan production. The 3D surface, along with the 2D contour plot, facilitates the identification of optimal conditions for maximizing β-glucan yield, emphasizing the importance of precise control over both temperature and potato powder concentration (**Figure 3(B)**).

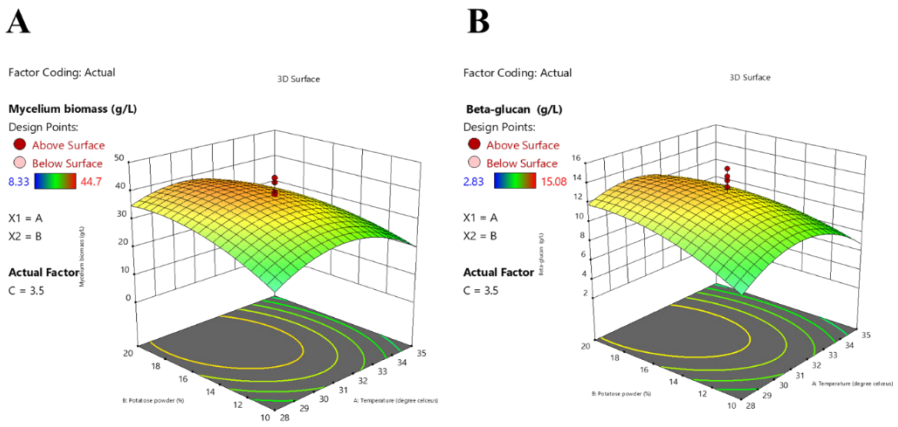

Figure 3 3D surface plot showing the effect of temperature and potato powder on mycelium biomass (A) and β-glucan production (B) of *L. rhinocerus* mycelium. The color gradient represents the predicted mycelium biomass, with blue indicating lower biomass and red indicating higher biomass.

A comparative analysis of β-glucan extraction between mycelium and fruiting bodies of mushrooms indicated that the β-glucan content in fruiting bodies ranges from approximately 24 - 40 %, whereas the β-glucan content in mycelium cultures is between 15 - 26 % [35].

However, for *L. rhinocerus*, differences were observed as the mycelium exhibited no significant β-glucan content compared to the fruiting body. The β-glucan levels across various developmental stages of *L. rhinocerus* were similar, ranging from 9.3 to 13.2 g/100 g dry weight. Previous reports also indicated no significant difference ($p < 0.05$) in β-glucan levels among the pileus, stipe, and sclerotium, although the highest levels were observed in the mycelium [36]. This study revealed that the optimal conditions for MB production were at a temperature of 31.5 °C, a potato powder concentration of 15 %, and a glucose concentration of 3.5 %. Under these conditions, the maximum β-glucan achieved was 15.08 g/L. Importantly, we found that the level of MB was related to the amount of β-glucan. Several studies highlight similar findings. The study on *Monascus purpureus* optimized using potato powder waste demonstrated the critical role of substrate concentration and temperature on biomass production, indicating non-linear effects and the necessity for precise control of these variables to maximize yield. This aligns with the observed quadratic effects in this study, emphasizing that very high and low concentrations of potato powder, along with deviations from the optimal temperature, can negatively impact biomass production [37].

The choice of carbon source plays a crucial role in the growth and metabolite production of fungal mycelium, as demonstrated in numerous studies, including those on *L. rhinocerus* and other fungi. Our study showed that glucose was particularly effective in promoting both biomass and β-glucan production in *L.*

rhinocerus mycelium. This aligns with previous findings, such as those by Usuldin *et al.* [38] where glucose concentration significantly impacted BM and EPS production in a stirred-tank bioreactor. Usuldin *et al.* [38] reported that increasing glucose concentration from 10 to 50 g/L resulted in a 1.7-fold increase in biomass and a 2.4-fold increase in EPS production in large-scale fermentation.

The role of nitrogen sources is equally critical in fungal mycelium growth and metabolite production, alongside carbon sources. Nitrogen is a key component in the synthesis of proteins, nucleic acids, and other cellular structures, and its availability can significantly influence biomass and polysaccharide production in fungi. While our study focused on glucose as the primary carbon source for optimizing β-glucan production in *L. rhinocerus*, the importance of nitrogen sources cannot be overlooked. In the study by Usuldin *et al.* [38], although the primary focus was on optimizing carbon sources like glucose for BM and EPS production, the media used for *L. rhinocerus* cultivation also contained nitrogenous compounds, such as peptone and yeast extract, which supported fungal growth and metabolite synthesis. Nitrogen sources are typically crucial for sustaining rapid fungal growth, particularly in large-scale bioreactor settings where efficient nutrient uptake is essential. This highlights the complementary role that nitrogen sources play alongside glucose in driving both biomass yield and secondary metabolite production.

The analysis of variance (ANOVA) indicated that both temperature and potato powder concentration significantly affected both MB levels, with quadratic effects observed for both variables. This suggests a non-linear relationship, where both very high and deficient concentrations of potato powder and deviations from the optimal temperature negatively impact biomass production [37]. These results underscore the importance of maintaining precise control over cultivation conditions to maximize mycelium yield.

The production of β-glucan in cultivating *L. rhinoceros* followed a similar trend to MB production, with the highest yield observed under the optimal conditions identified through RSM. The maximum β-glucan yield recorded was 15.08 g/L. The analysis of variance (ANOVA) results demonstrated that temperature and potato powder concentration significantly influenced β-glucan production, with significant linear and quadratic effects for both variables. This indicates a non-linear relationship where moderate levels of these factors are crucial for optimal β-glucan production.

Research has shown that temperature plays a critical role in β-glucan production in various fungal species. The study optimized the bioconversion of potato starch to β-glucan using *P. eryngii*, highlighting that the β-glucan content increased significantly under optimal temperature conditions [39]. Similar findings were reported in a study on *Ganoderma lucidum*, the research employed high-pressure steaming and enzymatic hydrolysis to optimize β-glucan extraction, revealing significant quadratic effects of substrate concentration on β-glucan yield. This supports the importance of maintaining moderate levels of potato powder for optimal β-glucan production [40]. Further evidence from studies on *Hericium erinaceus* demonstrated significant quadratic effects of temperature and substrate on polysaccharide production. These studies confirmed that deviations from optimal conditions negatively impacted yield, supporting the idea that both very high and low concentrations of potato powder and temperature deviations adversely affect β-glucan production [41].

Characterization of β-glucan

Field Emission Scanning Electron Microscope (FESEM)

Samples of β-glucan extracted of *L. rhinocerus* mycelium was used to study the structure. Microscopic level using a Field Emission Scanning Electron Microscope (FESEM) (Merlin compact, Zeiss, EDX (Oxford, Aztec), EBSD (Oxford, Nordlys Max)) with a magnification of 1-10 K X times. The results of the study show the structural characteristics of β-glucan. The extracted β-glucan has relatively fine particles held together to form a thin, smooth surface. β-glucan samples extracted from *L. rhinocerus* mycelium had a similarly surface with β-glucan extracted from commercial yeast β-glucan (Innovacan: Thailand) (**Figure 4**).

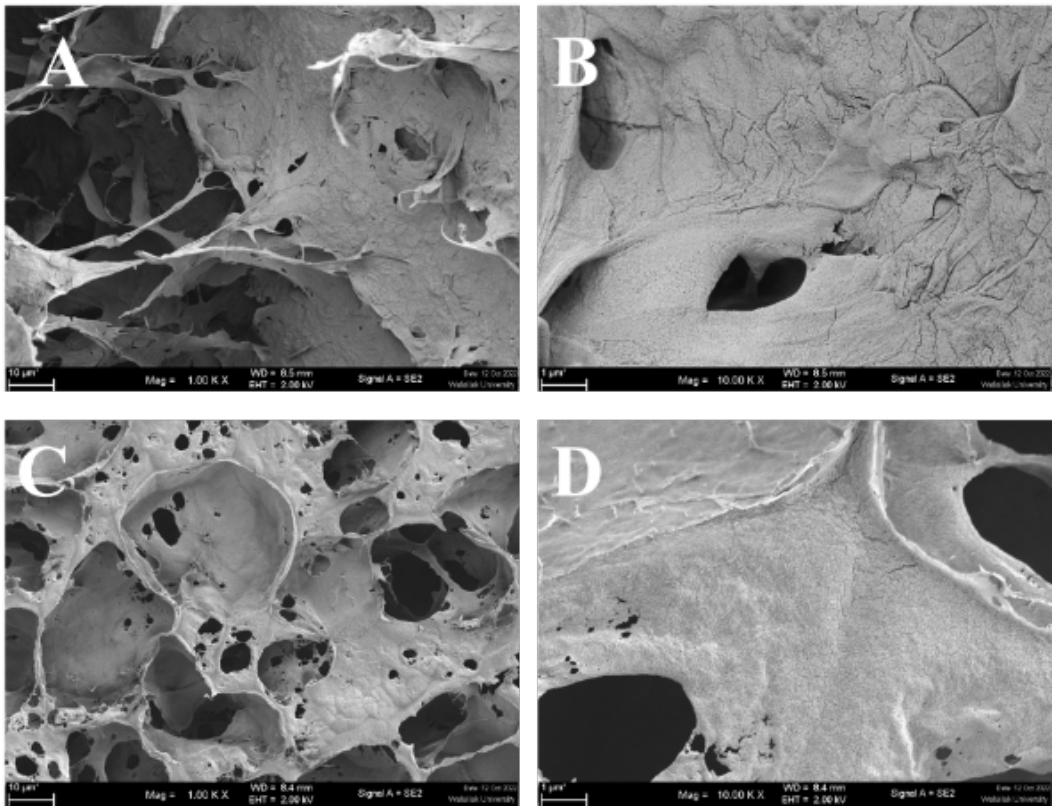

Figure 4 Field Emission Scanning Electron Microscopy (FESEM) images showing the morphological structure of β-glucan. (A) and (B) β- glucan samples extracted from *L. rhinocerus* mycelium, demonstrating relatively fine particles held together to form a thin, smooth surface. (C) and (D) Commercial yeast β-glucan (Innovacan, Thailand), exhibiting a similar surface morphology to the β-glucan extracted from *L. rhinocerus* mycelium.

Nuclear Magnetic Resonance (NMR) spectroscopy

The ^{13}C NMR spectra of β-glucan were analyzed to compare the structural characteristics of the β-glucan extracted from mushroom mycelium with the commercial β-glucan (Innovacan, China). ^{13}C NMR spectrum of commercial β-glucan (Glucan control 4.5 mg DMSO), and ^{13}C NMR spectrum of β-glucan extracted from *L. rhinocerus* mycelium (Glucan 04, 0.46 mg DMSO). Both spectra display characteristic chemical shifts, δ (ppm), which indicate the presence of (1-3,1-6)-β-D-glucan structures. The signal around 100 ppm is attributed to the (1-3) linkage at the C1 position, while signals between 55 - 85 ppm correspond to carbons C3, C5, C2, and C4. The signal around 60 ppm is associated with the (1-6) linkage at the C6 position (**Figure 5**).

The commercial β-glucan extracted exhibits stronger signal intensities compared to the β-glucan extracted from *L. rhinocerus* mycelium. This suggests that the concentration of β-glucan in the commercial sample is higher, likely due to more efficient extraction and preparation processes.

The signals in both spectra correspond to similar chemical shifts, indicating that the structural components of the β-glucans are consistent. However, the extracted β-glucan sample shows lower signal intensity, which might be due to a lower yield or purity of the β-glucan in this sample.

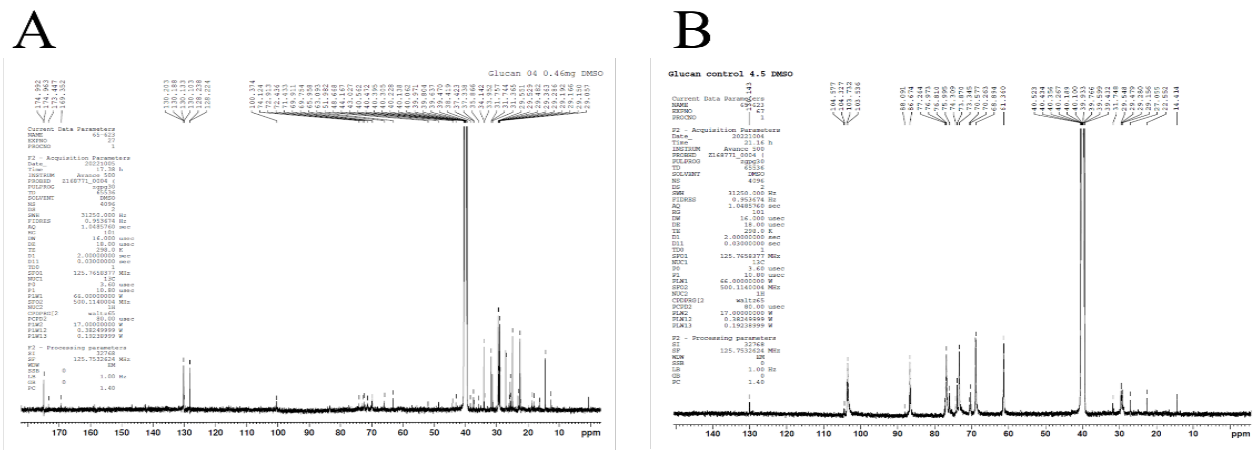

Figure 5 ^{13}C NMR Spectra of β-glucan extracted from *L. rhinocerus* mycelium (A), ^{13}C NMR spectrum of commercial β-glucan (Glucan control 4.5 mg DMSO) from Innovacan, China (B). Both spectra show characteristic chemical shifts (δ, ppm) indicating the presence of (1-3,1-6)-β-D-glucan structures.

Function group characterization by Fourier Transform Infrared (FTIR) spectroscopy

The spectra in the range of 3268 - 3303 cm^{-1} and 2850 - 2970 cm^{-1} indicated the presence of O-H and C-H stretching vibrations typical of polysaccharides. Absorption bands between 1627-1659 cm^{-1} were attributed to water and protein interactions, suggesting incomplete removal of proteins bound to the polysaccharides. The absorption in the range of 1000 - 1200 cm^{-1} corresponded to glycosidic bonds in the pyranose ring, indicating C-O-C and C-OH stretching vibrations. Distinct spectra in the range of 800 - 950 cm^{-1} were characteristic of β-(1,6) glucan molecules, with specific bands at 802 - 932 cm^{-1} indicating α- and β-glycosidic linkages found in (1→6)-β-D-glucan, confirming the presence of both α- and β-glucan in the samples. Additionally, the range of 1019-1098 cm^{-1} was indicative of β-(1,3) glucan, while bands around 1160 cm^{-1} signified β-(1,4) glucan. Sample analysis also showed bands in the range of 1141 - 1252 cm^{-1}. The frequency range of 1308 - 1376 cm^{-1} suggested the presence of cellulose, a common polysaccharide in plant and fungal cell walls.

The absorption bands at 802 - 932 cm^{-1}, 1019 - 1098 cm^{-1}, and 1141 - 1252 cm^{-1} collectively indicated the presence of β-(1,3), β-(1,6), and β-(1,4) glucans (**Figure 6**). These findings confirm that the β-glucan extracted from *L. rhinocerus* mycelium primarily consists of these structures, similar to the commercial yeast β-glucan.

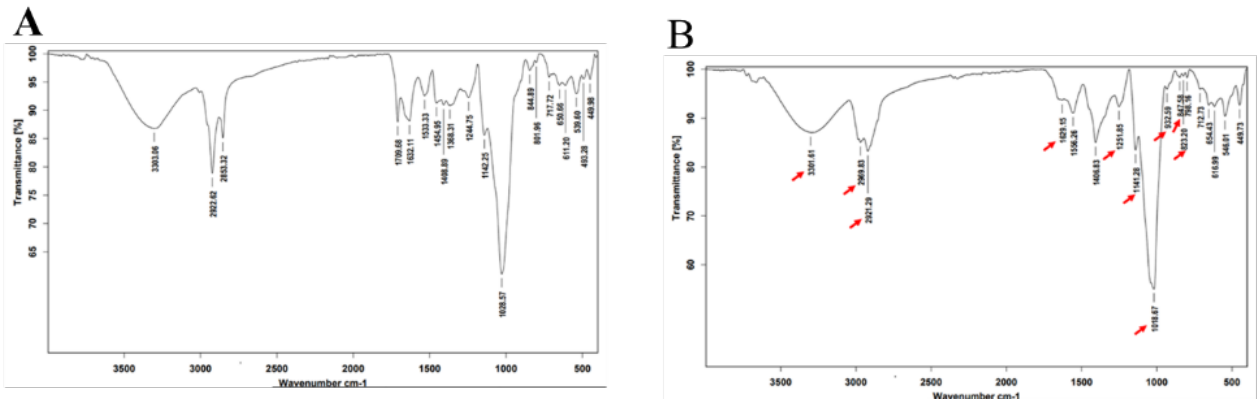

Figure 6 (A) β-glucan extracted from *L. rhinocerus* mycelium and (B) commercial β-glucan showing characteristic absorption bands. Key features include O-H and C-H stretching vibrations, glycosidic bonds, and cellulose, confirming the presence of β-(1,3), β-(1,6), and β-(1,4) glucans.

The use of microwave-assisted extraction proved to be an efficient method for isolating β-glucan from *L. rhinocerus* mycelium. This technique not only reduced the extraction time but also increased the yield compared to traditional methods. The findings support previous studies that highlight the efficacy of microwave extraction in enhancing the solubilization and recovery of polysaccharides from mushrooms [21]. The extraction of β-glucan from oats and barley is significantly influenced by the temperature used during the process. When high temperatures are applied during the shipping or processing stages, the concentration of β-glucan increases in a linear relationship with the temperature. Specifically, the extraction efficiency of β-glucan is markedly improved at elevated temperatures of 100, 120 and 140 °C, resulting in larger quantities and higher efficiency in the β-glucan yield [42,43]. This is consistent with previous studies on oyster mushroom samples, which found that using microwaves resulted in higher beta-glucan yields in a shorter time than using chemical methods alone. These findings underscore the importance of fine-tuning reaction parameters to enhance the yield and quality of oligosaccharides from grey oyster mushrooms, highlighting the advantages of microwave-assisted extraction techniques [21].

For the FESEM analysis, the result showed that the β-glucan extracted from *L. rhinocerus* mycelium consisted of fine particles forming a thin, smooth surface. This morphology was consistent with commercial yeast β-glucan, suggesting that the structural properties of β-glucan are maintained across different sources. Similar findings were reported indicated that the surface morphology of *L. rhinocerus* β-glucan was analyzed and found to have fine, smooth particles indicative of high purity and consistent extraction processes [44].

These findings are consistent with previous studies on *L. rhinocerus*, which also reported similar chemical shifts in the NMR spectra, confirming the presence of (1-3, 1-6)-β-D-glucan linkages in their β-glucan extracts [44]. The absorption bands observed, such as those between 1000-1200 cm^{-1} (glycosidic bonds) and 800 - 950 cm^{-1} (β-(1,6) glucan molecules), were consistent with those reported in previous studies indicated that β-glucan from *L. rhinocerus* also identified similar FTIR absorption bands, confirming the presence of β-(1,3), β-(1,6), and β-(1,4) linkages in their samples [16]. The consistency of these absorption bands across different studies suggests that the β-glucan structure in *L. rhinocerus* is inherently stable and reproducible, regardless of the extraction method used. This stability is crucial for ensuring that the functional properties of β-glucan, such as its immune-modulating and anticancer activities, are preserved in different preparations.

Antioxidant activity of β-glucan extract from *L. rhinoceros*

The antioxidant activities of β-glucan extract from *L. rhinocerus* were assessed using DPPH and ABTS radical scavenging assays. Vitamin C (ascorbic acid) at a concentration of 100 µg/mL was used as a positive control to benchmark the antioxidant activity. The results, demonstrate that the β-glucan extract exhibits notable antioxidant activity in both assays, although its efficacy is lower than that of the standard Vitamin C. Specifically, the DPPH radical scavenging activity of the β-glucan extract was measured at approximately 53.31 %, while its ABTS radical scavenging activity was around 47.31 %. In comparison, Vitamin C showed a significantly higher antioxidant activity, with a radical scavenging rate of around 71.94 % in both assays (**Figure 7**). These results are consistent with previous studies that report.

the antioxidant properties of β-glucans, which are attributed to their ability to neutralize free radicals [5]. The antioxidant assays have revealed that β-glucan from *L. rhinocerus* mycelium possesses notable antioxidant activity, though slightly lower than ascorbic acid. This finding is particularly significant as comparative analyses of the antioxidant capacity of mushrooms and mycelium from different morphological and developmental stages are scarce, with many findings lacking consistency. Previous studies have compared the antioxidant capacity and cytotoxic effects of *L. rhinocerotis* cultured under various liquid fermentation conditions to the sclerotium produced by solid-substrate fermentation. The aqueous methanol extracts of the mycelium and culture broth have demonstrated higher or comparable antioxidant capacities to the sclerotium extract based on their radical scavenging abilities, reducing properties, metal-chelating activities, and inhibitory effects on lipid peroxidation. Furthermore, the fruiting bodies of several cultivated mushrooms have shown higher antioxidant activity than their mycelium,

and all extracts have exhibited low cytotoxicity (IC50 > 200 µg/ml, 72 h) against selected mammalian cell lines [16].

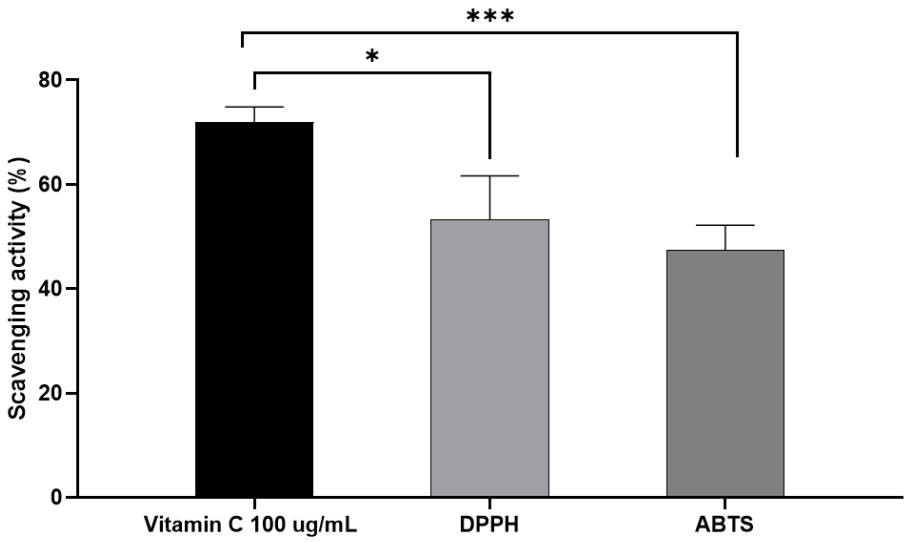

Figure 7 Scavenging activities of Vitamin C (100 µg/mL) and β-glucan extract from *L. rhinocerus* measured by DPPH and ABTS assays. Data are presented as mean ± standard deviation (n = 3). Significant differences are indicated as follows: *$p < 0.0109$, ***$p < 0.0008$.

Conclusions

This study successfully optimized the laboratory cultivation conditions for *L. rhinocerus* mycelium using Response Surface Methodology (RSM) and Central Composite Design (CCD). The optimal conditions were identified as a temperature of 31.5 °C, 15 % potato powder, and 3.5 % glucose concentration, which yielded a maximum mycelial biomass of 44.7 g/L and β-glucan production of 15.08 g/L. The use of a household microwave for β-glucan extraction proved to be efficient, enhancing both yield and cost-effectiveness. The extracted β-glucan demonstrated significant antioxidant activity, with DPPH and ABTS assays showing radical scavenging activities of 53.31 and 47.31 %, respectively. These values, while slightly lower than those of ascorbic acid, indicate that β-glucan from *L. rhinocerus* mycelium is a potent antioxidant. Comparative analyses revealed consistent antioxidant capacities across different morphological stages, with mycelium and culture broth extracts showing higher or comparable activities to sclerotium extracts. Structural characterization using Field Emission Scanning Electron Microscopy (FESEM) and Fourier Transform Infrared (FTIR) spectroscopy confirmed the integrity and composition of the β-glucan extracted. These findings highlight the potential of using *L. rhinocerus* mycelium as a sustainable and efficient source for β-glucan extraction, providing an alternative to traditional fruiting body extraction methods.

Acknowledgments

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