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Isolation of bioactive beta-glucans from mycelium of *Pleurotus ostreatus* mushroom

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ABSTRACT

Background: Beta-glucan, a compound found in higher fungi, possesses significant properties important for the human body, including immunomodulatory effects. Developing efficient extraction technology is crucial to ensure a high yield of beta-glucan component while preserving its bioactive properties.

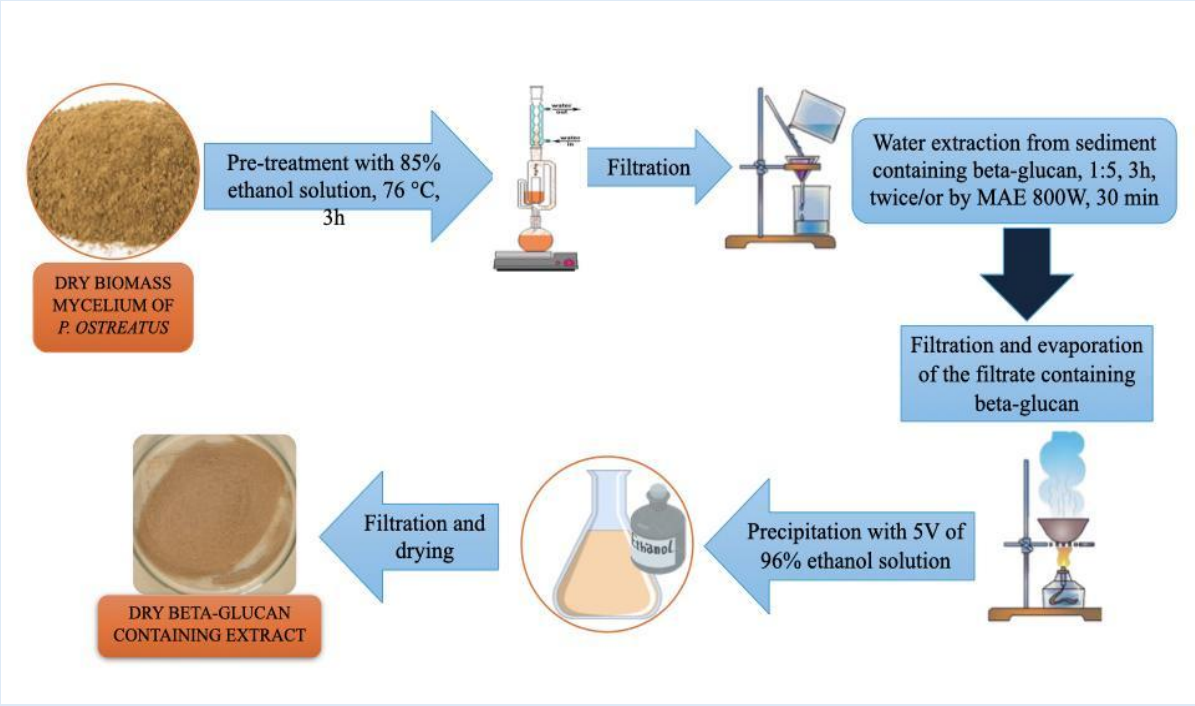
Context and purpose of this study: This study aimed to evaluate the extraction efficiency of beta-glucans from *Pleurotus ostreatus* mycelium powder using microwave-assisted extraction and to compare its performance with traditional extraction methods—specifically water and ethyl alcohol-based extractions.

Objective: The aim of this study is to increase the yield of the aqueous extract of beta-glucan from the mycelium of the fungus *Pleurotus ostreatus*, while ensuring its biological activity is preserved for subsequent use in the fortifying food products. To achieve this, various extraction methods were employed.

Results: Traditional methods of beta-glucan extraction, including water and ethyl alcohol-based processes, yielded the largest amount of beta-glucan of 1.45 ± 0.02 g of beta-glucan. The stimulation index, a measure of beta-glucans on the human phagocytosis system, reached 1.28 under optimal conditions — double treatment with ethanol followed by a double extraction with water, each extraction lasting three hours. When extracting beta-glucan using microwave radiation, 2.97 g of beta-glucan was obtained—2.05 times greater than traditional methods. The simulation index also improved by 13%, reaching 1.47% while reducing the extraction time.

Conclusions: Based on the conducted research, it has been found that the extraction of beta-glucan from the mycelium of the *Pleurotus ostreatus* fungus is advisable to carry out using microwave processing, which significantly reduces the extraction time while maintaining the biological activity of beta-glucan.

Keywords: Basidiomycetes, Pleurotus ostreatus, beta-glucan, water extraction, ethyl alcohol extraction, microwave radiation, immunomodulatory properties.



Graphical Abstract: Isolation of bioactive beta-glucans from mycelium of *Pleurotus ostreatus* mushroom

INTRODUCTION

Back in the 1st century BC, the ancient Roman scientist Dioscorides described the medicinal properties of larch polypore for gastrointestinal diseases. In traditional Chinese and Japanese medicine, mushrooms have played a central role for over 2,000 years, with medical literature describing the use of more than 1,000 varieties. The most frequently used species for medicinal purposes were *Lentinus edodes, Ganoderma lucidum, Trametes versicolor, Cordyceps sinensis,*

Auricularia auricula-judae, Grifola frondose, and *Pleurotus ostreatus. Pleurotus ostreatus* (oyster mushroom) belongs to the class Basidiomycetes and the orders of Hymenomycetes. It is one of the most widely produced commercial species and the most studied due to its medicinal properties. These mushrooms are a well-established industrial species valued for its β-1,3/1,6-D-glucan content, associated immunomodulatory properties [1-5], antimicrobial [6,7] and antioxidant activity [8-11], and hypocholesterolemic and hypolipidemic effects [12-14]. Research has extensively explored the biological effects of beta-glucans, with over 9,000 published studies, making them the most studied immunomodulators [15]. Beta-glycogens have demonstrated the ability to induce TRIM, offering protection against a range of viral infections through altered immune responses [16]. These large molecules are not subject to enzymatic fragmentation in the gastrointestinal tract. Upon ingestion, beta-glucans are captured by the cells of the intestinal mucosa, and actively transported to the submucosal layer. It is there that macrophages are activated, which then activate lymphocytes responsible for protecting the endothelium for local immunity [17,18]. Activated lymphocytes from the intestinal mucosa are spread to the mucous membranes of various organs, and this leads to their protection from infections [19].

It is widely recognized that natural or processed foods containing effective and safe quantities of bioactive compounds offer clinically validated and documented health benefits using specific biomarkers. These foods enhance human health, reduce the risk of chronic and viral diseases, and aid in managing symptoms of diseases [20-21]. Because beta-glucans are polysaccharides with immunomodulatory properties with GRAS (Generally Recognized as Safe) status by the FDA (Food and Drug Administration), this study worked on increasing the extraction yield for its subsequent utilization in the fortification of food products.

Traditional extraction methods involve multi-stage sequential ethanol and aqueous extractions [22], often resulting in suboptimal yields. This study evaluated the efficacy of microwave-assisted extraction (MAE) as a novel approach for the isolation of β-glucan from *P. ostreatus mycelia*.

Furthermore, MAE offers several advantages over traditional methods [23]. By reducing the number of extraction stages and requisite processing time, MAE significantly minimizes energy consumption, becoming a more sustainable and efficient approach for large-scale β-glucan extraction.

Based on these results, MAE demonstrates significant potential for β-glucan extraction from P. ostreatus. Its superior yield, enhanced immunomodulatory activity, and efficient processing suggest its broad applicability across food and pharmaceutical industries.

MATERIALS AND METHODS

Materials: Dry extract of the mycelium of the mushroom *Pleurotus ostreatus* was used for this experiment. The cultivation of *Pleurotus ostreatus* was conducted on a semi-synthetic culture media (based on glucose and peptone) using 0.75 L Erlenmeyer flasks containing 100 mL of medium on a shaker at 230 rpm, at a temperature of 23 ±2°C and humidity of 90 ±3 %.

After cultivation, the obtained mycelial biomass was separated from the native solution and dried in a drying oven (ES-4620), at 50°C until the moisture content did not exceed 10%. The dried biomass was ground into a powder using a RED solution RCD-M1608

grinder (China) with a rotor speed of 20,500 rpm for 200 seconds, achieving particle sizes of 0.3 - 0.5 mm.

Processing of the mycelium powder of the mushroom *Pleurotus ostreatus* with water and ethyl alcohol: This procedure was performed to remove lipids, abstract low-molecular-weight substances, and increase the permeability of the mycelial cell wall. Finely ground mycelium of P. ostreatus was mixed with 85% v/v ethyl alcohol in a ratio of 1:2.5 and the mixture was boiled at a temperature of 75±2°C for 3 hours. The mixture was then filtered through a 'red ribbon' paper filter (pore size: 8-12 µm), at a temperature of 23 ±2°C to separate the filtrate from the solid residue (single extraction). The solid residue was subsequently mixed with 85% v/v ethyl alcohol in a ratio of 1:2.5, and a second extraction was conducted to remove lipids and low-molecular-weight substances (double extraction). The third removal of lipids and low-molecular-weight substances from the solid residue was carried out in a similar manner (triple extraction).

Obtaining a water-soluble fraction of beta-glucan: The solid residue obtained after filtration was mixed with water in a ratio of 1:5. Using this mixture, beta-glucan was extracted for 3 hours at a temperature of 100±2°C (single extraction). The second and third extractions of beta-glucan from the solid residue with water were conducted in a similar manner. After the extraction and filtration were completed, the filtrate was collected. The resulting aqueous extracts were separated from the residue by filtration, concentrated, and high-molecular-weight compounds, including beta-glucans, were precipitated by adding 5 volumes of 96% v/v ethanol, leaving the mixture at a temperature of 4 ±2°C allowing for sediment formation. The precipitate was filtered through a 'red ribbon' paper filter (pore size: 8-12 µm) at a temperature of 23 ±2°C, dried at 70°C in a stream of air (6 hours with a layer thickness of 1.5 +/- 0.5 mm), and finally, ground into a powder.

Microwave radiation treatment of mycelium powder: Two variants of microwave-assisted extraction were evaluated in this study. In the first variant, the mycelium powder of the mushroom Pleurotus ostreatus was pre-treated with water and ethyl alcohol as described above. A suspension of the mycelium powder and water was then prepared in a ratio of 1:5. The water-soluble fraction was extracted through microwave treatment of the aqueous suspension of the dry biomass powder for 30 minutes. In the second variant, a suspension of the mycelium powder of *Pleurotus ostreatus* and water was prepared in a ratio of 1:5. The water-soluble fraction was extracted by microwave treatment of the aqueous suspension of the dry biomass powder for 30 minutes, without prior treatment of ethanol. For both variants, microwave treatment was performed using an LG model MB4049F/01 microwave oven with a power of 800 W and a frequency of 2450 MHz. The extracts obtained from both variants were concentrated, and high-molecular-weight compounds, including beta-glucans, were precipitated by adding 5 volumes of 96% v/v ethanol and allowing the mixture to stand at a temperature of 4°C for sediment formation. The precipitate was filtered through a 'red ribbon' paper filter (pore size: 8-12 µm), at a temperature of 23 ±2°C, dried at 70°C in a stream of air (6 hours with a layer thickness of 1.5 +/- 0.5 mm), and ground into a powder. The particle size distribution of the obtained powdered preparations was assessed using the sieve method.

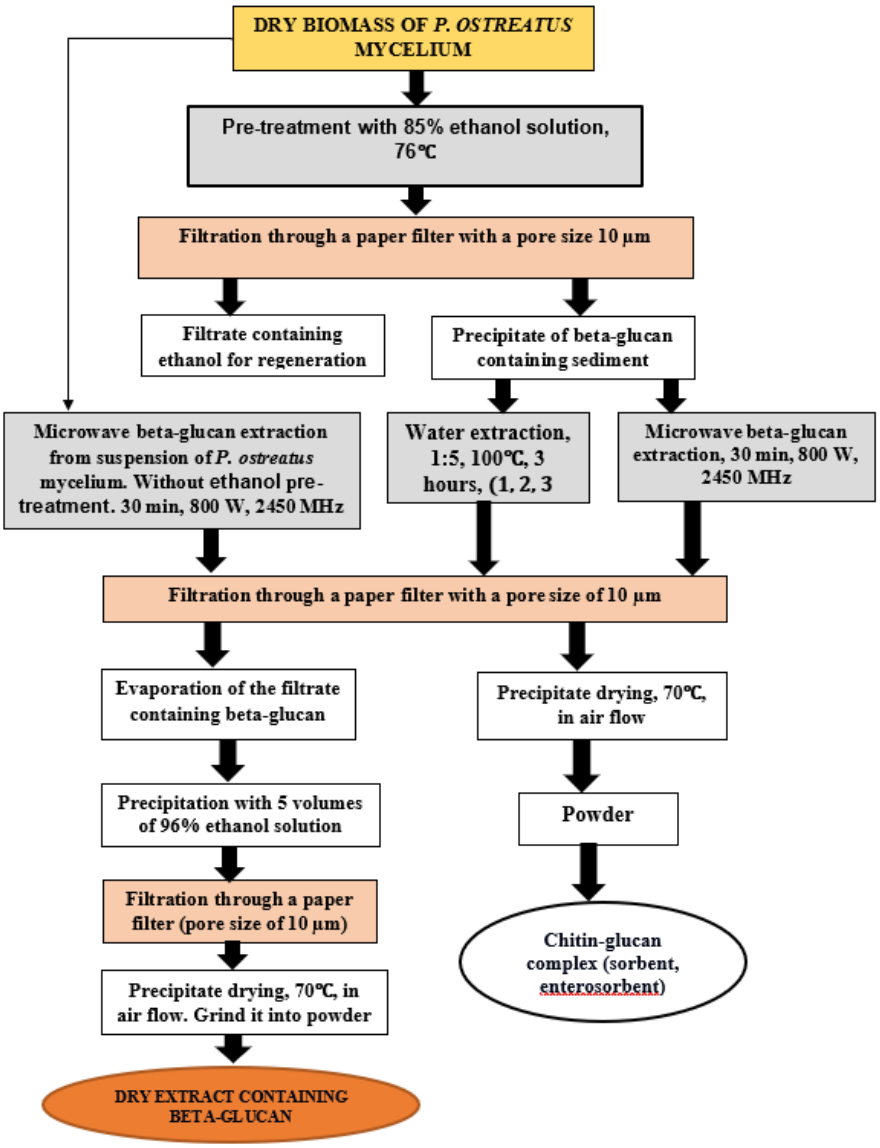


Figure 1. Scheme for obtaining beta-glucan containing isolates.

The content of mycelial beta-glucan in the resulting powder was determined by the enzymatic method [24]. This method consists of two main stages - determining the content of total glucan and α-glucan. The difference in the concentrations of total and alpha-glucans was used to determine the concentration of beta-glucan. Using luminol-dependent chemiluminescence, the assessment of the biological activity of the studied extracts was carried out by test analysis of reactive oxygen species generated by human peripheral blood cells. The test was chosen based on the assumption of the stimulating effect of *Pleurotus ostreatus* mycelium extracts on the phagocytosis system. When blood phagocytes were activated, a cascade of chain reactions was launched. When combined with luminol, the resulting product, which contained oxygen species at a wavelength of 425 nm, cause luminescence. Because of this, the production of reactive oxygen species was determined by luminol-dependent chemiluminescence.

The experiment used fresh whole donor blood, Hank's salt solution from Sigma, and a luminol solution with a concentration of 10 M (prepared by dissolving 1.77 mg of luminol in 10 ml of dimethyl sulfoxide). The level of chemiluminescence was measured using a Victor-2 chemiluminometer from Pribori Oy (Finland), connected to a personal computer that provided sequential registration of chemiluminescence from 96 samples.

The quantity of dry extract from the mycelium of the *P. ostreatus* fungus and the resultant dry extract containing beta-glucan was quantified using a

gravimetric method on VIBRA HT-224RCE balance with an accuracy of four decimal places.

To calculate the stimulation index, the average of duplicates/triplicates was first calculated, and then the background value (without cells) of each sample and control was substracted. The values of samples (stimulated cells) were then divided by the negative control (untreated samples).

The result is the arithmetic mean value of three parallel determinations. The statistical significance of the experiment was determined using the student's t-test.

RESULTS

Analysis of traditional methods for beta-glucan extraction using water and ethyl alcohol: Various solvents, including water and ethanol, are employed for the extraction of biologically active compounds. The extraction is conducted with varying extraction multiplicity. In the initial phase of the study, the objective was to compare the methods of extracting beta-glucan from the mycelium of *Pleurotus ostreatus* using water and ethyl alcohol extraction. The results are presented in Table 1.

Table 1. The influence of aqueous and hydroalcoholic extraction on the amount of beta-glucan extracted from 100 g of dry mycelium powder of *P. ostreatus*.

Extraction method	The amount of dry extract obtained from the mycelium of the fungus *P. ostreatus*, g	Concentration of beta-glucans in the dry preparation, %	Amount of beta-glucan in dry extract from the mycelium of the fungus *P. ostreatus*, g	Stimulation Index
Water extraction (1 time)	3.2± 0.01	7.4± 0.02	0,23± 0.02	0.51
Ethanol treatment (1 time) + water extraction (1 time)	4.7± 0.02	20.8± 0.03	0,98± 0.02	1.23

Based on the results presented in Table 1, it is demonstrated that performing extraction with water with extraction using 85% ethyl alcohol increases the amount of the dry extract containing beta-glucan derived from the mushroom *P. ostreatus* by 46 %, increasing its concentration threefold. Consequently, the total amount of extracted beta-glucan using both solvents is 4.2 times greater compared to the total amount as beta-glucan that was extracted using only water.

In the next stage of experiments, a study was conducted to investigate the effect of increasing the number of water extractions: one, two, and three times using a single extraction with 85% ethyl alcohol. The results of these experiments are presented in Table 2.

Table 2. Effect of increasing the number of water extractions on the amount of beta-glucan contained in 100 g of dry powder of *P. ostreatus* mycelium.

Extraction method	The amount of dry extract obtained from the mycelium of the fungus P. ostreatus, g	Concentration of beta-glucans in the dry preparation, %	Amoun of beta-glucan in dry extract from the mycelium of the fungus P. ostreatus, g	Stimulation Index
Ethanol treatment (1 time) + water extraction (1 time)	4.7± 0.02	20.8± 0.02	0,98± 0.02	1.23
Ethanol treatment (1 time) + water extraction (2 times)	5.7± 0.01	23.4± 0.01	1.33± 0.02	1.21
Ethanol treatment (1 time) + water extraction (3 times)	5.8± 0.02	22.8± 0.02	1,32± 0.02	1.22

The results presented in Table 2 indicate that performing a two-fold extraction with water alongside 85% ethyl alcohol from the mycelium powder of *P. ostreatus* yields a 21% increase in the dry mushroom extract compared to the single extraction with water and ethyl alcohol. Additionally, this approach increases the concentration of beta-glucan by 12.5% and increases the amount of extracted beta-glucan by 36%; however, it is accompanied by a slight decrease in the stimulation index. Further increasing the number of water extractions to three does not lead to an increase in the concentration of beta-glucan in the preparation or the total yield of extracted beta-glucan.

In the next stage of the experiments, a study was conducted to investigate the effect of increasing the number of extractions with the 85% ethyl alcohol: one, two, and three times, combined with a two-fold extraction with water. The results of these experiments are presented in Table 3.

Table 3. Effect of increasing the number of ethyl alcohol extractions on the yield of beta-glucan contained in 100 g of dry powder of *P. ostreatus* mycelium.

Extraction method	The amount of dry extract obtained from the mycelium of the fungus P. ostreatus, g	Concentration of beta-glucans in the dry preparation, %	Amoun of beta-glucan in dry extract from the mycelium of the fungus P. ostreatus, g	Stimulation Index
Ethanol treatment (1 time) + water extraction (2 times)	5.7± 0.01	23.4± 0.01	1.33± 0.02	1.21
Ethanol treatment (2 times) + water extraction (2 times)	6.1± 0.02	23.7± 0.01	1,45± 0.03	1.28
Ethanol treatment (3 times) + water extraction (2 times)	6.3± 0.02	23.2 ± 0.02	1,46± 0.02	1.25

The results presented in Table 3 indicate that performing a two-fold ethanol extraction combined with a two-fold water extraction from the mycelium powder of *P. ostreatus* increases the yield of the dry mushroom extract by 7% compared to a single ethanol extraction and a two-fold water extraction. This method also increases the concentration of beta-glucan in the powder by 1.3%, the total amount of extracted beta-glucan by 9%, and the stimulation index by 5.8%. Further increasing the number of ethanol extractions to three results in a slight increase in the extracted beta-glucan but causes a decrease in the stimulation index.

Comparing the results obtained from different beta-glucan extraction methods, it can be concluded that most effective approach to extract beta-glucan is a two-fold extraction with 85% ethyl alcohol combined with a two-fold extraction with water.

Application of microwave radiation for beta-glucan extraction: To intensify the extraction process of beta-glucan from the mycelium powder of *P. ostreatus*, two experimental approaches were tested. In the first case, beta-glucan was extracted using 85% ethyl alcohol for 3 hours, followed by treatment with microwave radiation at 800 W and a frequency of 2450 MHz for 30 minutes. In the second case, the mycelium powder of *P. ostreatus* was subjected to microwave radiation at 800 W and a frequency of 2450 MHz. For comparison, a two-fold extraction of beta-glucan using 85% ethyl alcohol and a two-fold extraction with water was performed. The results of these experiments are presented in Table 4.

Table 4. Application of microwave radiation for the extraction of beta-glucan contained in 100 g of dry powder of *P. ostreatus* mycelium.

Extraction method	The amount of dry extract obtained from the mycelium of the fungus P. ostreatus, g	Concentration of beta-glucans in the dry preparation, %	Amoun of beta-glucan in dry extract from the mycelium of the fungus P. ostreatus, g	Stimulation Index
Ethanol treatment (2 times) + water extraction (2 times)	6.1± 0.02	23.7± 0.01	1,45± 0.03	1.28
Ethanol treatment (once for 3 hrs.) + Microwave treatment	7.7± 0.02	39.5± 0.02	3,04 ± 0.01	1.83
Microwave treatment	9.8± 0.03	30.3± 0.02	2,97± 0.02	1.47

The results presented in Table 4 indicate that microwave treatment of the mycelium extract powder of *P. ostreatus* increases the amount of beta-glucan powder by 60%, raises the concentration of beta-glucan in the powder by 26%, and doubles the amount of extracted beta-glucan. Additionally, the stimulation index increases by 15% compared to the sample processed using a two-fold water extraction and a two-fold water treatment. Notably, this method reduced the extraction time from 12 hours to 30 minutes.

The exclusion of ethanol extraction and relying solely on microwave treatment of the mycelium of *Pleurotus ostreatus* leads to a 27% increase in the amount of the dry extract containing beta-glucan. However, this procedure leads to a 24% reduction in the concentration of beta-glucan within the powder and a 20% decrease in the stimulation index. This reduction in beta-glucan content and stimulation index is likely because the omission of alcoholic treatment does not facilitate the removal of the lipid fraction.

DISCUSSION

The experiments conducted demonstrates the effectiveness of microwave radiation for extracting beta-glucan from the mycelium powder of the fungus compared to traditional extraction methods using water and ethyl alcohol.

Mathematical processing and data analysis showed that treating a mixture of mycelium powder and water with microwave radiation at a power of 800 W and a frequency of 2450 MHz for 30 minutes, compared to a two-fold extraction with 85% ethyl alcohol (3 hours for each extraction) and a two-fold extraction with water (3 hours for each extraction), allows for a two-fold increase in the yield of beta-glucan. This method also achieved a 15% increase in the stimulation index, which reflects the immunomodulatory properties of beta-glucan, while significantly reducing the extraction time from 12 hours to 30 minutes.

Additionally, a comparative analysis of beta-glucan extraction from the mycelium of *P. ostreatus* using water and ethyl alcohol showed that ethyl alcohol enhances the biological activity of beta-glucan, but slightly reduces the yield of beta-glucan. A two-fold extraction of beta-glucan using both ethyl alcohol and water provides an advantage over single-extraction methods, while further increasing the number of extractions to three or more times is deemed impractical.

In conclusion, maximizing the yield of beta-glucan from the mycelium of *P. ostreatus* requires microwave radiation. However, to obtain beta-glucan with enhanced biological properties, extraction with ethyl alcohol is also necessary.

The results obtained from the study of beta-glucan extraction from the mycelium of *P. ostreatus* are significant for both the pharmaceutical and food

industries, particularly for the development of function food products containing beta-glucan- powder. The market for these products is steadily growing worldwide due to increasing public interest in healthy nutrition.

An important direction for further research is to optimize the microwave radiation regime and establish the relationship between the power and frequency of microwave radiation on the yield of beta-glucan from the mycelium powder of *Pleurotus ostreatus* and its biological activity (stimulation index). Additionally, it is necessary to investigate the functional and technological properties of beta-glucan extracted from the mycelium powder of *Pleurotus ostreatus* and their effects on quality and safety parameters of food products for incorporation into baked goods, confectionery, and meat products. Clinical trials will also need to be conducted on functional food products containing beta-glucan powder from the mycelium of *Pleurotus ostreatus* to demonstrate the beneficial effects of such products on human health.

Abbreviations: MAE: Microwave-Assisted Extraction, GRAS: Generally Recognized as Safe, FDA: Food and Drug Administration.

Author's Contributions: All authors contributed to this study.

Conflict of Interest: The authors declare no conflict of interest.

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