INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
25–528/2025/34:340512
http://www.fspublishers.org

Full Length Article

Effects of Agitation on *β*-glucan Production and Antioxidant Activity in *Lentinus polychrous* Mycelial Extract from Thailand

Chanida Kupradit*, Araya Ranok, Seksan Mangkalanan, Chompoonuch Khongla, Sumalee Musika and Kungnang Bunsroem

Department of Applied Biology, Faculty of Science and Liberal Arts, Rajamangala University of Technology Isan, Nakhon Ratchasima 30000, Thailand

*For correspondence: Chanida.ku@rmuti.ac.th

Received 26 May 2025; Accepted 15 July 2025; Published online 14 August 2025

Editor: Arshad Javaid

Abstract

Lentinus polychrous Lév. is a widely cultivated mushroom in Thailand. Submerged mycelial mushroom cultivation under agitation conditions can enhance mycelial biomass production. This study was carried out to investigate the effects of agitation on mycelial biomass and bioactive compound production in *L. polychrous*. Mycelia were cultivated in potato dextrose broth at agitation speeds of 0, 50, 100 and 150 rpm for 7 days at 35 ± 2°C, followed by extraction with water and 70% ethanol. The extracts were then analyzed for bioactive compounds and antioxidant activity. Results showed that mycelial biomass increased significantly at higher agitation speeds. High levels of *β*-glucan (11.22 ± 1.63% w/w), ABTS radical cation scavenging activity (42.11 ± 2.36 mg trolox eq per g extract), and ferric reducing antioxidant power (11.75 ± 4.09 mg trolox eq per g extract) were observed in the alcoholic extract of mycelia cultivated under optimal conditions at 100 rpm. The mycelial extract from the scaled-up cultivation was evaluated for cytotoxicity against human embryonic kidney (HEK 293) cells and macrophage (RAW 264.7) proliferation using an MTT assay. Results showed that the alcoholic extract at a concentration of 0.11 mg mL^{-1} promoted Raw 264.7 cell proliferation, increasing by 1.32 times compared to the control, with low toxicity. These findings conclude that the *β*-glucan content in *L. polychrous* mycelium positively influences macrophage proliferation.

Keywords: Agitation; Antioxidant activity; *β*-glucan; *Lentinus polychrous* Lév.; Submerged cultivation

Introduction

Mushrooms have been utilized worldwide for their pharmaceutical and nutraceutical properties (Bakratsas *et al*. 2021). Several edible mushrooms with valuable nutraceutical properties have been reported, including the *Pleurotus*, *Lentinus*, *Agaricus*, *Cordyceps*, *Ganoderma*, *Lactarius*, *Grifola*, and *Flammulina* (Kour 2022). Among them, *Lentinus* is recognized for its medicinal properties and rich nutritional composition (Fangkrathok *et al*. 2013; Dulay *et al*. 2020; Dulay *et al*. 2021; Sysouphanthong *et al*. 2023). In Thailand, *L. polychrous*, also known as "Hed Kra Dang" is an edible widely consumed in the northeastern regions. This mushroom thrives in tropical areas due to its ability to grow rapidly at high temperatures (Thetsrimuang *et al*. 2011). The chemical constituents of *L. polychrous* have been reported to include proteins, carbohydrates, polysaccharides, reducing sugars, and phenols (Mekjaruskul *et al*. 2015). Crude polysaccharide extracts from *L. polychrous* mycelium have demonstrated high free radical scavenging activity and strong reducing power (Thetsrimuang *et al*. 2011). These findings suggest that *L. polychrous* mycelium represents a valuable source of natural bioactive ingredients for use in health-related products.

Cultivating mushroom fruiting bodies has several disadvantages, including being time-consuming and requiring large substrates and considerable space. In contrast, submerged cultivation of mycelia offers several advantages, including shorter cultivation periods, safer end products, and improved reproducibility (Dulay *et al*. 2020; Kupradit *et al*. 2020; Bakratsas *et al*. 2021). Several studies have reported optimizing mycelial biomass yield and bioactive compound production in various mushroom species (Kała *et al*. 2021; Zhu *et al*. 2023; Pilafidis *et al*. 2024). In submerged mushroom cultures, agitation of the culture broth is necessary to ensure proper mixing, enhance heat and mass transfer, and increase biomass yield (Nursid *et al*. 2015; Veiter *et al*. 2018). However, agitation also generates shear force, which may damage hyphae and cause morphological changes in the mycelium. Therefore, designing an effective cultivation process under agitation conditions requires a thorough investigation of the relationship between morphology, metabolism, and productivity (Veiter *et al*. 2018). For *Lentinus* species, the optimal conditions for mycelial biomass and production in submerged cultivation vary depending on the species and isolate (Dulay *et al*. 2021). To date, available research on *L. polychrous* mycelial has focused on bioactive compounds extracted from mycelia isolated from various locations and cultivated only under static conditions (Thetsrimuang *et al*. 2011; Jiamworanunkul, 2019; Dulay *et al*. 2021). Although agitation can enhance mycelial biomass in submerged cultures, there is limited information on the effects of agitation on mycelial morphology, bioactive compound production, and biological activity, particularly in *L. polychrous*. Therefore, the effects of agitation on biomass production, mycelial morphology, and the production of bioactive compounds of *L. polychrous* should be further studied.

This study aimed to evaluate the effects of agitation speeds on biomass yield, bioactive compound production, and antioxidant activities of *L. polychrous* during submerged cultivation. Additionally, extracts from scale-up cultures were assessed for toxicity and macrophage proliferation activity to explore their application in health products.

Materials and Methods

Pure culture preparation of mycelial mushroom

Fresh *L. polychrous* was harvested from a mushroom farm in the northeastern region of Thailand. A small piece of fresh mushroom was placed on potato dextrose agar (PDA) and incubated at 35 ± 2°C for 5 days. The resulting mycelium was re-isolated on PDA medium and maintained on a PDA slant. For inoculum preparation, the mycelium from the slant was transferred to fresh medium and incubated at 35 ± 2°C for an additional 5 days.

To cite this paper: Kupradit C, A Ranok, S Mangkalanan, C Khongla, S Musika, K Bunsroem (2025). Effects of agitation on *β*-glucan production and antioxidant activity in *Lentinus polychrous* mycelial extract from Thailand. *Intl J Agric Biol* 34:340512. https://doi.org/10.17957/IJAB/15.2397

Effect of agitation on morphology and biomass of *L. polychrous* mycelium

The effects of agitation on mycelial morphology and biomass production were investigated under submerged cultivation. Mycelial inoculum was prepared by cutting 10 mm diameter discs using a sterile corkborer. Two discs were transferred into a 500 mL shake flask containing 200 mL of potato dextrose broth (PDB), prepared with 200 g of potato, 20 g of dextrose, and 1 L of water. Cultures were incubated at 35 ± 2°C for 7 days under various conditions, including static (0 rpm) and shaking at 50, 100, and 150 rpm. At the end of the cultivation period, the mycelium from each culture was harvested by filtration through filter paper, dried at 45 ± 2°C for 72 h and weighed using a digital balance. The dried mycelium was ground using a blender and stored in airtight plastic bags at 4 ± 2°C for further extraction and analysis.

Extraction of *L. polychrous* mycelium

Mycelial powder was added to water or 70% ethanol at a 1:49 (w/v) ratio. The mixtures were incubated in a water bath at 65 ± 2°C for 6 h. The soluble fractions were filtered through filter paper (Whatman No. 1). The solvents were evaporated at 65 ± 2°C for 48 h in a hot air oven, and the resulting dry extracts were weighed using a digital balance. The extraction yield (% w/w) was calculated as (weight of dry extract /weight of dry biomass) × 100. Finally, the extracts were re-suspended in sterile water (water extract) or 70% ethanol (alcoholic extract) at a final concentration of 100 mg mL^{-1}.

Bioactive compound analysis

Total phenolic compound assay: The total phenolic content was determined using a method adapted from Matthaus (2002), with slight modification. Two mL of 2% Na_2CO_3 was mixed with 100 μL of the mycelial extract and left at room temperature for 2 min. Subsequently, 100 μL of Folin-Ciocalteu solution (Merck, Darmstadt, Germany) was added, and the mixture was incubated at room temperature for 30 min. The absorbance was measured at 750 nm. The total phenolic compounds were reported as mg of gallic acid equivalents per gram of extract (mg GAE per g extract).

***β*-glucan assay:** The *β*-glucan content was measured using the Mushroom and Yeast *β*-glucan Assay kit (K-YBGL 2021) (Megazyme International, Wicklow, Ireland). The total glucan in mycelial extracts was determined through acid hydrolysis, beginning with a 2 h treatment in ice-cold 12 *M* sulfuric acid, followed by boiling in 2 *M* sulfuric acid for an additional 2 h. After neutralization with 8 *M* NaOH, enzymatic hydrolysis was performed using exo-1, 3-*β*-glucanase and *β*-glucosidase. The mixture was maintained at 40 ± 2°C for 1 h. For *α*-glucan analysis, the extract was hydrolyzed with 1.7 *M* NaOH solution and incubated on ice for 20 min. Then, the reaction was mixed with invertase and amyloglucosidase. Glucose generated from both the total glucan and *α*-glucan reactions was analyzed by adding glucose oxidase plus peroxidase and 4-aminoantipyrine (GOPOD) reagents and incubating at 40 ± 2°C for 20 min. The glucose contents were measured for absorbance at 510 nm. The *β*-glucan content was determined by calculating the difference between *α*-glucan and total glucan and expressed as grams of *β*-glucan per 100 g of extract (% w/w).

Antioxidant activities assay

ABTS radical cation scavenging activity (ABTS assay): The ABTS assay was determined using the method described by Wiriyaphan *et al*. (2012), with slight modifications. Briefly, 1.980 mL of the ABTS working solution (absorbance 0.70 ± 0.20 at 734 nm) was mixed with 20 μL of the mycelial extract. After incubating in the dark for 5 min, the absorbance was measured at 734 nm. Results were expressed as milligrams of trolox equivalent per gram of extract.

Ferric reducing antioxidant power (FRAP): The antioxidant activity of the mycelial extracts was assessed using the FRAP assay, following the method described by Benzie and Strain (1996), with slight modifications. Briefly, 100 μL of the mycelial extract was mixed with 1 mL of FRAP reagent and incubated at 37 ± 2°C for 15 min. The absorbance was measured at 593 nm. FRAP activity was expressed as milligrams of trolox equivalent per gram of extract.

Metal chelating activity: The metal chelating activity was analyzed using a modified version of the method described by Decker and Welch (1990). Deionized water (2,400 μL) was added to 100 μL of the mycelial extract. Then, 50 μL of 2 m*M* $FeCl_2$ was added to the reaction mixture and mixed immediately, followed by the addition of 100 μL of 5 m*M* ferrozine. The mixture was thoroughly mixed and incubated for 20 min. Absorbance was measured at 562 nm. The metal chelating activity was expressed as milligrams of EDTA equivalent per gram of extract.

Scale-up cultivation of *L. polychrous* mycelium

The inoculum mycelium was prepared by transferring 2 discs from PDA to a 500 mL shake flask containing 150 mL of PDB. The inoculum was cultivated at 35 ± 2°C with shaking at the designated agitation speed for 4 days. Then, 150 mL of the inoculum culture was transferred into a 2 L shake flask containing 850 mL of PDB to achieve a final volume of 1 L. The resulting culture was then incubated at 35 ± 2°C with shaking at the same agitation speed for 7 days. Next, the mycelium from the 1 L culture was harvested by filtration and dried at 45 ± 2°C for 72 h. The dried biomass was weighed and then ground using a blender for further extraction and analysis. The resulting mycelial extracts were analyzed for bioactive compounds and antioxidant activity. In addition, their toxicity and ability to promote macrophage cell proliferation were also evaluated to assess their potential application in health products.

Toxicity of the mycelial extract

The mycelial extract obtained from a 1 L-scale cultivation was tested for cytotoxicity against human embryonic kidney cell line (HEK 293) using an MTT assay, with minor modifications based on the method described by Bunsroem *et al*. (2022). Briefly, cells were cultured into 96-well plates (5,000 cells per well) in DMEM medium containing 1% FBS and 1% penicillin/streptomycin. The cultivation was carried out at 37 ± 2°C under 5% CO_2 for 24 h as a pre-cultivation step. The mycelial extract was diluted with DMEM medium to obtain various concentrations. Subsequently, the cell culture was mixed with 100 µL of the diluted mycelial extract. After 24 h incubation, the cell culture was mixed with 100 μL of MTT solution to achieve a final concentration of 0.5 mg mL^{-1}, followed by incubation in the dark for 2 h. Next, the supernatant was removed, and 100 μL of dimethyl sulfoxide (DMSO) was added. The absorbance of all samples was measured at 570 nm using a microplate reader. The viability of HEK293 cells was determined using the following equation.

Cell viability (%) = [Absorbance of treated cells ×100]/ Absorbance of control cell (1)

Macrophage proliferation assay

The proliferation activity of Raw 264.7 macrophage cells was evaluated using an MTT assay method, with minor modifications based on the method described by Fang *et al*. (2020). Briefly, Raw 264.7 cells were inoculated into DMEM medium at a final concentration of 7,500 cells per well in 96-well plates. The cells were incubated at 37 ± 2°C for 24 h under a 5% CO_2 atmosphere. Various concentrations of mycelial extracts (100 μL total volume) were added to each well and incubated for 24 h.

After incubation, 100 μL of MTT solution (0.5 mg mL^{-1}) was added and kept in the dark for 2 h. The supernatant was then discarded, and 100 μL of DMSO was added. The absorbance of each sample was measured at 570 nm using a plate reader. The viability of Raw 264.7 cells was calculated using the above-mentioned equation.

Statistical analysis

All experimental data were collected in duplicate and are presented as means ± standard deviations. Significant differences ($P < 0.05$) between groups were determined using one-way analysis of variance (ANOVA). The statistical analyses were performed using PASW Statistics 18 Release 18.0.0 software.

Results

Effect of agitation on morphology and biomass of *L. polychrous* mycelium

The *L. polychrous* mycelium was grown in PDB under various agitation speeds using submerged cultivation. Distinct morphological differences were observed at different agitation speeds, as shown in Fig. 1. Under static conditions (0 rpm), the mycelium grew on the surface of the culture broth as a layer of fungal mat with aerial mycelium on top. At 50 rpm, mycelial growth predominantly occurred on the surface of the culture broth, with a few large pellets observed beneath the broth. At agitation speeds of 100 and 150 rpm, most of the mycelium formed spherical pellets with short hairy surfaces submerged below the culture broth (Fig. 1). A comparison of mycelial biomass at various agitation speeds is presented in Fig. 2. The results indicate that mycelial biomass significantly increased at higher agitation speeds. Notably, the cultures shaking at 100–150 rpm yielded significantly higher biomass compared to those under static or lower conditions (0–50 rpm). The highest biomass yield, 5.93 ± 0.38 g L^{-1}, was observed at 150 rpm (Fig. 2).

Effect of agitation on the extraction yield

The effects of agitation on the concentration of mycelial extracts are shown in Fig. 3. The concentrations of both water and alcoholic extracts obtained from mycelium cultivated at 100 and 150 rpm were significantly higher than those from static or lower agitation conditions (0–50 rpm) (Fig. 3). Notably, high yields of water extracts, 558.29 ± 63.73 and 557.32 ± 18.76 mg per g cell dry weight, were obtained from mycelium cultivated at 100 and 150 rpm, respectively, over a period of 7 days. However, there was no significant difference in the yields of water and alcoholic extracts between mycelia cultivated at 100 and 150 rpm.

Effects of agitation on β-glucan content in mycelial extracts

The β-glucan content in the water and alcoholic extracts of mycelial mushrooms cultivated under different agitation conditions were analyzed and compared (Fig. 4). At agitation speeds ranging from 50 to150 rpm, the β-glucan content in alcoholic extracts, which ranged from 11.22 ± 1.63 to 12.16 ± 0.24% (w/w), was significantly higher than that in water extracts (Fig. 4).

Effect of agitation on the antioxidant activity of mycelial extracts

The effects of agitation on the antioxidant profiles of mycelial extracts are summarized in Fig. 5. The highest total phenolic content was observed in the water extract obtained from mycelium cultivated at 50 rpm (94.36 ± 7.56 mg GAE per g extract), which was significantly higher than that observed under other conditions ($P < 0.05$) (Fig.

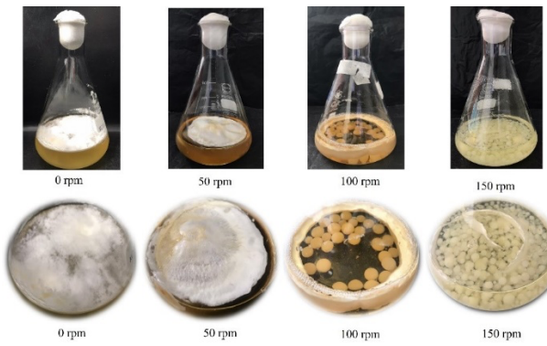

Fig. 1: Effect of agitation speed on morphology of *Lentinus polychrous* mycelium in submerged culture. The mycelium was cultivated in 200 mL PDB at 0, 50, 100 and 150 rpm at 35 ± 2°C for 7 days

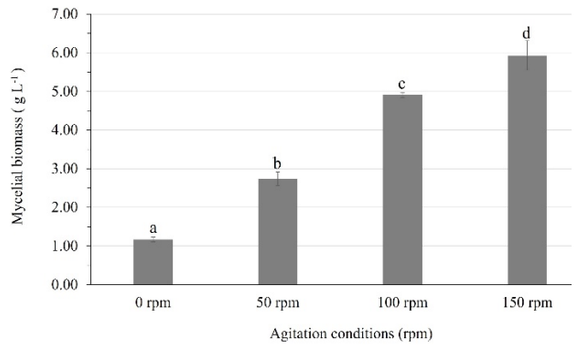

Fig. 2: Effect of agitation speed on mycelial biomass. The mycelium was cultivated in 200 mL of PDB at speeds of 0, 50, 100 and 150 rpm at 35 ± 2°C for 7 days in shake flasks. The standard deviation of the data is represented by error bars. Bars labeled with different letters indicate statistically significant differences in biomass at various agitation speeds ($P < 0.05$)

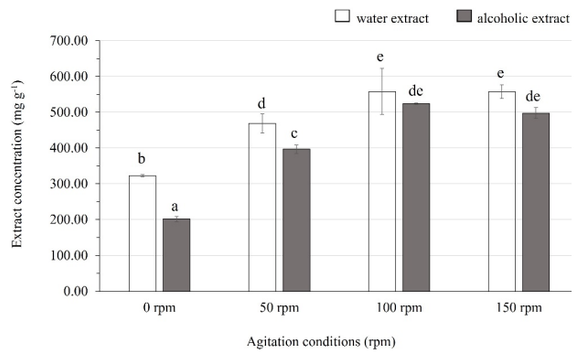

Fig. 3: Effect of agitation speed on mycelial extract concentration. The mycelium was cultivated in 200 mL PDB under shaking conditions at 0, 50, 100 and 150 rpm for 7 days. Error bars represent the standard deviation of the data. Different letters above the bars indicate significant differences in extract concentration among various agitation speeds and extraction conditions ($P < 0.05$)

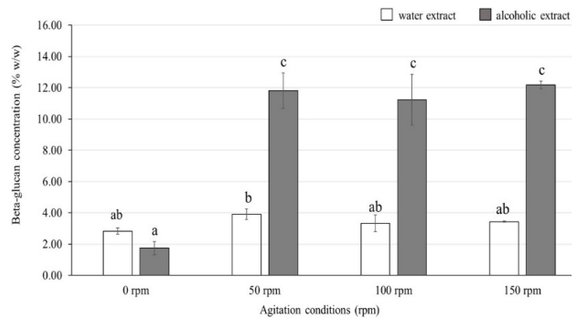

Fig. 4: Effect of agitation speed on β-glucan content in mycelial extracts of *Lentinus polychrous*. The mycelium was cultivated in 200 mL PDB at agitation speeds of 0, 50, 100 and 150 rpm, maintained at 35 ± 2°C for 7 days. The standard deviation of the data is indicated by error bars. Bars with different letters indicate significant differences in β-glucan content based on agitation speed and extraction conditions ($P < 0.05$)

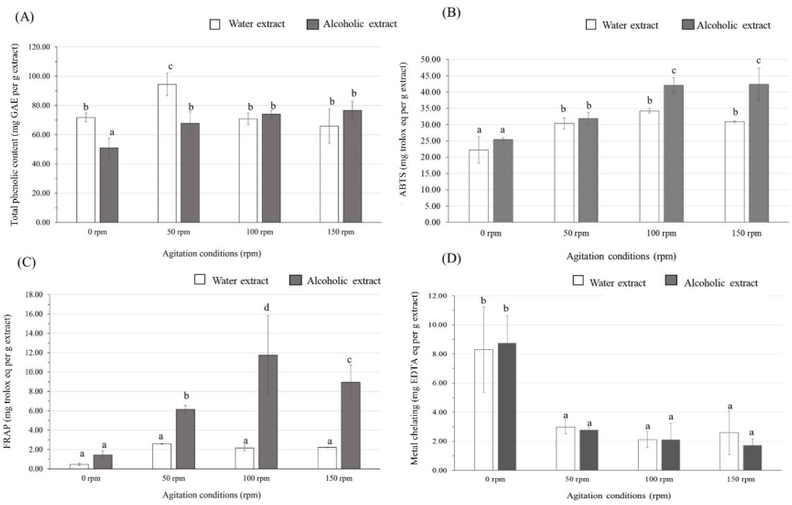

Fig. 5: Effect of agitation on the antioxidant profiles of mycelial extracts. The mycelium was cultivated in 200 mL of PDB at agitation speeds of 0, 50, 100 and 150 rpm, at 35 ± 2°C for 7 days. Error bars represent the standard deviation of the data. Different letters above the bars indicate significant differences in (**A)** total phenolic compounds, (**B**) ABTS, (**C**) FRAP and (**D**) metal chelating among the agitation speed rates and extraction conditions ($P < 0.05$)

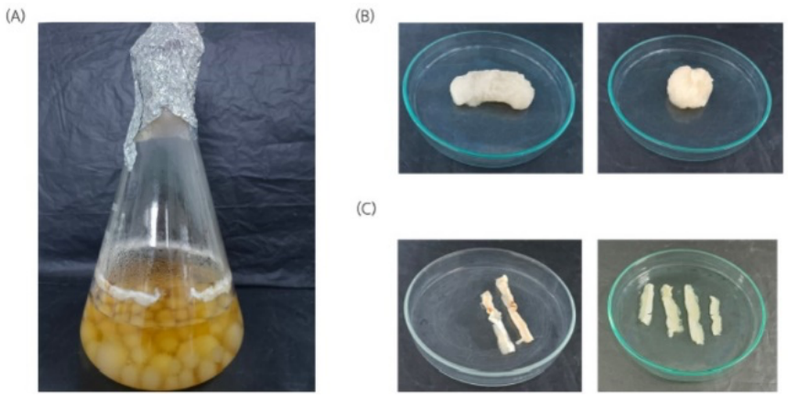

Fig. 6: Mycelial morphology of *L. polychrous* at 1 L cultivation. The mycelial cultivation was agitated at 100 rpm and 35 ± 2°C for 7 days. (**A**) Mycelial morphology observed in 1 L shake flask, (**B**) The shape of the mycelial pellet in the submerged culture broth and (**C**) Surface growth showing an O-ring structure formed by the mycelium

5A). In contrast, at higher agitation speeds 100–150 rpm, there was no significant difference in total phenolic content between water and alcoholic extracts (65.86 ± 11.68–76.63 ± 6.33 mg GAE per g extract) (Fig. 5A). Mycelial cultivation under shaking at 100–150 rpm exhibited significantly higher ABTS activity (42.11 ± 2.36–42.37 ± 4.95 mg trolox eq per g extract) in alcoholic extracts compared to other conditions (Fig. 5B). Similarly, a high antioxidant activity using the FRAP assay (11.75 ± 4.09 mg trolox eq per g extract) was observed in the alcoholic extract from mycelial cultivated at 100 rpm (Fig. 5C). In contrast, the metal chelating activity was highest in both water and alcoholic extracts from mycelium cultivated under static conditions (8.30 ± 2.93 and 8.75 ± 1.90 mg EDTA eq per g extract, respectively). These values were significantly higher than those obtained under other conditions (Fig. 5D). Based on the results for biomass, β-glucan content, and antioxidant profiles, the optimal agitation speed for cultivation of *L. polychrous* mycelium was determined to be 100 rpm.

Production of the bioactive compounds and antioxidant activity in 1 L of cultivation

Scaled-up cultivation (1 L) was performed at 100 rpm for 7 days (Fig. 6A). The mycelial morphology observed during submerged culture is illustrated in Fig. 6. The mycelium exhibited various sizes and shapes, forming both spherical and cylindrical mycelial pellets (Fig. 6B). The pellet morphology in the scale-up culture (1 L) were similar to that observed in the smaller-scale (200 mL) culture and appeared to depend on the degree of mycelial fragmentation in the inoculum. Both aerial mycelium and a polysaccharide mat were observed on the surface of the culture broth, forming an O-ring structure of mycelial growth pattern (Fig. 6C). The mycelial biomass and extraction yield of 1 L cultures are presented in Table 1. The biomass in the 1 L culture decreased to 2.03 ± 0.17 g L^{-1}, and the extraction yield in the 1 L culture was also lower compared to that observed from the 200 mL culture (52.39 ± 0.26–55.83 ± 6.37% w/w).

Bioactive compounds and antioxidant activity of the scale-up culture

The bioactive compound content and antioxidant profiles from scale-up (1 L) cultivation were analyzed (Table 2). The content of *β*-glucan, FRAP and metal chelating activity in the alcoholic extract was significantly higher than in the water extract. Conversely, the total phenolic compound in the water extract was significantly higher than in the alcoholic extract (Table 2). The ABTS was not significantly different between water and alcoholic extracts. Compared to the 200 mL cultivation in Fig. 5, the concentration of *β*-glucan in the alcoholic extract from the small-scale culture (11.22 ± 1.63 % w/w) was higher than in both the water and alcoholic extracts from the scale-up culture. Similarly, the total phenolic content (70.78 ± 3.99–74.06 ± 2.33 mg GAE per g extract) and ABTS activity (34.21 ± 0.72 – 42.11 ± 2.36 mg trolox eq per g extract) in both the water and alcoholic extracts from small-scale culture were higher than those obtained from the scaled-up culture. However, antioxidant activity measured by the metal chelating assay was higher in the alcoholic extract from scaled-up culture compared to both the water and alcoholic extracts from the small-scale culture (2.11 ± 1.12 – 2.13 ± 0.56 mg EDTA eq per g extract).

Table 1: The biomass and extraction yield of *L. polychrous* cultivated in submerged culture at 1 L, under agitation at 100 rpm for 7 days

Biomass and Extraction Yields	Content
Biomass (g L^{-1})	2.03 ± 0.17
Water extraction yield (% w/w)	32.33 ± 2.15
Alcoholic extraction yield (% w/w)	18.17 ± 1.00

Table 2: Bioactive compounds and antioxidant activities in mycelial extracts cultivated at 1 L, under agitation at 100 rpm for 7 days

Bioactive compounds/Antioxidant activity	Extraction	
	Water extract	Alcoholic extract
β-glucan content (% w/w)	1.28±0.13 [a]	2.64±0.08 [b]
The total phenolic compound (mg GAE per g extract)	65.67±1.83 [b]	57.83±1.12 [a]
ABTS (mg trolox eq per g extract)[ns]	26.76±1.28	27.47±1.06
FRAP (mg trolox eq per g extract)	7.58±0.08 [a]	10.73±0.68 [b]
Metal chelating activity: (mg EDTA eq per g extract)	1.96±0.09 [a]	3.86±0.39 [b]

All data are presented as mean ± S.D. from duplicate experiments. Different superscript letters within each row indicate significant differences among extraction solvents, measured by the T-test at $P < 0.05$, ns indicate not significantly different

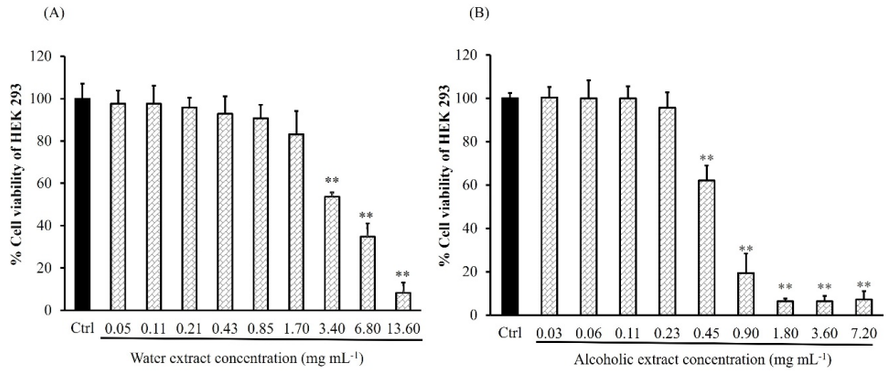

Fig. 7: (**A**) Viability of the HEK 293 cells treated with various concentrations of water extract and (**B**) alcoholic extract from mycelial cultivation at 1 L. Experiments were performed in triplicate, and error bars represent standard deviation. Asterisks (*) above the bars indicate significant differences between the treated and control samples, measured by the T-test at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

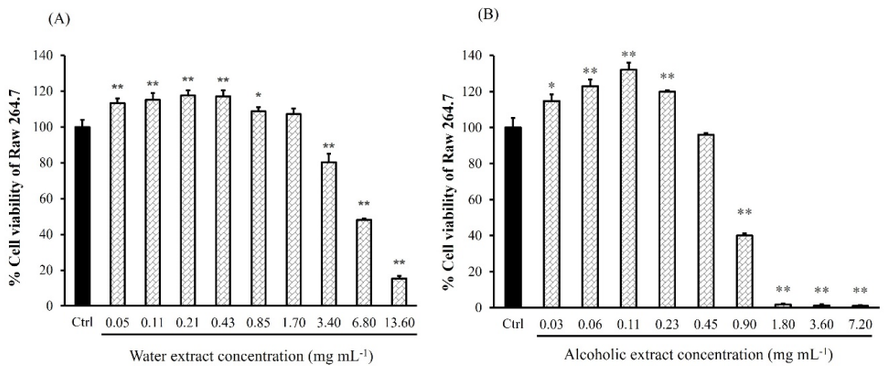

Fig. 8: (**A**) Viability of the Raw 264.7 cells treated with water extracts and (**B**) alcoholic extracts from mycelial cultivation at 1 L. Experiments were performed in triplicate, and error bars represent the standard deviation. Asterisks (*) indicate significant differences among the treated samples and the control, as assessed by the T-test at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

Toxicity of the mycelial extract

The toxicity of water and alcoholic extracts from 1 L mycelial cultivation was evaluated using HEK 293 cells. The results of cell viability are shown in Fig. 7. Treatment with 3.40 mg mL^{-1} of the water extract and 0.45 mg mL^{-1} of the alcoholic extract significantly reduced HEK 293 cell viability to 53.61% (Fig. 7A) and 62.02% (Fig. 7B), respectively, compared to the control.

Macrophage proliferation

RAW 264.7 cells were treated with mycelial extracts at concentrations ranging from 0.03-13.6 mg mL^{-1} for 24 h, and cell viability was assessed, as shown in Fig. 8. The proliferation of RAW 264.7 cells increased following treatment with water extracts at concentrations ranging from 0.05 to 0.85 mg mL^{-1} (Fig. 8A) and with alcoholic extracts at concentrations of 0.03 to 0.23 mg mL^{-1} (Fig. 8B). Notably, treatment with 0.11 mg mL^{-1} of alcoholic extract and 0.21 mg mL^{-1} of water extract increased proliferation by 1.32 and 1.18 times, respectively, compared to the control.

Discussion

In this study, *L. polychrous* mycelium was cultivated in submerged culture under varying agitation conditions. High agitation speeds are known to generate shear stress, leading to mycelium fragmentation and pellet formation in the culture broth. Similar results have been reported in previous studies (Purwanto *et al*. 2009; Nursid *et al.* 2015). Hyphal fragments can grow and serve as the center for new biomass production (Žnidaršič-Plazl and Pavko 2001; Veiter *et al*. 2018). Our results confirm that agitation significantly enhanced the biomass production of *L. polychrous*. Agitation plays a critical role in nutrient mixing, mass transfer, and heat transfer during submerged cultivation (Purwanto *et al*. 2009; Nursid *et al.* 2015). Culturing mycelial mushrooms under vigorous agitation resulted in the formation of smaller pellets. However, the effect of agitation on biomass production is species-dependent (Pilafidis *et al.* 2024). In this research, the extraction yield of mycelium cultivated at 100-150 rpm was 49.8-55.9%, which is higher than the 5.9% yield obtained from hot water extract, as reported by Thetsrimuang *et al*. (2011). These results suggest that cultivation conditions and extraction

methods have a significant impact on the yield of mycelial extraction. Shear force typically induces morphological changes, resulting in variations in growth and product formation. In this study, *β*-glucan content significantly increased under high agitation speed conditions. This increase in *β*-glucan content was positively correlated with the increased biomass observed at agitation speeds between 50–150 rpm. *β*-glucans are the dominant polysaccharides present in cell walls and can be found both intracellularly and as extracellular products (Kour *et al*. 2022). Therefore, increased agitation speed results in higher levels of biomass and increased *β*-glucan content. Similarly, Pilafidis *et al*. (2024) reported that the highest *β*-glucan content in *Ganoderma lingzhi* was observed under agitated cultivation. Moreover, agitation was found to elevate the total phenolic compound levels in the mycelial extract of *L. polychrous*. In this study, total phenolic compound content in both the water and alcoholic extracts from mycelium cultivated at 100–150 rpm was significantly higher than that reported under static conditions for 14 days by Jiamworanunkul (2019). Phenols are diverse compounds characterized by one or more hydroxyl groups attached to a benzene ring. Catechin, a major group of phenolic compound, has been identified in *Lentinus squarrosulus*, *L. polychrous* and *L. edodes* (Attarat and Phermthai 2015; Kour *et al*. 2022). In this study, the antioxidant activity, as measured by ABTS and FRAP assays, significantly increased when mycelium was cultivated under high agitation rates and extracted using 70% ethanol solution. In contrast, the metal chelating activity was higher in both water and alcoholic extracts when the mycelium was cultivated under static conditions. The mycelial extracts can disrupt the Fe^{2+} and ferrozine complex formation during the reaction (Boonsong *et al*. 2016). These findings suggest that the agitation speed of the mycelium influences the composition of *L. polychrous* and the mechanisms of its antioxidant activity.

The cultivation scale is another critical factor influencing biomass and bioactive compound production under agitation conditions. In this research, scaling up the culture volume to 1 L with agitation at 100 rpm resulted in decreased biomass yield, bioactive compounds and ABTS activity compared to the 200 mL culture. A decline in exopolysaccharide production at large scales has been previously reported in *Lentinus*. In 2015, Anike *et al*. (2015) reported that while the mycelial mass of *L. squarrosulus* Mont. increased in larger culture volumes under shaking conditions, the crude exopolysaccharide yield decreased. In submerged mycelial cultivation, the size of mycelial pellets and biomass varied with both agitation speed and cultivation time (Cui *et al*. 1997). The limitations associated pellet formations in fungi are based on the intraparticle mass transfer. Such limitations may create regions (microenvironments) with different growth patterns and substrate availability. In large pellets, the external zones are metabolically active, while the inner regions exhibit low viability due to low dissolved oxygen concentrations and nutrient availability (García-Soto *et al*. 2006). For this reason, smaller pellets are generally more advantageous for the production of bioactive compounds and biomass (Petre and Petre 2016). Thus, the observed reduction in biomass yield, bioactive compound production, and antioxidant activity in this research may be attributed to the increased pellet size associated with the scale-up cultivation volume. Another limitation of scale-up in submerged cultivation observed in this research was the use of a shake flask system. In such systems, control of essential parameters, such as pH, O_2 supply, nutrients, and heat transfer, is limited (Bakratsas *et al*. 2021). These factors, particularly the oxygen supply, significantly influence cell morphology and metabolite production (Pilafidis *et al.* 2024). Thus, insufficient oxygen and nutrient transfer likely contributed to the low extraction yield and metabolite production observed in large-scale cultivation. Based on the limitations of shake flask cultivation on a larger scale, the use of an airlift bioreactor with low shear force should be considered for future mycelial biomass production. However, scaling up to 1 L in this study was found to enhance the antioxidant activity of the alcoholic extract, particularly metal chelating activity.

The potential application of extracts from mycelial cultivation in health products was further evaluated through toxicity and macrophage proliferation assays. The immune system plays an essential role in the survival of organisms (Fangkrathok *et al*. 2014). Polysaccharides derived from microbial cell wall components are effective immunomodulators (Cui *et al*. 2015; Fang *et al*. 2020). The polysaccharide from *Collybia radicata* mushrooms has been shown to stimulate the proliferation of macrophages and enhance phagocytosis (Wang *et al*. 2018). In this research, the alcoholic extract stimulated Raw 264.7 cell proliferation more effectively compared to the water extract, which may be attributed to the high *β*-glucan content found in the alcoholic extract. Similarly, Fangkrathok *et al*. (2013) reported the anti-inflammatory effects of the ethanolic extract from *L. polychrous* mycelium. Consuming diets rich in *β*-glucans could enhance immunity. *β*-glucans derived from mushrooms are considered safe for human consumption (Yadav and Negi 2021). These results suggest that *β*-glucan content plays a critical role in promoting macrophage proliferation, which correlates with improved immune function.

Conclusion

This study investigated the effects of agitation on the production of mycelial biomass and bioactive compounds in *L. polychrous* under submerged cultivation. The mycelial biomass, *β*-glucan content, and antioxidant compounds significantly increased at higher agitation rates. Although scale-up cultivation in shake flasks presented limitations in biomass and metabolite production, it underscored the potential of *L. polychrous* for functional applications. Future research should focus on using an airlift bioreactor to improve biomass production on larger scales. The mycelial extracts from scale-up culture in this research were found to promote the proliferation of macrophage Raw 264.7 cells while exhibiting low toxicity toward HEK 293 cells. These results suggest that the *β*-glucan content in *L. polychrous* mycelium plays a key role in enhancing macrophage proliferation, thereby contributing to enhanced immune system function. Therefore, the mycelial extract of *L. polychrous* could be further utilized to produce safe bioactive additives for health-related products.

Acknowledgments

This research was supported by the Science Research and Innovation Fund under Contract No. FF66-P1-054.

Author's Contributions

CK designed the experiment, collected the data and wrote the original draft of the manuscript. AR analyzed the data, SM interpreted the results, CKh validated the manuscript, SMu and KB reviewed and edited the manuscript.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

References

Anike FN, OS Isikhuemhen, D Blum, H Neda (2015). Nutrient requirements and fermentation conditions for mycelia and crude exo-polysaccharides production by *Lentinus squarrosulus*. *Adv Biosci Biotechnol* 6:526–536

Attarat J, T Phermthai (2015). Bioactive compounds in three edible *Lentinus* mushrooms. *Walail J Sci Technol* 12:491–504

Bakratsas G, A Polydera, P Katapodis, H Stamatis (2021). Recent trends in submerged cultivation of mushrooms and their application as a source of nutraceuticals and food additives. *Future Foods* 4: 100086

Benzie IFF, JJ Strain (1996). The ferric reducing ability of plasma as a measure of antioxidant power the FRAP assay. *Anal Biochem* 239:70–76

Boonsong S, W Klaypradit, P Wilaipun (2016). Antioxidant activities of extracts from five edible mushrooms using different extractants. *Agric Nat Resour* 50:89–97

Bunsroem K, W Prinyawiwatkul, S Thaiudom (2022). The influence of whey protein heating parameters on their susceptibility to digestive enzymes and the antidiabetic activity of hydrolysates. *Foods* 11:1-13

Cui HY, CL Wang, YR Wang, ZJ Li, YN Zhang (2015). The polysaccharide isolated from *Pleurotus nebrodensis* (PN-S) shows immune-stimulating activity in RAW264.7 macrophages. *Chin J Nat Med* 13:355–360

Cui YQ, RGJMD Lans, KCAM Luyben (1997). Effect of agitation intensities on fungal morphology of submerged fermentation. *Biotechnol Bioeng* 55:715–726

Decker EA, B Welch (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *J Agric Food Chem* 38:674–677

Dulay RMR, EC Cabrera, SP Kalaw, RG Reyes (2021). Optimization of submerged culture conditions for mycelial biomass production of fourteen *Lentinus* isolates from Luzon Island, Philippines. *Biocatal Agric Biotechnol* 38:102226

Dulay RMR, EC Cabrera, SP Kalaw, RG Reyes, CT Hou (2020). Nutritional requirements for mycelial growth of three *Lentinus* species from the Philippines. *Biocatal Agric Biotechnol* 23:101506

Fang J, S Gao, R Islam, Y Teramoto, H Maeda (2020). Extracts of *Phellinus linteus*, Bamboo (*Sasa senanensis*) leaf and Chaga mushroom (*Inonotus obliquus*) exhibit antitumor activity through activating innate immunity. *Nutrients* 12:1-12

Fangkrathok N, J Junlatat, K Umehara, H Noguchi, B Sripanidkulchai (2014). Cytotoxic and immunomodulatory effects of polyhydroxyoctane isolated from *Lentinus polychrous* mycelia. *J Nat Med* 68:302–309

Fangkrathok N, J Junlatat, B Sripanidkulchai (2013). *In vivo* and *in vitro* anti-inflammatory activity of *Lentinus polychrous* extract. *J Ethnopharmacol* 147:631–637

García-Soto MJ, E Botello-Alvarez, H Jimenez-Islas, J Navarrete-Bolanos, E Barajas-Conde, R Rico-Martínez (2006). Growth morphology and hydrodynamics of filamentous fungi in submerged cultures. *Adv Agric Food Biotechnol* 2:17–34

Jiamworanunkul S (2019). Effective antioxidant production through submerged fermentation of edible mushrooms. *Thai J Pharmaceut Sci* 43:213–218

Kała K, A Krakowska, A Szewczyk, B Ostachowicz, K Szczurek, A Fijałkowska, B Muszy´nska (2021). Determining the amount of potentially bioavailable phenolic compounds and bioelements in edible mushroom mycelia of *Agaricus bisporus*, *Cantharellus cibarius*, and *Lentinula edodes*. *Food Chem* 352:129456

Kour H, D Kour, S Kour, SS Singh, AJ Hashmi, AN Yadav, K Kumar, YP Sharma, AS Ahluwalia (2022). Bioactive compounds from mushrooms: Emerging bioresources of food and nutraceuticals. *Food Biosci* 50:102124

Kupradit C, A Ranok, S Mangkalanan, C Khongla, S Musika (2020). Effects of natural carbon sources and temperature on mycelium cultivations of *Lentinus squarrosulus* (Mont.), *Lentinus polychrous* Lev., *Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm and *Volvariella volvacea* (Bull.) Singer. *Thai J Sci Technol* 9:539–553

Matthaus B (2002). Antioxidant activity of extract obtained from residues of different oil seeds. *J Agric Food Chem* 50:3444–3452

Mekjaruskul C, N Fangkrathok, B Sripanidkulchai (2015). Acute toxicity investigation of polysaccharide extracts of *Lentinus polychrous* in rats. *Songklan J Sci Technol* 37:433–439

Nursid M, M Manulang, J Samiadji, E Marraskuranto (2015). Effect of agitation speed and cultivation time on the production of the emestrin produced by *Emericella nidulans* marine fungal. *Squal Bull Mar Fish Postharv Biotechnol* 10:73–78

Petre M, V Petre (2016). *Biotechnology of mushroom growth through submerged cultivation. In: Mushroom Biotechnology: Developments and Applications*, pp:1-18. Berovic M, BB Podgornik, M Petre (Eds.). Elsevier Incorporation doi:10.1016/B978-0-12-802794-3.00001-1

Pilafidis S, E Tsouko, G Sougleri, P Diamantopoulou, K Gkatzionis, Z Ioannou, D Sarris (2024). Submerged cultivation of selected macro–fungi to produce mycelia rich in *β*–glucans and other bioactive compounds, valorizing side streams of the food industry. *Carbon Resour Convers* 7:100198

Purwanto LA, D Ibrahim, H Sudrajat (2009). Effect of agitation speed on morphological changes in *Aspergillus niger* hyphae during production of tannase. *World J Chem* 4:34–38

Sysouphanthong P, W Taliam, N Wannasawang, N Thongklang (2023). The genus *Lentinus* in Thailand: Taxonomy, cultivation, tests, nutritional analysis and screening for the biological activity of wild strains. *Curr Res Environ Appl Mycol* 13:136–161

Thetsrimuang C, S Khammuang, K Chiablaem, C Srisomsap, R Sarnthima (2011). Antioxidant properties and cytotoxicity of crude polysaccharides from *Lentinus polychrous* Lev. *Food Chem* 128:634–639

Veiter L, V Rajamanickam, C Herwig (2018). The filamentous fungal pellet–relationship between morphology and productivity. *Appl Microbiol Biotechnol* 102:2997–3006

Wang Y, Y Tian, J Shao, X Shu, J Jia, X Ren, Y Guan (2018). Macrophage immunomodulatory activity of the polysaccharide isolated from *Collybia radicata* mushroom. *Intl J Biol Macromol* 108:300–306

Wiriyaphan C, B Chitsomboon, J Yongsawadigul (2012). Antioxidant activity of protein hydrolysates derived from threadfin bream surimi byproducts. *Food Chem* 132:104–111

Yadav D, PS Negi (2021). Bioactive components of mushrooms: Processing effects and health benefits. *Food Res Intl* 148:110599

Zhu H, Z Chen, Y Hu, G Li, X Yao, L Cao (2023). Nutritional value, elemental bioaccumulation and antioxidant activity of fruiting bodies and mycelial cultures of an unrecorded wild *Lactarius hatsudake* from Nanyue mountainous region in China. *Food Res Intl* 173:113358

Žnidaršič-Plazl P, A Pavko (2001). The morphology of filamentous fungi in submerged cultivations as a bioprocess parameter. *Food Technol Biotechnol* 39:237–252