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Purification, initial characterization and immune activities of polysaccharides from the fungus, *Polyporus umbellatus*

Ye Sun [a,*], Xiaoyan Zhou [b]

[a] *Department of Biology, Shanghai Institute of Technology, Shanghai 201418, PR China*
[b] *Shanghai Institute of Industrial Microbiology, Shanghai 200233, PR China*

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Abstract

Polyporus umbellatus (formerly *Grifola umbellatus*), an edible fungus, has been used as traditional Chinese medicine. The present investigation aimed to uncover the active ingredients in this fungal species, with the emphasis on its polysaccharides. Two polysaccharides, one from fermented mycelium and the other from the fruiting body, were chromatographically purified. Molecular weights, anti-microbial and immune activities of the polysaccharides were determined. The mycelium polysaccharide was found to have a molecular weight of 857 kDa and consist of glucose and galactose at a molar ratio of 1.57:1, while the one from the fruiting body, 679 kDa in molecular weight, was also made up of glucose and galactose but at a molar ratio of 5.42:1. In immune assays, these fungal polysaccharides increased the killing potency of natural killer (NK) and lymphokine-activated killer (LAK) cells from mouse spleen and promoted proliferation of mouse B and T cells. Additionally, both *P. umbellatus* mycelium and its extract were found to inhibit *Escherichia coli* and *Staphylococcus aureus*, but had no effects on *Aspergillus niger* or *Aspergillus nidulans*. These results indicated that fermented *P. umbellatus* mycelium is indeed immune active, most probably due to its ingredients of polysaccharides. The consumption of this fungus may be beneficial for human health.

Keywords: *Polyporus umbellatus*; Fermented mycelium; Polysaccharide; Immune activities

1. Introduction

Fungal polysaccharide, a kind of active organic compound, is found in the mycelium, fruiting bodies, and fermentation broth of large medicinal and edible fungi [1]. In recent years, polysaccharides derived from fungi have become an important source of bioactive substances. A polysaccharide from the fungus, *Polyporus umbellatus*, a polymer of glucose primarily linked by β $(1 \rightarrow 3)$ glycosidic bond, has been shown to be capable of decimating tumor cells [2]. Further investigations revealed that the tertiary structure, rather than the primary structure, is the major determinant of the pharmacological activities of this fungal polysaccharide. It has been suggested that a polysaccharide with both β $(1 \rightarrow 3)$ and β $(1 \rightarrow 6)$ or with both β $(1 \rightarrow 4)$ and β $(1 \rightarrow 6)$ glycosidic bonds, instead of purely β $(1 \rightarrow 4)$ glycosidic bond, is generally more pharmacologically active [3,4].

The structures of polysaccharides from several edible and pharmacologically active fungi have been elucidated. The main chain of lentinan polysaccharide is linked by β $(1 \rightarrow 3)$ glycosidic bond, while the side chains branch out at C_6. There are three types of side chains, namely, side chains with β $(1 \rightarrow 6)$ glycosidic bond only, short side chains with β $(1 \rightarrow 3)$ glycosidic bond only and side chains with a non-reducing end [5]. The polysaccharide of fruiting body from the glossy ganoderma, *Ganoderma lucidum*, is made up of fucose, xylose and mannose at a molar ratio of 1:1:1. The main chain is a polymer of D-mannose linked by $(1 \rightarrow 4)$ glycosidic bond, and the side chains, which are polymers of fucose and xylose, branch out at C_3 of the mannose on the main chain [2]. The insoluble polysaccharide from the Chinese caterpillar fungus, *Cordyceps sinensis*, is a glucosan with a molecular weight of 632 kDa [2], while the acidic tremellan consists of a main chain made up of mannose linked by α $(1 \rightarrow 3)$ and side chains that are polymers of glucuronic acid and xylose [6]. However, to our best knowledge, there has been no report on biochemical properties of the polysaccharide from the mycelium of *P. umbellatus*.

* Corresponding author at: Tel.: +86 21 60873190; fax: +86 21 54487207.
E-mail address: sunye@sit.edu.cn (Y. Sun).
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The anti-microbial activities of extracts from fermented fungi have been reported for numerous fungal species, such as *Cordyceps sinensis* [7], *Ganodorma lucidum* [8], and *Lactarius deliciosus* [9]. However, very little is known about the anti-microbial activities or pharmacological activities of extracts of fermented mycelium of *P. umbellatus*. Hence, the objectives of the present investigation were to characterize the biochemical properties of polysaccharides purified from the mycelium and fruiting body of *P. umbellatus* and test anti-microbial and immune activities of these polysaccharides.

2. Materials and methods

2.1. Preparation of polysaccharides

P. umbellatus was obtained from the Institute of Microbiology, Chinese Academy of Sciences. The fungus was cultured in a medium (75 mL) containing 3% glucose, 2% broth, 0.2% yeast extract, 0.15% $MgSO_4$, and 0.3% KH_2PO_4 and at pH 5.5. The fungus culture was incubated at 90 r/min and 25 °C for 4 d. In order to extract polysaccharides, *P. umbellatus* (both the mycelium and fruiting body) was mixed with water at a mass ratio of 1:15. This mixture was treated with a VC 750 ultrasonic processor (Sonics & Materials Inc., Newton, CT, USA) for 25 min to disrupt the cells. The ultrasonic power and frequency were 500 W and 18 kHz, respectively. Then, an equal volume of hot water (98 °C) was added and the mixture was incubated at 98 °C for 1.5 h. Polysaccharides were precipitated by the addition of ethanol (final concentration 80%, *v/v*). The crude extract of fungal polysaccharides was dried, and the polysaccharides content was assayed using the phenol–sulfuric acid method [10].

2.2. Purification of polysaccharides

The crude polysaccharides were dissolved in water at a concentration of 0.5 g/L. This solution was then chromatographically resolved with Sephadex G-100 (Pharmacia, Swedish) at an elution rate of 0.5 mL/min. The presence of polysaccharides was detected using the phenol–sulfuric acid method. The elution fractions containing purified polysaccharides were freeze-dried for 24 h. Two polysaccharides, symbolized as PUm-C and PUf-C, were obtained. These two polysaccharides were first hydrolyzed by trifluoroacetic acid (TFA), and then the hydrolysis products were resolved by HPLC (LC-20A, Shimadzu, Japan) to characterize monosaccharide components. Additionally, the molecular weights of these two polysaccharides were determined using HPLC (LC-20A, Shimadzu, Japan). The conditions were injection volume: 20 μL; mobile phase: 3 mmol/L NaAc; flow rate: 0.5 mL/min. The molecular weights were then calculated by reference to the calibration curve made using standard dextrans (Sigma–Aldrich, St. Louis, MO, USA) with known molecular weights.

2.3. Immune activities

Male mice (age 5–6 weeks) purchased from the Institute of Zoology, Chinese Academy of Sciences, were fed with a single formula containing mycelium polysaccharides of *P. umbellatus* or a mixed formula with three fungal polysaccharides from *P. umbellatus, Ganoderma lucidum* and *Cordyceps sinensis* at a mass ratio of 8:1:1 (Table 1). The control animals were given cyclophosphamide (CTX) (Jiangsu Hengrui Medicine Co., Ltd., Jiangsu, China). At the end of the treatment (day 15), mice were sacrificed and the spleen was dissected under sterile condition. The spleen was then crushed and lymph cells were obtained with Ficoll (density 1.088). These cells included T cells, B cells, natural killer (NK) cells and lymphokine-activated killer (LAK) cells. The induction of LAK cells was carried out according to the method of Zhuang et al. [11]. Spleen lymph cells were cultured in a medium containing 10% FCS1640 and 500 U/mL IL-2 for 24 h. Cytotoxicity assay was conducted using the MTT colorimetric method as described by Cai [12]. The YAC mouse leukemia cells, P815 Mouse mast leukemia cells, Lewis lung cancer cells and HAC liver cancer cells were used as target cells. To investigate the impact of dietary administration of fungal polysaccharides on lymph cell proliferation, 200 μL spleen extract containing 2×10^6 cells was introduced to the 96-well plates. T cells and B cells were sensitized with 5 μg/mL ConA (Sigma–Aldrich, St. Louis, MO, USA) or 5 μg/mL lipid polysaccharide (LPS; Sigma–Aldrich, St. Louis, MO, USA) (n Y = 5). Control cells were not treated with ConA or LPS. Both B and T cells were cultured at 37 °C and 5% CO_2 for 3 d for ConA-treated cells, and 5 d for LPS-treated cells. Sixteen hours before the termination of the treatment, ^{3}H-tritiated thymidine (TdR) was added to each well. Cells were collected with a filter paper, and then treated with 0.5 mL scintillation solution. The amount of radiation in count per minute (CPM) was determined with a scintillation counter (Model XH-6925, Shanghai Precision Instrument Co., Ltd., China). The stimulation index (SI) and the ratio between the CPMs of treated and control cells were determined.

Table 1
Treatment scheme for immune assays.

Group	Number of mice	Feeding dose[a]
Saline (blank)	3	0.2 mL/each
CTX inhibition control	3	0.6 mg/each
Single formula	3	100 μg/each
Mixed formula (high-dose)	3	500 μg/each
Mixed formula (medium-dose)	3	100 μg/each
Mixed formula (low-dose)	3	50 μg/each

[a] The mice were fed on day 1, 7 and 14.

2.4. Anti-microbial activities

Two methods were used to test anti-microbial activities of *P. umbellatus* extract. In one method, blocks of fermented mycelia were placed on the nutrient broth (NB) or potato dextrose agar (PDA) mixed with 0.1 mL tested bacteria- or molds-containing culture. The other was the double-layer plate method, wherein the lower layer of the medium contained NB for bacteria and PDA for fungi, while the upper layer was a mixture of soft agar and 0.1 mL bacteria- or molds-containing culture. After agar became solidified, holes were drilled, and 1 mL the

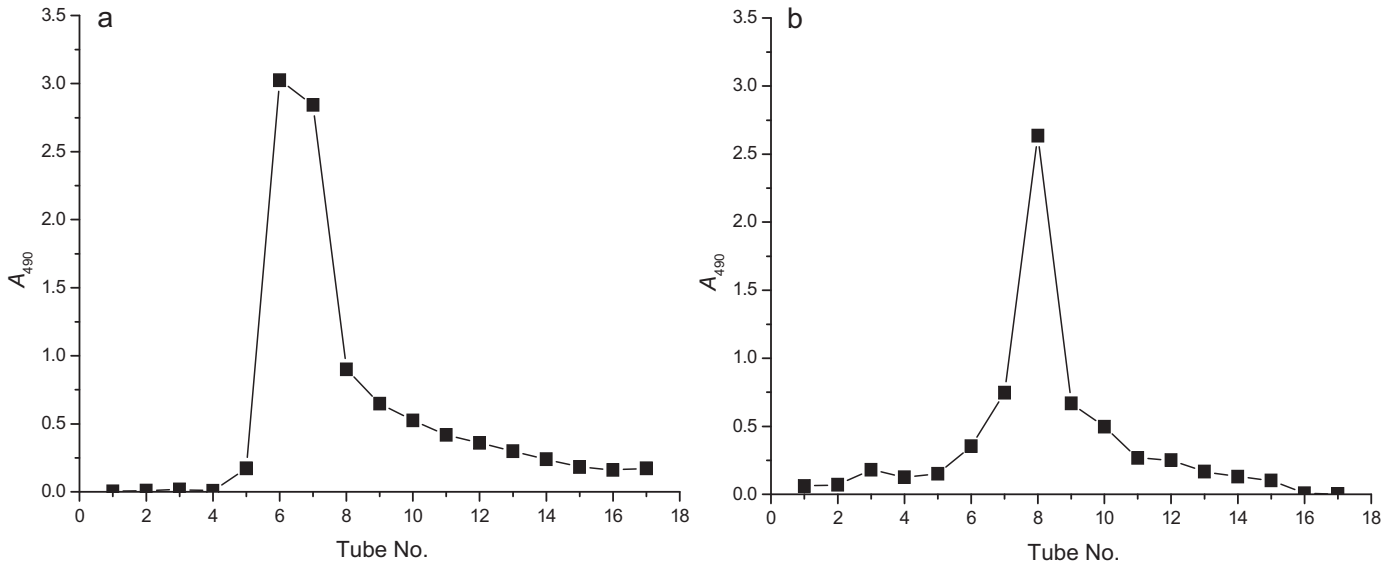

Fig. 1. Elution profiles of polysaccharides from the mycelium (a) and fruiting body (b) of *P. umbellatus*.

polysaccharide extract from fermented mycelium was added. The size of inhibition zone was measured after 20 h and 48 h. Norfloxacin was used as a positive control.

2.5. Data analysis

Data are expressed as the means ± standard deviation. Dunnett's *t*-test (SAS 8.0) was used to analyze the results. A *P*-value of less than 0.05 was considered significant.

3. Results and discussion

The elution profile was shown in Fig. 1. UV scan at 260 nm and 280 nm exhibited no absorption, suggesting that there was no nucleic acids or proteins. HPLC analysis of hydrolysis products of these two polysaccharides (Fig. 2) showed that the polysaccharide from the fruiting body of *P. umbellatus* consisted of glucose and galactose at a molar ratio of 5.42:1, and the one from mycelium of this fungus was also made up of glucose and galactose but at a molar ratio of 1.57:1. The molecular weights of these two polysaccharides were 679 kDa for the one from the fruiting body and 857 kDa for mycelium polysaccharide according to the HPLC results (Fig. 3). It has been noted that the anti-tumorogenicity of a polysaccharide is related to its molecular weight, with the minimum molecular weight for anti-tumorogenicity of 10 kDa [2]. The molecular weights of the two polysaccharides were greater than 10 kDa, suggesting that these two fungal polysaccharides may possess anti-tumor activity.

NK and LAK cells play an important role in immune defense. In the present investigation, alterations in the potency of these cells in killing NK-specific target cells (YAC-1 cells), NK-non-specific cells (P815), Lewis lung cancer cells (Lewis) and liver cancer cells (HAC) after dietary treatments with *P. umbellatus* polysaccharides were examined. The potency in killing P815 cells of NK cells from mice fed with the single formula containing *P. umbellatus* polysaccharide was 19%, greater than that

Table 2
Killing activities of NK cells and LAK cells.

Treatment	Killing rate (%)							
	YAC-1		P815		Lewis		HAC	
	NK	LAK	NK	LAK	NK	LAK	NK	LAK
Saline (blank)	34.04 ± 2.04	32.84 ± 1.20	13.06 ± 0.78*	26.53 ± 0.78	27.50 ± 0.71	32.86 ± 1.20	28.33 ± 0.49	30.46 ± 2.19
CTX inhibition control	22.08 ± 1.53*	27.25 ± 1.06*	10.63 ± 0.85*	19.94 ± 1.34*	21.74 ± 1.77*	26.12 ± 1.56*	18.35 ± 0.92*	21.05 ± 1.13*
Single formula	23.85 ± 2.62*	33.23 ± 0.89	19.00 ± 0.99*	13.76 ± 1.06*	19.04 ± 1.06*	33.01 ± 1.41	17.68 ± 0.99*	23.26 ± 0.61*
Mixed formula high-dose	35.61 ± 0.85	33.57 ± 2.19	21.77 ± 1.06	23.77 ± 1.34	21.46 ± 1.34*	28.55 ± 2.19	19.89 ± 1.27*	26.99 ± 2.83
Mixed formula medium-dose	31.45 ± 1.34	33.09 ± 1.56	16.70 ± 1.70*	18.50 ± 2.12*	19.26 ± 1.77*	33.03 ± 1.48	13.07 ± 0.64*	21.83 ± 1.17*
Mixed formula low-dose	25.72 ± 1.56*	35.53 ± 2.12	16.12 ± 0.85*	19.83 ± 1.91*	13.63 ± 0.92*	31.65 ± 0.78	17.06 ± 1.49*	20.01 ± 1.15*

Note: Data represent mean ± standard deviation.
* Significant at $P < 0.05$. Similarly hereinafter unless otherwise stated.

Table 3
Multiplication activation of T and B-lymphocytes as indicated by the stimulation index (SI).

Group	CPM		SI	CPM		SI
	Control	ConA		Control	LPS	
Saline (blank)	120 ± 67	49,676 ± 2459*		129 ± 35	4287 ± 947*	
CTX inhibition control	1620 ± 608	28,992 ± 7227*	1.0	1648 ± 238	2271 ± 953	1.0
Single formula	792 ± 392	60,411 ± 2789*	2.2	482 ± 621	4330 ± 257*	1.5
Mixed formula (high-dose)	2425 ± 1160	32,485 ± 10,545*	1.1	872 ± 621	4732 ± 2508*	1.8
Mixed formula (medium-dose)	2279 ± 893	42,763 ± 3699*	1.3	878 ± 383	5089 ± 629*	1.3
Mixed formula (low-dose)	1112 ± 612	79,556 ± 9230*	2.9	3200 ± 1065	3126 ± 913	1.1

* Significant at $P < 0.05$.

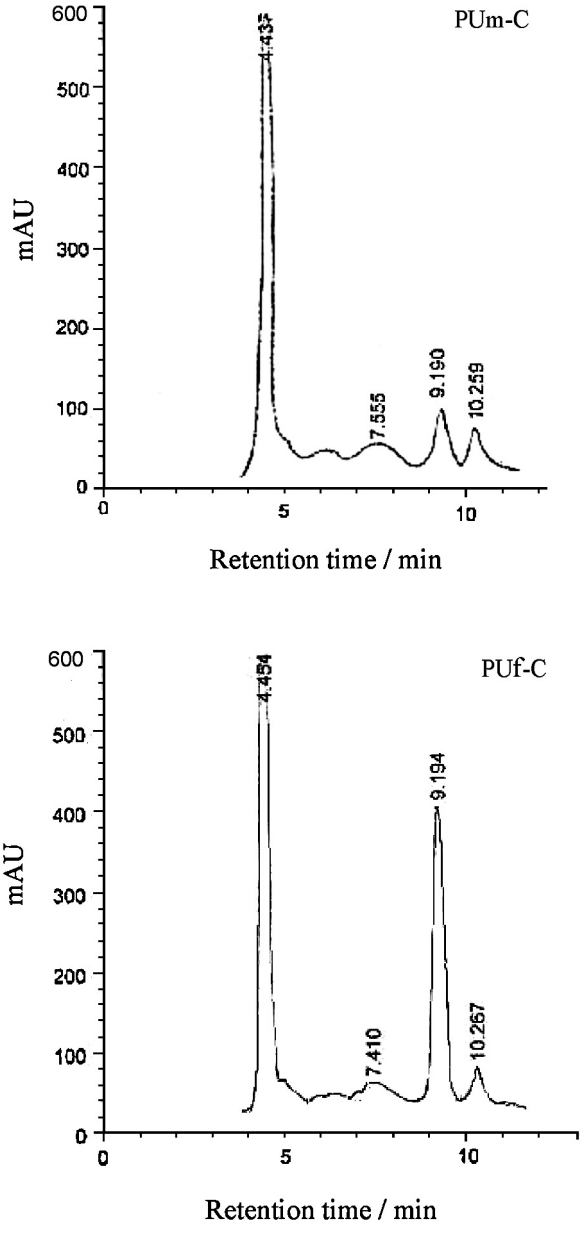

Fig. 2. HPLC spectra of hydrolysis products of polysaccharides from mycelium (PUm-C) and fruiting body (PUf-C).

Table 4
Anti-microbial activities of fermented mycelium of *P. umbellatus* as indicated by the size (diameter in cm) of the inhibition zone.

Inhibition zone	Dilution		
	10^{-1}	10^{-2}	10^{-3}
Control	+++	+++	+++
Escherichia coli (20 h)	3.9 ± 0.1	4.5 ± 0.3	+++
Staphylococcus aureus (20 h)	4.2 ± 0.1*	5.0 ± 0.2*	+++
Aspergillus niger (24 h)	–	–	–
Aspergillus nidulans (48 h)	–	–	–

Note: Control: norfloxacin. –, no inhibition zone; +++, inhibition zone diameter >6 cm. Similarly hereinafter.
* Significant at $P < 0.05$.

of the blank group (13.06%) (Table 2). In other words, dietary treatments with the mycelium polysaccharide resulted in a 60% increase in the killing potency of NK cells. But this dose of mycelium polysaccharide had no effect on the killing potency of LAK cells. Dietary treatments with three doses of the mixture formula resulted in greater P815-killing potencies of LAK relative to the control, with the highest dose being the most potent (Table 2). Treatments with polysaccharide mixture increased the potency of LAK cells in killing HAC, Lewis and YAC-1 in a dose-response manner. These results strongly suggest that *P. umbellatus* polysaccharides, when given alone or in mixture with other fungal polysaccharides, increase the activity of immune cells.

Results of cell proliferation experiment (Table 3) showed that dietary treatments with *P. umbellatus* polysaccharides alone or in combination with other polysaccharides resulted in an increase in SI value, suggesting that these fungal polysaccharides are capable of stimulating proliferation of T and B cells.

As shown in Table 4, the fermented mycelium of *P. umbellatus* was found to be capable of inhibiting the proliferation of *Escherichia coli* and *Staphylococcus aureus* with the size of inhibition zone increasing with the dilution of the seed bacteria. Treatments with the fermented *P. umbellatus* had no effect on the growth of *Aspergillus niger* or *A. nidulans*. The antibacterial activity of *P. umbellatus* mycelium was further supported by the additional results that the polysaccharide extract of *P. umbellatus* mycelium was also capable of inhibiting bacterial proliferation, with the size of inhibition zone increasing when the dilution of the seed bacteria (Table 5). But this fungal extract was inactive on either *A. niger* or *A. nidulans*. Similarly, Hou

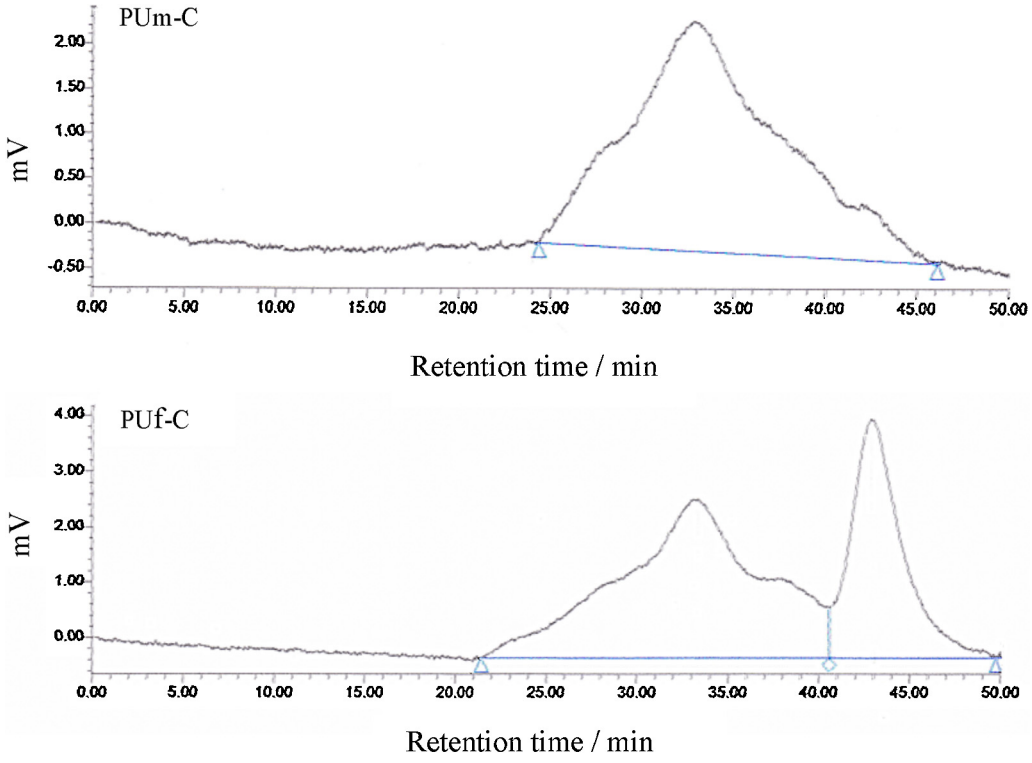

Fig. 3. HPLC spectra of polysaccharides from mycelium (PUm-C) and fruiting body (PUf-C).

Table 5
Anti-microbial activities of extraction from polysaccharides of *P. umbellatus* as indicated by the size (diameter in cm) of the inhibition zone.

Inhibition zone	Dilution		
	10^{-1}	10^{-2}	10^{-3}
Escherichia coli (20 h)	3.7 ± 0.1	3.9 ± 0.2	+++
Escherichia coli (38 h)	3.2 ± 0.4	3.7 ± 0.1	+++
Staphylococcus aureus (20 h)	4.1 ± 0.3	5.3 ± 0.1	+++
Staphylococcus aureus (38 h)	2.8 ± 0.1	3.9 ± 0.3	+++
Aspergillus niger (24 h)	–	–	–
Aspergillus nidulans (48 h)	–	–	–

et al. [13] evidenced that a novel polysaccharide (TMP-A) isolated from *Tricholoma matsutake* showed antibacterial activity against *E. coli* and *Staphylococcus albus*. And TMP-A did not exhibit any antifungal activity against target strains, including *A. niger*.

4. Conclusion

The results of the present study provide experimental evidence that fermented *P. umbellatus* mycelium is indeed immune active, with active ingredients most likely being its polysaccharides, and the consumption of this fungus may be beneficial for human health. Hence, the polysaccharide productions of *P. umbellatus* from the submerged mycelium are worth further exploration. Nevertheless, further investigations are necessary to determine the anti-tumor and antioxidant activities of the polysaccharide isolated from fermented *P. umbellatus* mycelium.

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