



Article Improved Production and Insulinotropic Properties of Exopolysaccharide by *Phellinus igniarius* in Submerged Cultures

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Abstract: Phellinu igniarius (P. igniarius), a basidiomycete belonging to the family Polyporaceae, is a medicinal basidiomycetous fungus belonging to the Hymenochaetaceae and is an excellent remedy with anticancer and antioxidant qualities. The mushroom has been used as traditional medicines for the treatment of cardiovascular disease, tuberculosis, liver or heart diseases, bellyache, bloody gonorrhea, and diabetes. However, the limited production and market shortage have been attributed to the slow growth and the difficult collection of the fruiting body as well as the rare natural resources. The problem can be solved through the effective approach of submerged culture to produce a high bioactivity polysaccharide of P. igniarius. The project was proposed to investigate the effect of a surfactant on the production of polysaccharide in submerged culture of *P. igniarius* and their insulinotropic properties. Eight different surfactants including PEG series (4000, 6000), Tween series (20, 40, 80, 85), and Span series (20, 80) all at a concentration of 0.5 g/L were supplemented in turn to the basal medium in shake flasks. Among the various surfactants tested, Tween 80 exhibited the greatest exopolysaccharide production of 128.43 mg/L, and PEG 6000 showed the maximum biomass of 6.76 mg/mL. To find the optimal Tween 80 concentration for biomass and exopolysaccharide production, different Tween 80 levels (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 g/L) were used in the medium. The maximal exopolysaccharide production of 132.76 mg/mL was achieved with the addition of 0.6 g/L of Tween 80 to the medium. The experimental results exhibited that the maximum of mycelia production in a stirred tank bioreactor was 3.01 mg/mL at Tween 80 0.2 g/L. In this study, their compounds, molecular weight, and protein content from fermentation product extracts were also tested. The average molecular weights of exopolysaccharide and intracellular polysaccharide were 1.715×10^6 Da and 4.87×10^5 Da, respectively. The protein contents of exopolysaccharide and intracellular polysaccharide were about 3.68% and 3.02%. The maximum RINm5F cell proliferations of exopolysaccharide and intracellular polysaccharide at 2 mg/mL were 142.3% and 120.07%, respectively. Cell proliferations of exopolysaccharide and intracellular polysaccharide increased with their concentrations. The maximum insulin secretion of exopolysaccharide at 2 mg/mL on RINm5F cell insulin was 0.615 μ g/L.

Keywords: *Phellinu igniarius*; submerged culture; polysaccharides; Tween 80; antioxidant activity; cell proliferation; insulin

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1. Introduction

Currently, about 14,000 mushroom species have been successfully identified and around 700 mushroom species are safe for oral consumption [1]. Mushrooms have attracted great attention in the latest decades for their nutritional and medicinal values. Mushrooms contain highly valuable nutrition including dietary fiber, polysaccharides, heteroglucans, peptidoglucans, proteoglucans, and vitamins such as thiamine and riboflavin [2–4]. Mushrooms also have beneficial effects on human health, as they contain bioactive compounds with high medicinal value, such as lectins, terpenoids, phenolics and polyphenolics, polysaccharides, and ergosterols. Bioactive compounds from mushrooms can promote healthy



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human to prevent and treat a variety of pathologies such as tumors, microbial and viral infections, immune disorders, diabetes, and neurodegenerative diseases [5–7]. Based on their antidiabetic, antihypercholesterolemic, antioxidant, antitumor, and antibacterial effects [8], many mushrooms can be used as a source for the development of drugs, functional food, and/or nutraceuticals [8–10].

Phellinus igniarius (P. igniarius) is a well-known fungus belonging to the genus Phellinus in the Polyporaceae family. P. igniarius has been used as a herbal medicine in Asia due to its high biological activities [11]. Recent studies have revealed that *P. igniarius* has a few important pharmacological activities including antitumor effects, inhibition of cholesterol synthesis, anti-influenza virus, antioxidative and anti-inflammatory effects, and immunomodulating activities [12–14]. Many investigators reported that polysaccharide extracted from partial *Phellinus* mushrooms confirmed the effects of stimulating cell-mediated and humoral immunity and inhibiting tumor growth and metastasis [15]. One of the most important bio-active compounds derived from *P. igniarius* is polysaccharides. The main polysaccharide compounds from P. igniarius, which include intracellular polysaccharides (IPS) and exopolysaccharide (EPS), have attracted great attention for many years. In recent years, pharmacological actions of *P. igniarius* polysaccharide have been extensively examined, and results told the positive effects such as antibacterial, antiviral, antioxidative, antitumor, and antimutagenic activities. Due to market demand, the efficient production with high-quality polysaccharides is a very important course. Accordingly, mushroom submerged culture is viewed as a promising alternative for the efficient polysaccharide production [16,17].

Physical and chemical conditions of *P. igniarius* polysaccharide by submerged culture have been extensively studied. Yang et al. (2021) reported that *Ganoderma lucidum* (G. lucidum) exopolysaccharide (EPS) production in submerged fermentation was stimulated by Tween 80 [18]. Liu and Wu (2012) pointed out that surfactants added to the medium inhibited pellet formation, resulting in smaller and looser pellets, and Tween 80 exhibited a remarkable promoting effect on EPS production by medicinal fungus *Cordyceps sinensis* Cs-HK1 [19]. However, few studies have been published on the effects of surfactants supplement on *P. igniarius* polysaccharide production by submerged culture. The EPS by *Laetiporus sulphureus var. miniatus* have been proven to be glucose-rich polysaccharides and were able to increase proliferation and insulin secretary function of rat insulinoma RINm5F cells, in a dose-dependent manner [20]. Although the pharmacological activities of *P. igniarius* polysaccharide has investigated by several researchers, little information has been published concerning insulinotropic properties of exopolysaccharide by *P. igniarius* in submerged cultures.

The objective of this research was to investigate the effects of surfactants supplement on *P. igniarius* polysaccharide production by submerged culture. Additionally, we examined insulinotropic properties of exopolysaccharide obtained from *P. igniarius* in submerged cultures. This research may provide a useful reference for effectual production and pharmacological application of *P. igniarius* exopolysaccharide.

2. Results

2.1. Effects of Surfactants in Shake Flask Cultures

The effects of various surfactants on biomass and EPS production were exploded in shake flask cultures. Amongst the various surfactants tested, PEG 6000 and Tween 80 showed the maximum biomass and EPS production of 6.76 and 0.128 g/L, respectively (Figure 1). As can be seen from Figure 1, several surfactants except for PEG 6000 and Tween 80 will inhibit cell growth as compared to control (without adding any surfactants). Based on the results obtained, Tween 80 might play an important role for promoting cell growth and EPS biosynthesis, and we chose accordingly it for further experiments.







(b)

Figure 1. The effects of various surfactants on mycelia growth (**a**) and EPS production (**b**) in shake –flask cultures.

2.2. Effects of Different Tween 80 Concentrations in Shake Flask Cultures

Different Tween 80 concentrations effects on biomass and EPS production proceeded in a 250 mL shake flask. As shown in Figure 2, impacts of various Tween 80 concentrations on cell growth and EPS formation were conspicuous. All Tween 80 concentrations tested will increase biomass yield. Especially, supplementation of Tween 80 concentration of 0.4 g/L to basal medium can obtain a maximum biomass of 7.82 g/L resulting in a 50% enhancement as compared to control. Influences of Tween 80 concentrations ranged from 0.2 to 1.2 g/L on EPS production were also remarkable and the maximum EPS production occurred at 0.6 g/L of Tween 80. Lower Tween 80 concentrations (0.2–0.6 g/L) can promote EPS formation; however, higher Tween 80 concentrations (>0.6 g/L) will inhibit EPS production compared with control. It is worth noting that the maximum biomass and EPS production appeared at distinct Tween 80 concentrations. The similar results were found in the other mushroom. In order to enhance EPS production, the Tween 80 concentrations of 0.6 g/L were chosen for further study.







(b)

Figure 2. The effects of different Tween 80 concentrations on mycelia growth (**a**) and EPS production (**b**) in shake–flask cultures.

2.3. Effects of Different Tween 80 Concentrations in Stirred Tank Bioreactor

Effects of different Tween 80 concentrations on cell growth and EPS production were also performed in a 5 L stirred tank bioreactor. The time-course data of stirred tank bioreactor culture at 0 g/L and 0.6 g/L Tween 80 concentrations are illustrated in Figures 3 and 4, respectively. The fermentation results under different Tween 80 concentrations in stirred-tank bioreactor are summarized in Table 1. With glucose consumption without any Tween 80 addition, cell growth increased with culture time until day 5 and then fell rapidly until the end of the fermentation (Figure 3). At 0.6 g/L Tween 80, the maximum biomass and EPS production were obtained at day 8 of culture time (Figure 4).

The fermentation time in the stirred-tank bioreactor cultures increased with an increase in Tween 80 concentration (Table 1). The fermentation kinetics for EPS biosynthesis showed a growth-associated pattern under all Tween 80 concentrations tested. For different Tween 80 concentrations tested, the maximum biomass (3.02 g/L) and EPS production (184.3 mg/L) appeared at 0.2 g/L and 0.6 g/L Tween 80, respectively. At 0.6 g/L Tween 80, a percentage enhancement for EPS production (from 110 mg/L to 184.3 mg/L) was achieved as compared to control (0 g/L Tween 80), resulting in the optimal values in productivity (Qp) of 23.04 mg/L/d and in product yield (Y_{P/S}) of 22.06 mg/g (Table 1). To optimum EPS production, 0.6 g/L Tween 80 was selected as an optimal concentration supplemented in culture medium for further scale-up experiments.



Figure 3. The effect of Tween 80 at 0 g/L on mycelia growth and EPS production in in a 5-L stirred tank bioreactor.



Figure 4. The effect of Tween 80 at 0.6 g/L on mycelia growth and EPS production in a 5–L stirred tank bioreactor.

conc. (g L ⁻¹)	μ ^a (d ⁻¹)	Q_X^{b} (gL ⁻¹ d ⁻¹)	Q_P^{c} (mgL ⁻¹ d ⁻¹)	X_{max} ^d (mgmL ⁻¹)	<i>P_{max}</i> ^e (mgmL ^{−1})	$Y_{P/X}$ g (mgg ⁻¹)	$Y_{X/S}$ h (gg ⁻¹)	$Y_{P/S}$ ⁱ (mgg ⁻¹)	t ^j (d)
0	0.45	0.42	15.75	2.14	0.11	51.40	0.22	11.24	5
0.2	0.48	0.43	16.93	3.02	0.12	39.28	0.33	12.83	7
0.4	0.51	0.38	17.26	2.65	0.14	52.00	0.27	13.89	7
0.6	0.57	0.32	23.04	2.55	0.18	72.22	0.31	22.06	8
0.8	0.41	0.18	21.73	1.41	0.15	107.58	0.16	17.43	8
1	0.42	0.10	18.10	0.93	0.14	156.32	0.11	16.59	9
1.2	0.43	0.11	17.82	0.87	0.12	142.91	0.09	13.14	8
0.6 (20L)	0.30	0.42	13.26	4.65	0.15	31.36	0.47	14.61	11

Table 1. Fermentation parameters of the batch experiments in a 5 L and a 20 L stirred tank bioreactor under different culture conditions.

^a μ : specific growth rate (d⁻¹), ^b Q_X : biomass productivity (gL⁻¹d⁻¹), ^c Q_P : EPS productivity (mgL⁻¹d⁻¹), ^d *Xmax*: maximum biomass concentration (mgmL⁻¹), ^e *Pmax*: maximum EPS product concentration (mgmL⁻¹), ^g $Y_{P/X}$: specific product yield (mg EPS g⁻¹ biomass), ^h $Y_{X/S}$: cell yield (g biomass g⁻¹ glucose), ⁱ $Y_{P/S}$: product yield (mg EPS g⁻¹ glucose), $t^j(d)$: culture time (day).

2.4. Scale-Up Fermentation

The scale-up fermentation in 20 L stirred tank bioreactor was investigated the performance of Tween 80 addition on EPS production. The profile of 20 L stirred tank bioreactor culture under 0.6 g/L Tween 80 is depicted in Figure 5 and its fermented results are listed in Table 1. The results showed that the maximum biomass Xmax and EPS production Pmax were 2-fold (from 2.14 g/L to 4.65 g/L) and 1.5-fold (from 110 mg/L to 150 mg/L) than those of control. In addition, level of the maximum EPS production in 20 L stirred tank bioreactor culture was similar with that of 5 L stirred tank bioreactor culture at the same Tween 80 concentration. The results confirm that the performance of Tween 80 effect on scale-up fermentation for yielding effectively EPS was proved in 20 L stirred tank bioreactor culture.



Figure 5. The effect of Tween 80 at 0.6 g/L on mycelia growth and EPS production in a 20–L stirred tank bioreactor.

2.5. Number-Average Molecular Weights and Protein Contents of Polysaccharides

The number average molecular weight of intracellular polysaccharide and exopolysaccharide obtained from 20 L stirred tank bioreactor culture were 1.71×10^6 Da and 4.87×10^5 Da, respectively, as shown in Table 2. The number average molecular weight of intracellular polysaccharide is significantly higher than that of exopolysaccharide. The protein content of intracellular polysaccharide and exopolysaccharide were 3.02% and 3.68%, respectively. On the contrary, the protein content of intracellular polysaccharide is lower than that of exopolysaccharide.

Table 2. Number-average molecular weights and protein contents of polysaccharides.

Samples	Protein/Polysaccharide (%, w/w)	Mn (Da)
Exopolysaccharide	3.68	$4.87 imes10^5$
Intracellular polysaccharide	3.02	$1.71 imes 10^6$

2.6. RINm5F Cell Proliferation

Cellular morphology of RINm5F cell is shown in Figure 6. The maximum RINm5F cell proliferations of exo-polysaccharide and intracellular polysaccharide at 2 mg/mL were 142.3% and 120.07%, respectively (Figure 7). Cell proliferations of exopolysaccharide and intracellular polysaccharide increased with their concentrations. In this study, cell proliferations of intracellular polysaccharide are not very apparent compared to exopolysaccharide. Therefore, exopolysaccharide was employed only to investigate for further RINm5F insulin secretion.



Figure 6. Cellular morphology of RINm5F cell grown in 96-well flat-bottomed plates.

2.7. RINm5F Insulin Secretion

The maximum insulin secretion of exopolysaccharide at 2 mg/mL on RINm5F cell was $0.615 \mu g/L$, as shown in Figure 8. Insulin secretion of exopolysaccharide increased when concentration of exopolysaccharide increased. These results indicate that exopolysaccharide has a positive effect on stimulating insulin secretion of RINm5F cell.



Figure 7. Effect of different concentration Phellinus igniarius polysaccharides on RINm5F cell proliferation.



Figure 8. Effect of different concentration *Phellinus igniarius* exopolysaccharides on RINm5F insulin secretion.

3. Discussion

This experimental study was designed to examine the effectiveness of exopolysaccharide by *Phellinus igniarius* in submerged cultures. Additionally, insulinotropic properties of exopolysaccharide were examined. These results indicate that an enhancement in production of exopolysaccharide was achieved by adding Tween 80 in submerged culture, and proved in scale-up cultures (20 L bioreactor). In this study, the exopolysaccharide produced by Phellinus igniarius submerged cultures in a 20 L stirred tank bioreactor has been demonstrated with high bioactivity in RINm5F insulin secretion.

Our results indicate that Tween 80 is the most suitable stimulant for exopolysaccharide production. For Tween 80 contraction effects in shake flask and stirred tank bioreactor cultures, lower and higher Tween 80 contractions are not beneficial to growth and ex-

opolysaccharide production of *Phellinus igniarius* in submerged cultures. Similar results were found in other mushroom *Grifola frondosa* reported by Hsieh et al. (2008) [21] and *Ganoderma lucidum* by Yang et al. (2021) [20], who found that Tween 80 addition can increase cell growth and exopolysaccharide production. Li et al. (2018) reported that with adding to Tween 80, the outputs of biomass and intracellular polysaccharide increased during the *Lentinus edodes* fermentation, respectively [22]. These results can be explained that Tween 80 can promote the absorption of nutrients from fermentation medium and accelerate the growth of mycelia and extend the growth cycle of fungus because it maintains the intact structure of the mycelia and alleviates the destructive power brought by the oscillatory force during shaking [10]. In addition, Tween 80 can play an important role acting as an efficient permeabilizer to improving bioactivator, and serving as an elicitor to promote physiological activator biosynthesis for increasing the yield of useful fungal metabolites, such as mycelial growth and exopolysaccharide yield [21].

In this study, exopolysaccharide exhibited significantly high bio-activity in RINm5F insulin secretion and followed a marked dose-dependent pattern. Moreover, *Phellinus igniarius* exopolysaccharide obtained from 20 L stirred tank bioreactor culture had high number-average molecular weight and protein/polysaccharide ratio. Exopolysaccharides is associated with protein as complexes. It is possible that the RINm5F insulin secretion capability of polysaccharides might be related to their number-average molecular weight and protein/polysaccharide ratio. Several researchers have reported that many bio-activities such as antitumor activity and TNF-release capability on RAW 264.7 were closely related to number-average molecular weight and protein/polysaccharide ratio of polysaccharide [4,23]. However, the correlation between RINm5F insulin secretion of exopolysaccharide and its structure properties will be demonstrated in our future study.

Overall, the findings of this study suggest that improvement of exopolysaccharide production by *Phellinus igniarius* submerged cultures can be achieved through supplementation of Tween 80 into the culture medium. Additionally, the exopolysaccharide produced possess an excellent RINm5F insulin secretion activity.

4. Materials and Methods

4.1. Microorganism and Seed Culture

Phellinus igniarius BCRC 35308 was obtained from Bioresources Collection and Research Center in Hsinchu, Taiwan. The culture was maintained in PDA (potato dextrose agar). The sub-culture was conducted by transferring grown mycelia to a fresh PDA every month. The three-week-old cells grown on the media agar plate were collected with 25 mL sterilized water mixed by mycelia. A total of 20 mL collected mycelia were then transferred to 250 mL seed culture flasks containing 50 mL culture medium (g/L) composed of PDB (potato dextrose broth), 12; malt extract, 5.0; peptone, 0.5. The seed culture was incubated at 28 °C on a rotary shaker at 150 rpm for 7 days.

4.2. Shake-Flask Cultures

All shake-flask cultures were performed in 250 mL Erlenmeyer flasks with 100 mL culture medium. The basal medium contained the following components (g/L): sucrose, 10; malt extract, 3; yeast extract, 3; peptone, 5; KH₂PO₄, 0.5 and MgSO₄, 0.5. The media were sterilized at 121 °C for 20 min. The 20 mL inoculums from seed cultures were transferred to culture flasks. Eight different surfactants including PEG series (4000, 6000), Tween series (20, 40, 80, 85) and Span series (20, 80) all at a concentration of 0.5 g/L were supplemented in turn to the basal medium in shake flasks. Different concentrations (0–1.0 g/L) of Tween 80 (given by screening) in the culture medium were incubated in the shake flasks for screening its suitable concentration. All shake-flask experiments were performed at 28 °C, 150 rpm, and initial pH 4.0. All experiments were performed in three replicates (n = 3).

4.3. Stirred-Tank Bioreactor Culture

Fermentations of Phellinus igniarius proceeded in a 5 L stirred-tank bioreactor to evaluate the effect of supplementation of Tween 80 on EPS production. Tween 80 concentration varies between 0.2 g/L and 1.0 g/L at the interval of 0.4 g/L and without adding Tween 80 in culture medium was regarded as control. The fermenter contained 3.0 L medium and 5% (v/v) inoculums from the seed culture. The culture medium in a 5 L stirred-tank bioreactor consisted of the following ingredients (g/L): sucrose, 10; malt extract, 3; yeast extract, 3; peptone, 5; KH_2PO_4 , 0.5 and MgSO₄, 0.5. The stirred-tank fermentations proceeded at 28 °C, controlled pH 4.0, 1vvm (volume of aeration per volume of bioreactor per minute) aeration rate, and 150 rpm agitation speed. The scale-up fermentation of Phellinus igniarius proceeded in a 20 L stirred tank bioreactors filled with 12 L culture medium and 5% (v/v) inoculums derived from seed cultures. The culture medium in the bioreactor was composed of 0.6 g/L Tween 80, 10 g/L sucrose, 3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 0.5 g/L KH₂PO₄, and 0.5 g/L MgSO₄. The scale-up stirred tank bioreactor culture was operated at same conditions with those of 5 L stirred tank bioreactor culture. The pH of culture medium was automatically controlled by adding 1 N HCl or 1 N NaOH. Mycelia were separated from fermented broth by centrifugation (4 °C, $8000 \times g$ for 15 min), then washed with distilled water and finally freeze-dried to powders. Biomass concentration was determined in dry weight per unit volume. Residual sugar in the supernatant was determined by the dinitrosalicylic acid method [24]. The polysaccharide concentration in the supernatant was determined by phenol-sulfuric acid assay [25]. All experiments were performed in three duplicates (n = 3).

4.4. Molecular Weights and Protein Contents of Polysaccharides

Molecular weights of polysaccharides were determined by gel permeation chromatography (GPC) of Waters (Milford, MA, USA) 600E system equipped with GPC column (Shodex OHpak SB-804HQ) and a model 410 RI detector. All chromatographic data were processed by Millennium (Milford, MA, USA) software. Polyethylene glycol (PEG) standards (Polymer Laboratories, Church Stretton, UK) with narrow polydispersity and with molecular weights ranging from 1.9 to 1260 kDa constructed a calibration curve. Deionized water was used as mobile phase at the flow rate of 0.6 mL/min. Protein contents of polysaccharides were determined by the Lowry method [26], with bovine serum albumin as standard.

4.5. RINm5F Cell Culture

RINm5F cell line BCRC 60410 was purchased from the Bioresources Collection and Research Center (Hsinchu, Taiwan). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and 100 U/mL penicillin and streptomycin in the Petri dishes. The cell culture was incubated at 37 °C in a humidified CO_2 incubator for two weeks where the culture medium was changed per 3 days.

4.6. Analysis of RINm5F Cell Proliferation

RINm5F cell proliferation was assessed by adding CCK-8 reagent for determination in absorbance of cell broth. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) in 96-well flat-bottomed plates (Sumitomo Baklite Co. Ltd., Tokyo, Japan) at 1×10^4 cells per well in 1ml of culture medium. The cell culture was incubated at 37 °C in a humidified CO₂ incubator for 24 h. The cells were then treated with different concentration of 20 µL crude exopolysaccharide at dosage of 0.5, 1, and 2 mg/mL for 24 h at 37 °C in 96-well flat-bottomed plates. After 24 h, 10 µL CCK-8 reagent were added to each well and incubated for 24 h. The absorbance was read at 450 nm by ELISA reader. The absorbance was proportional to the number of live cells [27]. The proliferation rate of cells was calculated as follows: (mean value of treated group/control group) \times 100%. Data are presented as mean values from three independent experiments.

4.7. Analysis of Insulin Secretion by RINm5F Cell

RINm5F cells were treated as described above. Briefly, the cells were transferred to 96well flat-bottomed plates and incubated in at 37 °C in a humidified CO_2 incubator for 24 h. various concentrations (0.5, 1, and 2 mg/mL) of crude *Phellinus igniarius* exopolysaccharide were added to the cell-grown wells for incubating 24 h to stimulate insulin secretion. Rat Insulin Kit reagents were added to culture solutions, and the absorbance was then measured spectrophotometrically at 450 nm [28]. Rat insulin was used as a standard constructed a calibration curve.

5. Conclusions

This study investigated the effects of surfactants supplementation into the culture medium on *Phellinus igniarius* exopolysaccharide biosynthesis. The RINm5F cell proliferation and secretion of exopolysaccharide were also determined. Our results provide evidence that *Phellinus igniarius* exopolysaccharide production can be obviously promoted by supplementation of Tween 80 into culture medium. The insulin secretion and proliferation were greatly increased by stimulation of exopolysaccharide. The results report here could be beneficial to biotechnology and pharmaceutical industry attempting to develop diabetes-related health-food and medicinal products.

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