



Effects of Tween series and agar additives on mycelia biomass and β -glucan production by *Hericium erinaceus* in submerged culture

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Abstract

Hericium erinaceus has been widely used in food and medicine because of its various promising bioactivities. This study investigated the effects of Tween series surfactant and agar addition on mycelial growth and β -glucan in liquid submerged culture of the medicinal mushroom *H. erinaceus*. The addition of Tween 80 in liquid medium resulted in higher dry cell weight (DCW) and β -glucan production, with values of 6.06 and 1.21 g/L for 3.0% (w/v) and 7.27 and 1.22 g/L for 4.0% (w/v), respectively. This corresponded to a 1.82 relative ratio of DCW to control and a 1.86 relative ratio of β -glucan to control for 3.0% (w/v) and a 2.18 relative ratio of DCW to control and a 1.87 relative ratio of β -glucan to control for 4.0% (w/v), respectively. Further, the mycelial pellets were altered by the addition of agar in liquid medium, forming small pellets. The pellet diameter decreased with increasing medium viscosity, and the smallest pellet diameter, 1.5 mm, was attained with the addition of 0.3% (w/v) agar. The medium viscosity was 48.7 mPa s. Moreover, β -glucan production increased with a decreasing pellet diameter, and the highest β -glucan production, 0.86 g/L, was observed with 0.3% (w/v) agar added to the medium. The influence of flask type on DCW and β -glucan production was investigated which were almost 1.2-fold higher in baffled Erlenmeyer flasks than in normal Erlenmeyer flasks. Finally, cultivation with agar addition in a baffled Erlenmeyer flask was determined to be most effective for DCW and β -glucan production, yielding the highest values of 7.74 and 1.43 g/L, respectively.

Keywords *Hericium erinaceus* · Mycelial biomass production · Medicinal mushroom · Surfactant · Agar

1 Introduction

Hericium erinaceus, known as Yamabushitake in Japan, is a medicinal mushroom [1]. Its polysaccharides can prevent, alleviate, or treat major diseases including cancer, gastric ulcer, diabetes, hyperlipidemia, hepatic injury, and neurodegenerative diseases [2–5]. In particular, β -glucan is a major bioactive compound known to have biological activities including anti-cancer, anti-inflammatory, and immune-modulating properties. Due to the specific physical properties of β -glucan, such as water solubility, viscosity, and gelation, it has been increasingly used by the food and other industries [6]. Therefore, it is required to be produced on a large scale and should be available throughout the year. Polysaccharides can be isolated from

the fruiting bodies of mushrooms, cultured mycelia, and cultured broth [7]. Fruiting body cultivation takes 2–3 months for harvesting based on solid cultures using substrates such as wood, grain, or sawdust [8]. Moreover, its growth is seasonal. To overcome these problems, mycelial cultivation using submerged culture techniques can be used as an alternative method of polysaccharide production at an industrial scale with a shorter cultivation period (8–10 days) [9]. Therefore, in our previous study, the optimal carbon source required to produce *H. erinaceus* mycelia in submerged cultivation was investigated. Furthermore, yogurt whey as an alternative nitrogen source was used, which was determined to be favorable for mycelial growth and β -glucan production [10]. However, there are only a few studies on mycelial growth and β -glucan production by *H. erinaceus* with submerged cultivation.

Tween 80 is one of the most favorable surfactants for mycelial growth and exopolysaccharide production with medicinal fungi, such as *Grifola frondosa* [11], *Schizophyllum*

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commune [12], and *Pleurotus tuber-regium* [13]. Some researchers have indicated that it significantly increases cell membrane permeability. This increased cell membrane permeability might be attributed to changes in the composition and content of unsaturated fatty acids in the cell membrane, which could facilitate the uptake of nutrients, as well as the secretion of metabolites such as β -glucan [14–16].

In this study, we evaluated the effects of Tween series surfactants and agar on *H. erinaceus* mycelial growth and β -glucan production in submerged culture. To prevent the aggregation of mycelia and to miniaturize the pellets, agar was added into the medium. Next, the effect of a culture reactor, specifically a baffled Erlenmeyer shake flask use, on mycelial growth and β -glucan production was investigated. Finally, cultivation with additives in a baffled Erlenmeyer flask was investigated.

2 Materials and methods

2.1 Microorganism

Fungal mycelia of *H. erinaceus* were kindly provided by Oze Trading Co., Japan, in this study and cultured on potato dextrose agar (Wako Pure Chemical Industries, Ltd.) plates at 25°C.

2.2 Culture media and cultivation

To preculture the fungus, fresh mycelia from potato dextrose agar plates were inoculated into 200-mL Erlenmeyer flasks containing 50 mL of medium consisting of the following ingredients buffered with 0.4 M phthalic acid (pH 5.0): glucose 30 g/L, polypeptone 6.3 g/L, yeast extract 0.2 g/L, KH_2PO_4 1.0 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g/L (basal medium). All nutrients were purchased from Wako Pure Chemicals. The flasks were incubated in a rotary shaker (TAITEC, Bio-Shaker BR-300LF) at 25°C with 100 rpm for 7 days.

The main culture was carried out in 300-mL Erlenmeyer flasks containing 60 mL of basal medium. The pre-culture was homogenized with a polytron homogenizer (Model PT-MR 2100, Kinematica AG, Switzerland) for 20 s at 1500 rpm. Two percent (v/v) homogenized pre-cultures were inoculated into the main culture media. The flasks were then incubated at 25°C with 100 rpm. The incubation periods (end of culture) were defined as the day at which the glucose concentration reached 0 g/L. The mycelial morphological characteristics were observed with the naked eye, and the average pellet diameter was measured with specimen samples taken from each flask. For each sample, at least 20 pellets were measured using a ruler to attain the average pellet diameter. The cultivation was performed in duplicate, and the mean results are shown.

2.3 Cultivation with additives

The effects of surfactant, specifically the Tween series (Tween 20, Tween 40, Tween 60, and Tween 80), or agar addition on *H. erinaceus* culture were studied. The concentrations of Tween series and agar used were 0.5, 1.0, 2.0, 3.0, and 4.0% (w/v) (Tween series) and 0.05, 0.1, 0.2, and 0.3% (w/v) (agar). These additives were supplemented in liquid media and the sample was cultivated at 25°C on a rotary shaker, as described previously herein, with shaking at 100 rpm.

2.4 Shake flasks

A comparison of cultivation conditions by type of flask used was also performed. Shake flasks used for cultivation were typical (normal) Erlenmeyer flasks with or without baffles. Each 300-mL baffled Erlenmeyer flask consisted of a conventional flask (SIBATA, Erlenmeyer flask, narrow neck, Borosilicate Glass, SIBATA SCIENTIFIC TECHNOLOGY, Japan) with three indentations, which were 7.0 cm high, 1.0 cm wide, and 0.8 cm deep.

2.5 Analytical methods

After cultivation, mycelia were filtered from the culture medium using ADVANTEC no.131 filter paper [10]. The filtered mycelial pellets were washed with 300 mL distilled water and air-dried for 2 days at 35°C. Subsequently, the sample was stored in a vacuum desiccator with silica gel at room temperature. The dry cell weight (DCW) was then determined. Glucose concentrations were determined by the mutarotase GOD method (Glucose C-Test; Wako Pure Chemicals, Osaka, Japan). To determine the β -glucan content in the DCW, generated glucose via lichenase treatment was determined by mutarotase GOD method. The glucose from β -glucan in the DCW was assayed using the Mushroom and Yeast β -glucan Assay Kit (Megazyme International Ireland Ltd., Bray Business Park, Bray, Co. Wicklow, Ireland). All experiments were carried out in triplicate, and individual assays were performed at least twice.

3 Results and discussion

3.1 Effects of Tween surfactants on cell growth and β -glucan production

The DCW and β -glucan are two of the most desired products resulting from the cultivation of *H. erinaceus*. To accelerate the production of these components, we previously reported the effects of various carbon sources and the effects of yogurt whey addition as an alternative nitrogen source on mycelia (DCW) and β -glucan production [10]. In this study, the

effects of surfactant Tween series addition on *H. erinaceus* in submerged cultivation were investigated. Liu et al. reported the effects of surfactant Tween 80 addition on mycelial growth and exopolysaccharide production in *Cordyceps sinensis* [17]; specifically, the DCW and exopolysaccharide yields were increased, and the highest exopolysaccharide yield was attained with the addition of 1.5% Tween 80, which was 2-fold higher than that in control conditions (without Tween 80).

Figure 1 shows the basal glucose consumption time course and growth curve of *H. erinaceus* in submerged culture. The initial glucose concentration of 20.7 g/L decreased to zero during 8-day cultivation period, and 3.33 g/L of DCW was achieved. The influence of Tween series (Tween 20, 40, 60, and 80) and concentrations (0.5, 1.0, 2.0, 3.0, and 4.0% (w/v)) on the DCW, β -glucan, and relative ratios of DCW and β -glucan to control values of *H. erinaceus* in the culture media are summarized in Table 1. The DCW of *H. erinaceus* was found to increase at all concentrations of Tween 40, 60, and 80, and the relative ratios of the DCW to control values were 1.16–2.18 (the highest, at 4.0% (w/v) Tween 80). In contrast, the remarkable increase in β -glucan was observed only with Tween 80 at 3.0% (w/v) and 4.0% (w/v), representing high concentrations; the relative ratios of β -glucan to control values were 1.86 at 3.0% (w/v) and 1.87 at 4.0% (w/v). From this result, it was concluded that Tween 40 and 60 were only accumulated in the fungus cell bodies; therefore, the addition of these contributed little to metabolite production, such as β -glucan production. Yang et al. also reported the effect of Tween 80 on metabolite production (exopolysaccharide production) in submerged mycelial cultures of *Ganoderma lucidum* [15]. The addition of 0.25% (w/v) Tween 80 on day 3 resulted in the maximum production

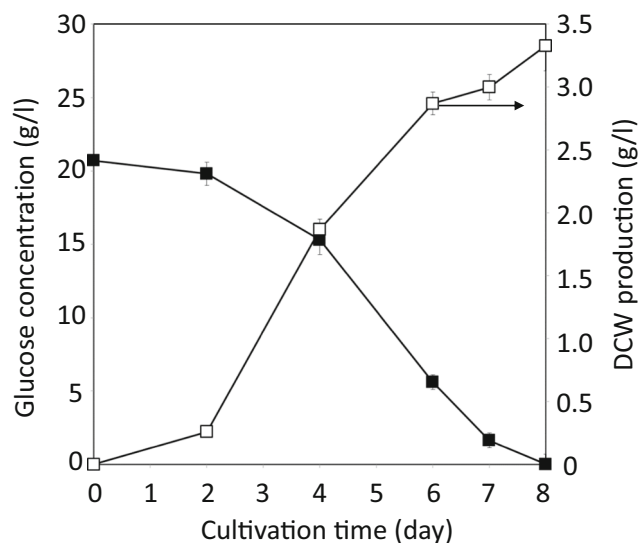


Fig. 1 Basal glucose consumption time course and growth curve of *Hericium erinaceus*. ■, glucose concentration (g/L); □, DCW production (g/L)

of DCW and exopolysaccharide, with an increase of 19.8 and 137.5%, respectively. Moreover, it was found that Tween 80 significantly increased cell membrane permeability. The increased cell membrane permeability could facilitate the uptake of nutrients, biosynthetic activity for exopolysaccharide production, and membrane permeability for exopolysaccharide secretion [14, 16]. Tween 80 is composed of fatty acid esters of polyoxyethylene sorbitan. The fatty acid composition is primarily oleic acid, but other fatty acids, such as palmitic or linoleic acid, may be included [18]. Zhan et al. reported that Tween 80 is one of the stimulatory agents for the submerged culture of mushroom (*P. tuber-regium*) mycelia [16]. With the addition of Tween 80 into the medium, the amount of total fatty acids in the mycelial lipids of *P. tuber-regium* was significantly enhanced; especially, the amount of oleic acid was significantly increased. Therefore, they suggested that the oleic acid contained in Tween 80 could be incorporated into the mycelial cell membrane of *P. tuber-regium*, increasing its fatty acid (oleic acid) composition and the cell membrane permeability. Xu et al. also reported that oleic acid and Tween 80 had favorable effects on mycelia and exopolysaccharide production in submerged culture of *Inonotus obliquus*. Tween 80 could be hydrolyzed by microbial enzymes, such as lipase, to release oleic acid [19]. The effect of Tween 80 might be partially attributed to its convert to oleic acid. Oleic acid was proved to increase the amount of mycelia and exopolysaccharides [20]. However, with the addition of 4% (w/v) of Tween 80 into the medium, the glucan content in the DCW decreased. This result indicated that more than 4% (w/v) of Tween 80 addition could increase the DCW, but might have no effect on β -glucan production. While, no effect of shortening the cultivation period (at the end of glucose consumption) was observed over the entire Tween series. With Tween 20 at concentrations of 2, 3, and 4% (w/v), marked inhibition of cell growth and no consumption of glucose were observed after 4 days of cultivation. Therefore, the cells were recovered from the medium in the middle of cultivation, and no β -glucan was observed in the mycelia. Similar phenomena were observed in submerged cultures of *Grifola frondosa* when Tween 20 was added to the medium (0.1–1.0% (w/v)) [11]. It was speculated that lower carbon chain lengths (12C side chain) of surfactants with high concentrations might damage the cell membrane or interact with other bio-compounds in the cell, resulting in low cell growth.

3.2 Effects of agar on cell growth and the production of β -glucan

Pellet morphology and size play a major role in metabolite production [21]. The pelletization of mycelia depends on culture conditions and is thought to be strain-specific [12]. Generally, autolysis of cells occurs in large pellets due to limitations oxygen and nutrients [22, 23]. Zmak et al. also

Table 1 Effects of Tween series surfactants on *Hericium erinaceus* cell growth and β -glucan production

Surfactant (% (w/v))	Cultivation period (day)	DCW ^a (g/L)	DCW productivity (g/L /day)	DCW yield (%) ^b	Relative ratio of DCW to control	β -Glucan (g/L) ^c	β -Glucan productivity (g/L/day)	β -Glucan yield (%) ^d	Glucan content(%) ^e	Relative ratio of glucan to control
Without (control)	8	3.33	0.42	16.1	-	0.65	0.081	3.1	19.52	-
Tween 20	0.5 10	3.48	0.35	1.05	16.8	0.74	0.074	3.6	21.26	1.14
	1.0 9	3.64	0.40	1.09	17.6	0.88	0.098	4.3	24.18	1.35
	2.0 14	0.74	0.053	0.22	3.6	n.d.	n.d.	n.d.	n.d.	n.d.
	3.0 12	1.01	0.084	0.30	4.9	n.d.	n.d.	n.d.	n.d.	n.d.
	4.0 18	1.78	0.098	0.53	8.6	n.d.	n.d.	n.d.	n.d.	n.d.
Tween 40	0.5 7	3.93	0.56	1.18	19.0	0.63	0.090	3.0	16.03	0.97
	1.0 7	4.90	0.70	1.47	23.7	0.77	0.110	3.7	15.71	1.18
	2.0 8	5.31	0.66	1.59	25.6	0.56	0.070	2.7	10.54	0.86
	3.0 10	5.24	0.52	1.57	25.3	0.82	0.082	4.0	15.65	1.26
	4.0 9	6.22	0.69	1.86	30.0	0.75	0.083	3.6	12.05	1.15
Tween 60	0.5 7	4.63	0.66	1.39	22.4	0.66	0.094	3.2	14.25	1.01
	1.0 8	4.04	0.51	1.21	19.5	0.66	0.083	3.2	16.33	1.01
	2.0 8	5.16	0.65	1.55	24.9	0.49	0.061	2.4	9.49	0.75
	3.0 9	5.48	0.61	1.64	26.5	0.80	0.089	3.9	14.60	1.23
	4.0 9	6.16	0.68	1.85	29.7	0.92	0.102	4.4	14.93	1.41
Tween 80	0.5 7	4.17	0.60	1.25	20.1	0.64	0.091	3.1	15.35	0.98
	1.0 7	3.87	0.55	1.16	18.7	0.71	0.101	3.4	18.35	1.09
	2.0 6	4.48	0.75	1.34	21.7	0.74	0.123	3.6	16.52	1.14
	3.0 8	6.06	0.76	1.82	29.3	1.21	0.151	5.8	20.00	1.86
	4.0 8	7.27	0.91	2.18	35.1	1.22	0.153	5.9	16.78	1.71

^a DCW, dry cell weight

^b DCW yield (%), [produced concentration of DCW (g/L) / glucose consumption (g/L)] \times 100

^c β -Glucan (g/L), produced amount of β -glucan (g) in the liquid medium (l)

^d β -Glucan yield (%), [produced concentration of β -glucan (g/L) / glucose consumption (g/L)] \times 100

^e Glucan content (%), [β -glucan (g/L) / DCW (g/L)] \times 100

found that autolysis occurs in the pellet center in all pellet sizes (6.1 mm, 11 mm, and 16 mm) of *Phanerochaete chrysosporium* after 150 h of cultivation, except for the smallest pellets (2.0 mm) formed in Erlenmeyer flasks [24]. Therefore, pellet miniaturization of less than 2 mm in size was investigated by adding agar to the medium to increase the viscosity of the medium. Yang et al. [25] investigated the effect of various polymer additives in the culture of *G. lucidum* on mycelial growth. With agar (0.4% (w/v)) addition, the viscosity of the medium increased and the pellets were minimized by reducing mycelium aggregation. In this study, 0.05, 0.1, 0.2, and 0.3% (w/v) agar concentrations were studied. Figure 2 shows the mycelial micromorphology of *H. erinaceus* in the medium with and without agar at the end of culture. An increase in agar concentrations resulted in a significant decrease in the pellet size. The influences of agar concentration on the DCW, β -glucan, and pellet size

(diameter) of *H. erinaceus* in the cultures are summarized in Table 2. The pellet diameters varied from 4.3 mm without agar to 1.5 mm with 0.3% (w/v) of agar. DCW and β -glucan production with 0.3% (w/v) agar were almost 1.3-fold higher than those of the control (without agar). Generally, as the size of the pellet increases, the diffusion of nutrients, particularly oxygen, into the center of the pellets becomes limited [25]. Pellet morphology and pellet diameter affect the amounts of secondary metabolites, and Supramani et al. reported that the morphology of *Ganoderma pfeifferi* mycelium was affected by medium pH and medium agitation speed. Pellets with larger diameter were associated with a higher DCW, whereas small dispersed pellets were associated with high exopolysaccharide, for example, β -glucan production [21]. Based on microscopic observations, it can be seen that there were many crystal particles (agar) in the medium with agar (data not shown), and the mycelium tips adhered to the crystal

Table 2 Effects of agar on *Hericum erinaceus* cell growth, the production of β -glucan, and pellet diameter

	Without agar (control)	With agar (% (w/v))			
		0.05	0.1	0.2	0.3
Cultivation period (day)	8	8	7	7	6
Medium viscosity (mPa s)	3.3	4.6	9.6	27.7	48.7
Pellet diameter (mm)	4.34±0.9	3.84±0.5	3.08±0.5	2.42±0.5	1.5±0.4
DCW (g/L)	3.33	3.7	3.8	3.7	4.3
β -Glucan (g/L)	0.65±0.01	0.70±0.01	0.76±0.03	0.73±0.06	0.86±0.05

DCW, dry cell weight

particles. The presence of crystal particles might also prevent the formation of mycelium aggregates [25]. In the study, as 1.5 mm of pellet size (less than 2 mm) was attained at 0.3% (w/v) of agar concentration, higher concentrations than 0.3% (w/v) were not investigated.

3.3 Comparison of culture efficiency using Erlenmeyer flasks with and without baffles

To investigate the influence of incubators on cell growth and metabolite production, we evaluated the submerged culture of *H. erinaceus* in normal and baffled Erlenmeyer flasks. DCW production, β -glucan production, and pellet size (diameter) of *H. erinaceus* in the cultures using normal and baffled Erlenmeyer flasks are summarized in Table 3. DCW production and β -glucan production in baffled Erlenmeyer flasks

were almost 1.2-fold higher than those in normal Erlenmeyer flasks. Moreover, the pellet size in a baffled Erlenmeyer flask (1.6 mm) was smaller than that in a normal Erlenmeyer flask (4.5 mm). Bermek et al. reported the morphology and ligninolytic enzyme production in the wood-degrading fungus *Trichophyton rubrum* in submerged cultures using normal and baffled Erlenmeyer flasks. In baffled Erlenmeyer flasks, fungal morphologies were notably different, the pellet size diameter was small (approximately 2–4 mm), and free mycelia were observed. In contrast, large ball formation was observed in normal Erlenmeyer flasks. It was clear that limited oxygen conditions were responsible for the formation of large pellets. Baffled Erlenmeyer flasks have a unique design, creating strong convection stirring, and thus allowing better mixing and air uptake into the medium [26]. Recently, Ángeles-Argáiz et al. also reported that mycelia of

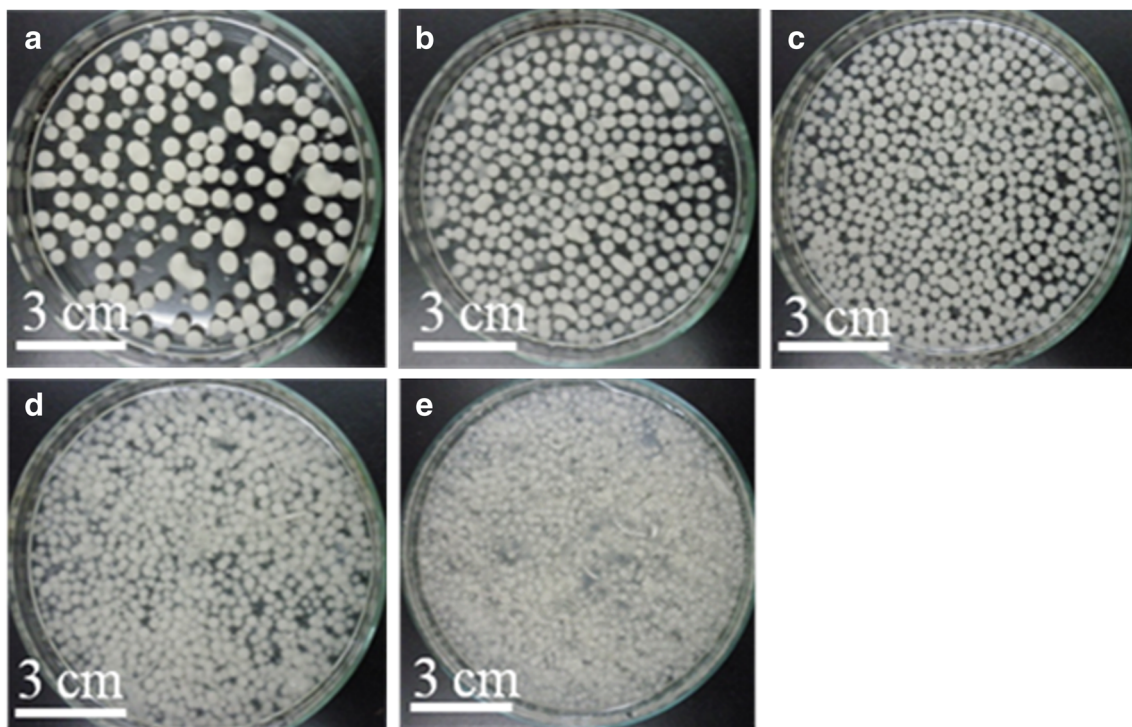


Fig. 2 Micromorphologies of *Hericum erinaceus* mycelia in submerged culture without agar (a) and with agar at concentrations 0.05% (w/v) (b), 0.1% (w/v) (c), 0.2% (w/v) (d), and 0.3% (w/v) (e)

Table 3 Comparison of cell growth, β -glucan production, and pellet diameter in submerged cultures of *Hericium erinaceus* between normal and baffled Erlenmeyer flasks

	Cultivation period (day)	DCW ^a (g/L)	DCW productivity (g/L/day)	DCW yield (%) ^b	β -Glucan (g/L) ^c	β -Glucan productivity (g/L/day)	β -Glucan yield (%) ^d	Pellet diameter (mm)
Normal Erlenmeyer flask	8	3.33	0.42	16.1	0.65	0.081	3.1	4.34
Baffled Erlenmeyer flask	6	4.76	0.79	24.7	0.89	0.15	4.6	1.61

^aDCW, dry cell weight

^bDCW yield (%), [produced concentration of DCW (g/L) / glucose consumption (g/L)] \times 100

^c β -Glucan (g/L), produced amount of β -glucan (g) in the liquid medium (L)

^d β -Glucan yield (%), [produced concentration of β -glucan (g/L) / glucose consumption (g/L)] \times 100

^eGlucan content (%), [β -glucan (g/L) / DCW (g/L)] \times 100

the ectomycorrhizal mushroom *Laccaria trichodermophora* in submerged culture in a baffled Erlenmeyer flask were fragmented and the pellets were compact with growing hyphae throughout their surface. In a normal Erlenmeyer flask, the mycelia formed larger and fewer filamentary pellets [27]. Larger pellets have deficiencies in oxygen and nutrient diffusion, which leads to low growth [28].

3.4 Effects of agar or Tween 80 on cell growth and the production of β -glucan in baffled Erlenmeyer flask

Figure 3 shows *H. erinaceus* DCW and β -glucan production in a baffled Erlenmeyer flask with and without 3.0% (w/v) of Tween 80 or 0.3% (w/v) agar. The highest β -glucan concentration, 1.89 g/L, was attained when 0.3% (w/v) agar was added to the medium with a shorter cultivation period (5

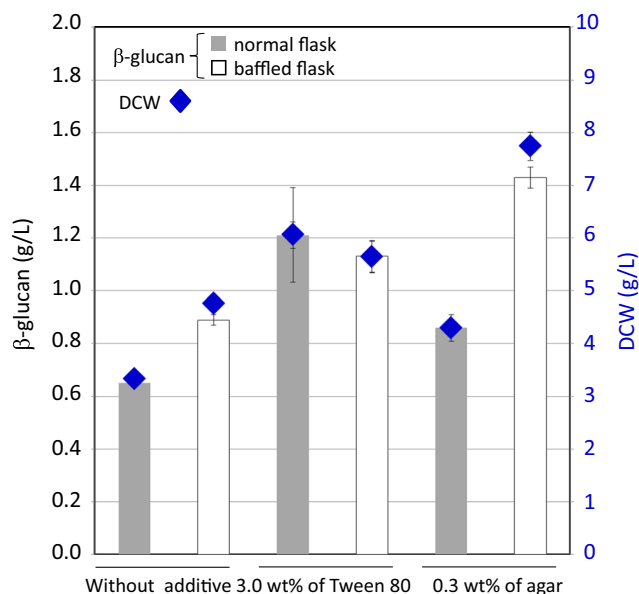


Fig. 3 Influence of Tween 80 (3.0% (w/v)) and agar (0.3% (w/v)) addition on *Hericium erinaceus* mycelial growth (DCW) and β -glucan production in normal and baffled Erlenmeyer flasks

days). However, no effect was observed when 3.0% (w/v) of Tween 80 was added to the medium. This is because the viscosity remained low (3.0 mPa s), which is similar to that without agar (3.3 mPa s), and there might have been limitations in oxygen uptake into the pellets [29].

4 Conclusion

In summary, the addition of Tween 80 or agar into the medium for *H. erinaceus* submerged cultivation could significantly increase cell growth and β -glucan production. Additionally, baffled Erlenmeyer flasks were found to be efficient for β -glucan production. In the future, a scale-up experiment is required. It will also be necessary to carry out a submerged cultivation experiment using tank batch culture and fed-batch continuous culture.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13399-021-01342-y>.

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