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Original Article

Mycelium and polysaccharide production of *Agaricus blazei* Murrill by submerged fermentation

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Background and Purpose: Over the last decade, *Agaricus blazei* Murrill has been studied and developed as a novel functional food in Japan, Korea, China, and Taiwan. Due to the low yields, the fruiting bodies of *A. blazei* Murrill are relatively expensive, and a cheap and stable source of *A. blazei* Murrill mycelium for commercial purposes is highly desirable. Culture media and conditions were investigated with a view to reducing the cost and improving the mycelium and polysaccharide production of *A. blazei* Murrill by submerged fermentation.

Methods: Thirty six isolates of *A. blazei* Murrill were isolated from 22 fruiting bodies produced in Taiwan, and 16 of them could be successfully cultivated on mannitol-egg yolk-polymyxin medium. The isolates were identified by species-specific polymerase chain reaction (PCR) and optimized for the culture media and conditions by submerged fermentation for mycelium and polysaccharide production. Some properties of polysaccharide extract were also investigated.

Results: All of the PCR products with species-specific primers showed high identity and matched the internal transcribed spacer 1 sequences of *A. blazei* Murrill. The phylogenic tree of *A. blazei* Murrill isolates generated from random amplified polymorphic DNAs arranged all samples into 3 groups and 2 independent cases. The optimal culture media of mycelium production in submerged fermentation were 5% malt extract, 0.1% yeast extract, and 0.5% peptone at pH 6.0, while the optimal culture conditions were 200 mL medium in 500 mL Hinton flask, shaking at 90 rpm for 3 days and then shifting to 105 rpm for 5 days at 27°C. Each liter of *A. blazei* Murrill M72 yielded 10.83 ± 0.24 g dried mycelia weight and each liter of *A. blazei* Murrill M152 produced 0.251 ± 0.004 g crude polysaccharide (3.03 ± 0.05% of dried mycelia weight). Crude polysaccharide of *A. blazei* Murrill M162 contained 82.27-99.14% of total sugar and less than 1.63% of protein; it had 4 major molecular weight components (274.1, 32.7, 7.5, and 2.1 kDa, respectively), with the 2.1 kDa portion possibly a beta-(1,3)-glucan.

Conclusions: These results show that selection of media and conditions can be employed in order to improve the mycelium and polysaccharide production of *A. blazei* Murrill M72 or M152 by submerged fermentation. Mycelia and polysaccharide production of *A. blazei* Murrill with submerged fermentation is potentially feasible.

Key words: *Agaricus*, fermentation, mycelium, polysaccharides, random amplified polymorphic DNA technique

Introduction

Edible and medicinal mushrooms are good materials for functional foods, having regulatory functions related to nutrition, taste and physiology. Many mushrooms have attracted the attention of the biotechnological industry as food supplements and materials for developing medicines [1]. *Agaricus blazei* Murrill is an edible mushroom originally used as a folk remedy and food in Brazil. It was also known for its potent anti-tumor activity [2-4]. Over the last decade, *A. blazei* Murrill has been studied and developed as a novel functional food in Japan, Korea, China and Taiwan [1]. The fruiting bodies of *A. blazei* Murrill are still quite expensive to obtain and thus a relatively cheap and stable source of *A. blazei* Murrill mycelium for commercial purposes is sought.

To reduce the cost and improve the productivity, numerous researchers have studied production of the mycelium and polysaccharide by submerged fermentation of *A. blazei* Murrill [5-9]. In this study, *A. blazei* Murrill was isolated from the fruiting bodies, identified by species-specific polymerase chain reaction

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(PCR) and optimized for the cultural media and conditions by submerged fermentation for mycelium and polysaccharide production. Some properties of the polysaccharide extract were also investigated.

Methods

Tested strains

A. blazei Murrill BCRC 36814 was purchased from the Bioresource Collection and Research Center, Hsinchu, Taiwan. *A. blazei* Murrill A321 was kindly provided by Professor R. S. Hseu, National Taiwan University, Taiwan. Other strains were isolated from fruiting bodies cultivated in Nantou County, Taiwan. Dried fruiting bodies were purchased from the markets in Brazil and Taiwan; fresh fruiting bodies were also obtained from Nantou County, Taiwan.

Culture media and conditions

Mannitol-egg yolk-polymyxin (MYP) medium containing 4% malt extract, 0.1% yeast extract, and 0.5% peptone at pH 6.0 was used for strain isolation and basal medium in submerged fermentation. The culture was incubated at 27°C with shaking at 90 rpm and 105 rpm.

Species-specific PCR identification

Dried fruiting bodies purchased from the markets in Taiwan and Brazil had significant morphological differences. DNA extraction and species-specific PCR of *A. blazei* Murrill were performed as described by Huang [10]. Dried fruiting bodies or mycelia were ground in liquid nitrogen, and 40-60 mg of powder was used for DNA extraction. Total DNA of *A. blazei* Murrill was amplified with species-specific primers NS7 (GAG GCA ATA ACA GGT TGC TGA TGC) and Bla (CAC ATC AAG GAC AGC AAA GC) by GeneAmp PCR system 2400 (Perkin-Elmer, Cetus, USA). Sequencing of PCR products was carried out by the Protech Technology Enterprise Co. Ltd., Taiwan. Multiple sequences alignment was performed by the software ClustalX 1.83 for Windows and Macintosh.

Morphological observation

Acridine orange is a fluorochrome used to stain nucleic acid, including DNA and RNA. When excited with blue light (490 nm), double-strand DNA was green (530 nm) and single-strand RNA was orange (640 nm). It has been widely used to study cell physiology [11,12], diagnostic fluorescent staining [13,14], and the viability of bacteria [15,16].

Mycelia of *A. blazei* Murrill were inoculated on MYP agar plate, incubated at 27°C for 6 days, cultivated with the slide culture method [17], and stained with 8 mg/mL acridine orange (Sigma-Aldrich, St. Louis, USA) in 0.1 M glycine-sodium hydroxide buffer at pH 9.5. Aniline blue, also a fluorochrome, is used to stain beta (β)-(1,3)-D-glucan, the light intensity having a linear correlation with the concentration of β-(1,3)-D-glucan [18]. The slide culture was stained with aniline blue and Calcofluor White M2R (Sigma-Aldrich, St. Louis, USA), observed under an Olympus BH-2 microscope with fluorescent microscopic accessories (BP495+IB, BA505), and photographed with an Olympus camera (C35AD-4) with Fuji X 400 film.

Random amplified polymorphic DNA technique

DNA extraction and random amplified polymorphic DNA (RAPD) PCR of *A. blazei* Murrill were performed as described by Huang [10]. Total DNA of *A. blazei* Murrill was amplified with arbitrary primers R4 (GGA GGG TGT T), OPB15 (CCA CAG CAG T) and OPB18 (CAA ACG GGT G). Gel electrophoresis of RAPDs was recorded by Polaroid film and all visible 43 bands were selected as phylogen analysis markers. Markers were converted as binary data matrix into Statistical Package for the Social Sciences (SPSS) for Windows (Version 12, SPSS Inc., Chicago, IL, USA) according to presence (1) or absence (0) of band. Heirarchical cluster analysis of RAPD data matrix was carried out with binary pattern difference and between-group linkage distance measurement.

Mycelia production

A. blazei Murrill was cultivated on MYP agar at 27°C for 6 days, blended with 10 mL sterilized distilled water using a Waring Blender (Dynamic Corporation, New Hartford, USA), transferred into 250 mL flask with 125 mL MYP medium as seed culture, and shaken at 90 rpm for 4 days. Seed culture was homogenized by blender and mixed with 125 mL sterilized distilled water. Five percent of mycelia suspension was transferred into a 500 mL Hinton flask as inoculum and cultivated at 27°C for 10 days. Mycelium of *A. blazei* Murrill was collected with a fine stainless steel sieve, washed with sterilized distilled water and lyophilized to constant weight.

Extraction and analysis of polysaccharide

Lyophilized mycelia or dried fruiting bodies were blended with 10 or 30 volumes of distilled water and

refluxed at 100°C for 24 h. Supernatant was filtered with Whatman no. 1 filter paper (Whatman Paper Ltd., Maidstone, England), 5-sulfosalicylic acid (SSA; Sigma-Aldrich, St. Louis, USA) was added to a final concentration of 2%, and the preparation cooled to 4°C for 30 min to precipitate proteins. The sample was consequently centrifuged at 3500 rpm and at 4°C for 1 h and then filtered by Whatman no. 1 and no. 42, and 0.45 μm membrane filters. Filtrate was poured into 3 volumes of 95% ethanol, incubated at 4°C overnight, and then centrifuged at 3500 rpm and at 4°C for 15 min. The pellet was redissolved into hot distilled water, dialyzed (molecular weight cut-off, 6000-8000; Spectrum Laboratories Inc., Rancho Dominguez, USA) overnight against distilled water and weighed after lyophilization [19]. Total carbohydrate content of crude polysaccharide extracts was determined by the phenol-sulfuric acid method [20], and protein content was measured by the Bradford method [21].

High-performance gel permeation chromatography analysis

High-performance gel permeation chromatography (HPGPC) analysis was carried out on a Shimadzu LC-9A high performance liquid chromatograph (Shimadzu Co., Japan). An injector Reodyne 7125 equipped with 20 μL sample loop, a PW_{XL} guard column, a G500 PW_{XL} gel filtration column (Tosoh Co., Japan), and a Shimadzu differential refractive index detector (RID)-10A were connected in series. The mobile phase was 0.01 N aqueous sodium hydroxide at a flow rate of 0.7 mL/min with detector operated at 40°C. Samples and calibration polyethylene glycols (molecular weight range, 2000 to 900,000) at 1 mg/mL were injected. The output signal from the RID-10A was recorded and processed by a Shimadzu CR-6A integrator.

Statistical analysis

Experiments were carried out in triplicate. Data were subjected to analysis of the coefficient of variance and the Duncan's multiple range test ($p \leq 0.05$) using the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA) [22].

Results and Discussion

Isolation, identification and morphology of *A. blazei* Murrill

The growth of mushroom depends on environmental conditions (such as light intensity, temperature, humidity and aeration) and culture media (such as pH, carbon source, nitrogen source and growth factors). Thirty six isolates were obtained from 22 fruiting bodies of *A. blazei* Murrill produced in Nantou County, Taiwan. Isolates M21, M22 and M23 were isolated from the same fruiting bodies. Isolates were cultivated on MYP agar plate at 27°C for 6 days; all the colony morphologies were white rhizomorph, uneven edge and cottony aerial hyphae. All isolated strains showed some differences in colony morphologies and growth rates, including isolates M21, M22 and M23, which were isolated from the same fruiting bodies. *A. blazei* Murrill A321 has shown some unique morphological characteristics, e.g. unusually slow growth rate and fragmentated hyphae. Both morphology and nuclei of mycelium were clearly observed by staining mycelia with fluorescent dye acridine orange. Mycelia of isolated strains were multi-nucleated with no clamp connection, with green nuclei and orange mycelia. Each cell contained 0 to 8 nuclei, with most having 3 or 4 nuclei. Some had fused mycelial cells with rearranged nucleus (Fig. 1). Morphological characteristics were similar to those observed by Huang [10].

Cell fusion and nuclear rearrangement may play important roles in the genetics of *A. blazei* Murrill, as with other basidiomycetes. When the mycelium was stained with aniline blue, most of the fluorescent materials were in the mycelia tip and the septa area and no fluorescence was found in the middle part; the mycelia stained with Calcofluor White had uniform color among the mycelium. This phenomenon showed that β-(1,3)-D-glucan was concentrated in the mycelia tip and septa area. Kippert and Lloyd [23] also found fluorescent material to be concentrated in the cell septa when *Schizosaccharomyces pombe* was stained

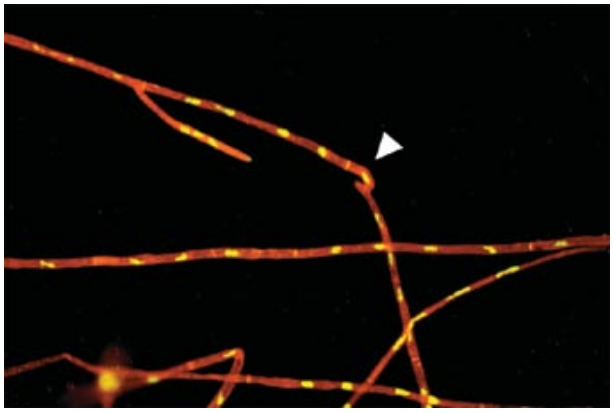

Fig. 1. *Agaricus blazei* Murrill M21 mycelium stained with acridine orange. The arrowhead indicates the fusant cell.

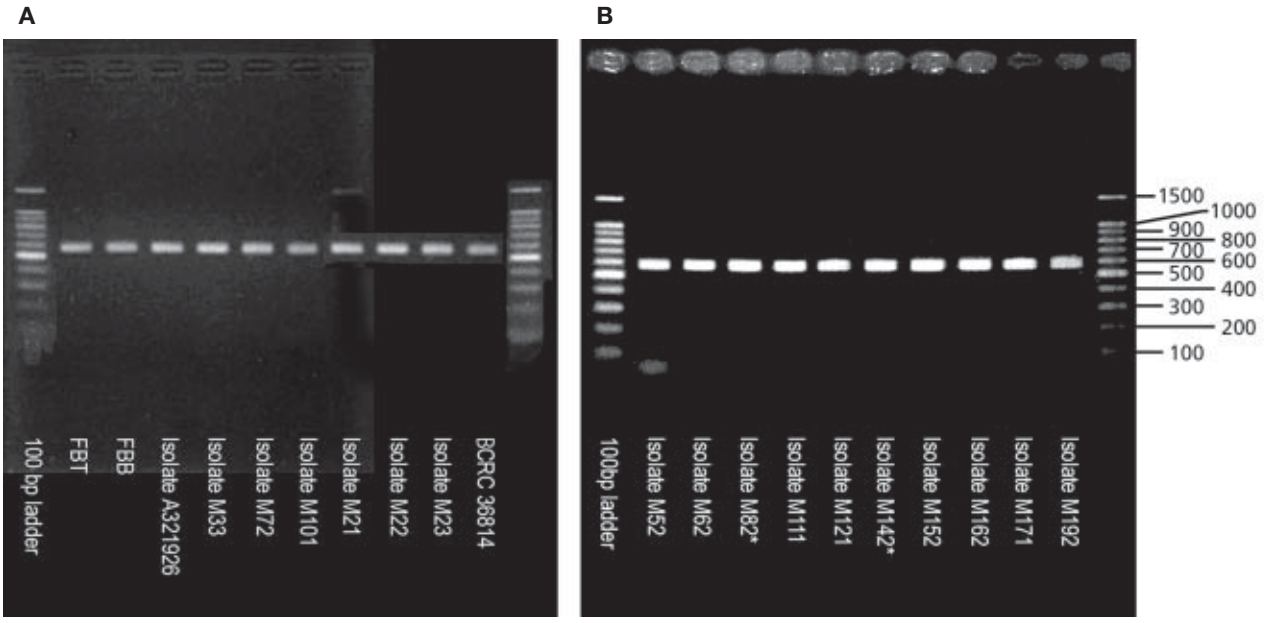

Fig. 2. Gel electrophoresis of species-specific polymerase chain reaction products. FBT = fruiting bodies from Taiwan; FBB = fruiting bodies from Brazil. * = discontinued strain.

with aniline blue. This might be due to the presence of β-(1,3)-D-glucan in the areas of cell growth or cell repair [24,25].

PCR amplification of the total DNA of *A. blazei* Murrill fruiting bodies and the isolated strains' mycelia with species-specific PCR primers had a single 520 bp product as expected (Fig. 2). Sequences of all PCR products showed high identity (96.58-100.00%) and matched the internal transcribed spacer 1 (ITS1) sequences of *A. blazei* Murrill (EMBL protein-accession code: AF081463). All fruiting bodies and the isolated strains were identified as *A. blazei* Murrill. The result suggested that the morphological differences among dried *A. blazei* Murrill fruiting bodies are due to differences of cultivation method, environmental condition, harvesting time, and processing method. Therefore, the morphological identification of *A. blazei* Murrill is not always possible; a reliable identification method of *A. blazei* Murrill is necessary.

Morphological differences between *A. blazei* Murrill and *A. blazei* Heinem at different cultivation areas also revealed that morphological identification of *A. blazei* Murrill was not always reliable [26]. Compared with the morphological method, a genetic approach such as species-specific PCR identification is more reliable in identification of *A. blazei*.

The phylogenic tree generated from RAPDs arranged samples into 3 groups and into 2 independent cases. *A. blazei* Murrill M52, M72 and M101 were in group 1 and the RAPDs of *A. blazei* Murrill M52 and M101 were found to be identical. *A. blazei* Murrill M21, M22, M23, M33 and M52 were in group 2. *A. blazei* Murrill M62, M111, M162, M171 and M192 were in group 3. *A. blazei* Murrill M152 and *A. blazei* Murrill A321 were independent from these groups (Fig. 3). *A. blazei* Murrill M152 was a unique strain with high polysaccharide yield. As mentioned above, *A. blazei* Murrill A321 was different from all other strains in morphology and matched its phylogenic tree position. *A. blazei* Murrill M21, M22 and M23 in group 2 were isolated from the same fruiting bodies and revealed the relevance of the phylogenic tree.

Effect of medium volume and shaking speed on mycelia growth

Shearing force and mass transfer are important factors of mycelia growth in submerged fermentation [19]. Medium volume and shaking speed were the major factors determining shearing force and mass transfer in shaking flask cultivation. The effects of medium volume, shaking speed and 2-step shaking speed on mycelia growth of *A. blazei* Murill M21 are shown in Fig. 4. The optimal medium volume was 200 mL MYP medium in 500 mL Hinton flask at shaking speed 90 rpm. The optimal medium volume was varied with the shaking speed. The balance between shearing force and mass transfer is very important in mycelia growth. Mycelia growth was almost inhibited at shaking speed

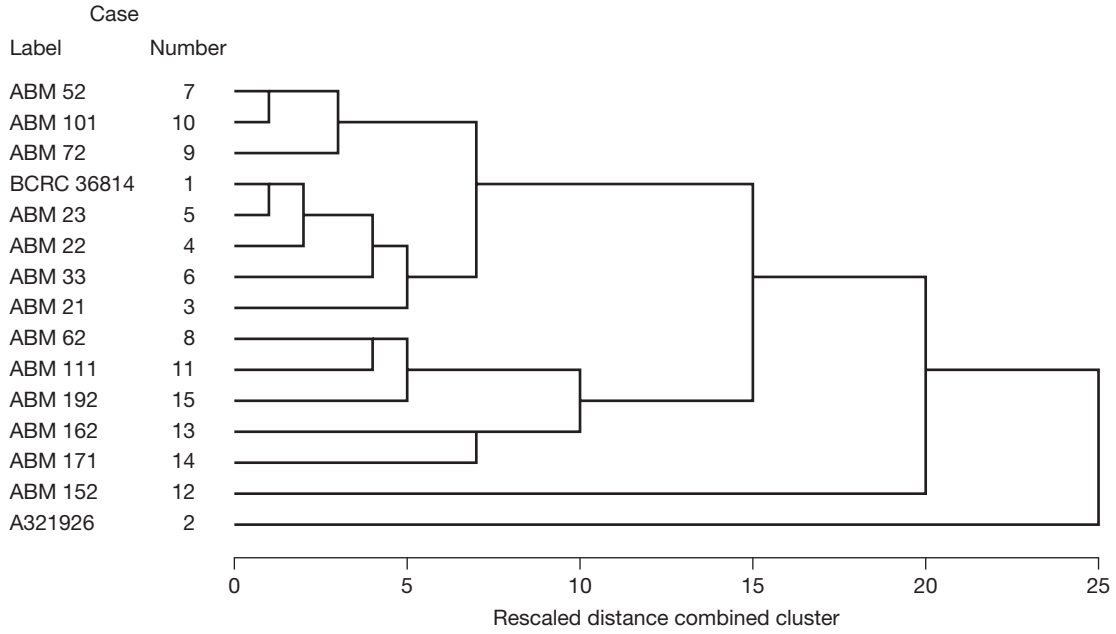

Fig. 3. Phylogenic tree generated from random amplified polymorphic DNAs. Dendrogram using average linkage between groups.

115 rpm; this phenomenon might be due to the high shearing force inhibiting mycelia growth. The lag phase of *A. blazei* Murrill M21 in submerged cultivation was 2 days; too high (>105 rpm) or too low (<65 rpm) a shaking speed prolonged the lag phase (Fig. 5). Log phase of *A. blazei* Murrill M21 was from day 2 to 6 at shaking speed 90 rpm, and mycelia growth decreased after day 8. Although the maximal mycelia growth rate at shaking speed 105 rpm was lower than that at 90 rpm, mycelia growth increased gradually after day 8.

To investigate the effect of shaking speed on mycelia growth, 2-step shaking speeds were used. *A. blazei* Murrill M21 was cultivated at 90 rpm for 3 or 6 days and then shifted to 105 rpm. Raising shaking speed on day 3 significantly increased the growth rate, but the mycelium yield remained same. The increasing growth rate might be due to the improvement of mass transfer; however, high growth rate resulted in a loose of mycelium pellet. Raising the shaking speed at day 6 had less effect on the mycelium yield, which remained constant until day 10.

Effect of initial pH and temperature on mycelia growth

The effects of incubation temperature (25, 27, 29, 31, and 33°C) and initial pH (5.02, 5.25, 5.51, 5.81, 6.02, and 6.09) on mycelia growth were investigated. The highest mycelium yield was at initial pH 6.02 and incubation temperature 27°C. *A. blazei* Murrill in submerged cultivation had optimal initial pH 5 to 6 [5] and *Agaricus* spp. had optimal initial pH 4 to 7 [27,28] and optimal incubation temperature was between 15 and 35°C for *Agaricus* spp. [28,29] and 29°C for *A. blazei* Murrill [8]. The optimal initial pH and incubation temperature depended on species and culture media, and both were very important determinants of the mycelia yield of mushrooms [29,30].

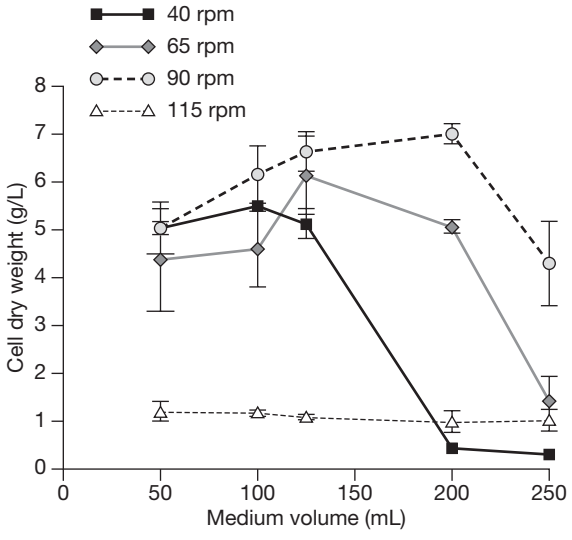

Fig. 4. Effect of medium volumes and shaking speeds on dried mycelia weight of *Agaricus blazei* Murrill M21.

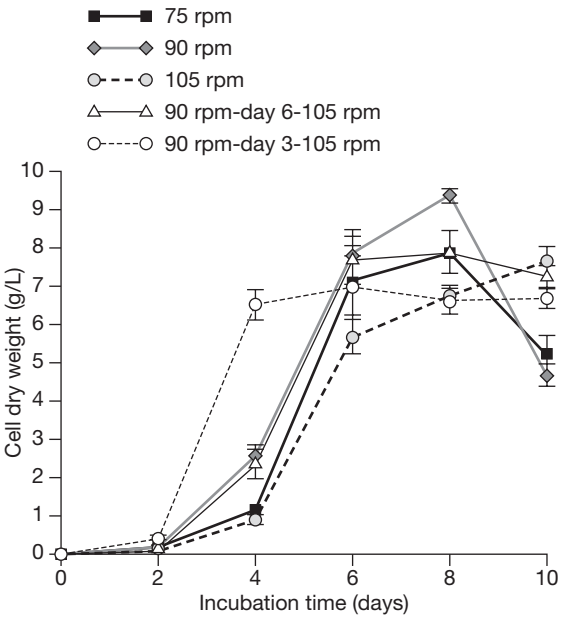

Fig. 5. Effect of shaking speeds and 2-step shaking on the dried mycelia weight of *Agaricus blazei* Murrill M21.

Effect of carbon source on mycelia growth

The effect of carbon sources on mycelia growth was studied by using glucose, sucrose, soluble starch, maltose, malt extract, and arabinose as supplementation in modified Barth's solution medium at 90 rpm and 27°C for 8 days, and the results are shown in Tables 1 and 2. Malt extract was the best carbon source. When the equivalent amount of carbohydrate as maltose, glucose, sucrose and soluble starch was used as carbon source instead of malt extract (carbohydrate content is between 70 and 80%) in de Man-Rogosa-Sharpe (MRS) medium, mycelia growth decreased.

Supplementation of arabinose to glucose as carbon source had a positive effect on mycelia growth, whereas sucrose and soluble starch as carbon source had high mycelia growth with 2 g/L of arabinose supplementation. In contrast, arabinose supplementation had an inhibitory effect on mycelia growth when maltose was used as the carbon source. Dried mycelia weight production with different carbon sources varied significantly. Yang et al [31] reported that the mixture of 3% corn starch and 2% sucrose was the best carbon source for *A. blazei* Murrill mycelia growth among potato starch, corn starch, corn mill, corn syrup, soybean cake flour, soybean flour, wheat flour, sucrose, and glucose. However, Yoneyama et al [6,7] indicated that arabinose as carbon source gave the highest dried mycelia weight of *A. blazei* Murrill in submerged cultivation; ribose, sucrose and trehalose were the next; and cellobiose and xylose were the lowest. In this study, it was found that 50 g/L of malt extract in MRS medium produced the highest mycelia weight, followed by 40 g/L of malt extract and 25 g/L of glucose, with 5 g/L of arabinose being the least efffective.

Although the major component of malt extract is maltose (around 70-80%), the mycelia growth of *A. blazei* Murrill M21 with the equivalent amount of maltose as carbon source was lower than that with malt extract. Therefore, some growth factors in malt extract might stimulate the mycelia growth of *A. blazei* Murrill M21. In addition, it was found that the mycelia growth increased with increasing malt extract from 40 g/L to 50 g/L.

Effect of nitrogen source on mycelia growth

The effect of nitrogen source on mycelia growth was determined by use of ammonium sulfate ($[NH_4]_2SO_4$), potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3), urea, and yeast extract to replace the peptone in MRS medium at 90 rpm and 27°C for 8 days. The results are summarized in Table 3. Different combinations of malt extract, peptone, and yeast extract were used to study the optimal concentration of organic nitrogen sources for mycelium yield. Organic nitrogen sources were better than inorganic nitrogen sources, except urea. Yeast extract was better than peptone, while NH_4NO_3 was the best inorganic source for mycelia growth of *A. blazei* Murrill M21, followed by KNO_3, $(NH_4)_2SO_4$, and urea. There was a significant difference in mycelia growth between NH_4NO_3 and $(NH_4)_2SO_4$.

Table 1. Effect of composition of de Man-Rogosa-Sharpe (MRS) media on mycelia growth of *Agaricus blazei* Murrill M21 (g/L)

Component	MRS	MRS-1	MRS-2	MRS-3	MRS-4	MRS-5	MRS-6	MRS-7
Malt extract	40	40	40	40	50	50	50	50
Peptone	5	5	3	3	5	5	8	2
Yeast extract	1	2	1	2	1	2	1	8
Dried mycelia weight	6.25 ± 0.27^{a}	$9.00 \pm 0.62^{d,e}$	7.33 ± 0.32^{b}	$7.73 \pm 0.26^{b,c}$	10.15 ± 0.48^{f}	$9.12 \pm 1.13^{d,e,f}$	$9.88 \pm 0.48^{e,f}$	$8.48 \pm 0.46^{c,d}$

$^{a–f}$Mean ± standard deviation (n = 3) in the same row not sharing the same superscripts were significantly different at 5% level according to Duncan's multiple range test.

Table 2. Effect of carbon source on mycelia growth of *Agaricus blazei* Murrill M21 (g/L)

Component	MRS	G	GA	GA-1	S	SA	SA-1	M	MA	MA-1	Ss	SsA	SsA-1
Malt extract	40	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	-	30	30	25	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	30	30	25	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	30	30	25			
Arabinose	-	-	2	5	-	2	5	-	2	5	-	2	5
Soluble starch	-	-	-	-	-	-	-	-	-	-	30	30	25
Peptone	5	5	5	5	5	5	5	5	5	5	5	5	5
Yeast extract	1	1	1	1	1	1	1	1	1	1	1	1	1
Dried mycelia weight	6.25 ± 0.27^{i}	4.81 ± 0.57^{f}	5.18 ± 0.27^{f,g}	5.77 ± 0.15^{h,i}	2.67 ± 0.01^{a,b}	3.67 ± 0.02^{d}	3.24 ± 0.01^{c}	5.42 ± 0.05^{g,h}	4.93 ± 0.03^{f}	4.37 ± 0.01^{e}	4.03 ± 05.4^{d,e}	3.03 ± 0.08^{b,c}	2.31 ± 0.18^{a}

Abbreviations: MRS = de Man-Rogosa-Sharpe medium; G = glucose (30 g/L); GA = glucose (30 g/L) + arabinose (2 g/L); GA-1 = glucose (25 g/L) + arabinose (5 g/L); S = sucrose (30 g/L); SA = sucrose (30 g/L) + arabinose (2 g/L); SA-1 = starch (25 g/L) + arabinose (5 g/L); M = maltose (30 g/L); MA = maltose (30 g/L) + arabinose (2 g/L); MA-1 = maltose (25 g/L) + arabinose (5 g/L); Ss = soluble starch (30 g/L); SsA = soluble starch (30 g/L) + arabinose (2 g/L); SsA-1 = soluble starch (25 g/L) + arabinose (5 g/L)

$^{a–i}$Mean ± standard deviation (n = 3) in the same row not sharing same superscripts were significantly different at 5% level according to Duncan's multiple range test.

In the submerged cultivation of *A. blazei* Murrill, yeast powder [31], yeast extract [6,7] and the mixture of yeast extract and peptone [8] were the best organic nitrogen sources. However, the mixture of 2 g/L yeast extract and 2 g/L peptone was the best nitrogen source for *Pleurotus tuber-regium* Fr. Singer [32] and 5 g/L yeast extract and 5 g/L peptone was the best nitrogen source for *Genoderma lucidum* [33,34]. In this study, the effect of nitrogen source on the mycelia growth of higher fungi depended on the species and cultivation conditions. Mycelia growth was high and the pellet of mycelia was loose at appropriate growth conditions and media, while the mycelia was slender and the pellet of mycelia was compact at unfavorable growth conditions (such as high or low temperature, poor media, etc.) [19]. Therefore, the mycelia growth rate and the degree of compactness of mycelia was dependent on the culture conditions and culture media.

Effect of phosphate supplementation on mycelia growth

The effect of phosphate supplementation on mycelia growth was evaluated using 0, 0.125, 0.250, 0.500 and 0.750 g/L potassium phosphate monobasic (KH_2PO_4). The results are shown in Table 4. Phosphate supplementation enhanced the mycelia yield and the effect was greatest with 0.25 g/L of KH_2PO_4. High concentration of phosphate supplementation inhibited mycelia growth. Several researchers also indicated the necessity of an appropriate amount of phosphate for microbial growth [6,7,20,33,34]. Yang et al [31] reported that 0.3% of KH_2PO_4 was the optimal concentration for the mycelia

Table 3. Effect of nitrogen source on mycelia growth of *Agaricus blazei* Murrill M21 (g/L)

Component	MRS	KN	AS	AN	U	MY	MY-1
Malt extract	40	-	-	-	-	40	40
Arabinose	-	-	2	5	-	-	-
Peptone	5	-	-	-	-	-	-
Yeast extract	1	1	1	1	1	1	6.35
Potassium nitrate	-	3.75	-	-	-	-	-
Ammonium sulfate	-	-	2.45	-	-	-	-
Ammonium nitrate	-	-	-	1.74	-	-	-
Urea	-	-	-	-	1.15	-	-
Dried mycelia weight	6.25 ± 0.27^{c}	7.12 ± 0.16^{d}	5.16 ± 0.07^{a}	7.25 ± 0.21^{d}	5.52 ± 0.17^{b}	5.29 ± 0.18^{a}	7.26 ± 0.14^{d}

Abbreviations: MRS = de Man-Rogosa-Sharpe medium; KN = potassium nitrate (3.75 g/L); AS = ammonium sulfate (2.45 g/L); AN = ammonium nitrate (1.74 g/L); U = urea (1.15 g/L); MY = malt extract (40 g/L) + yeast extract (1 g/L); MY-1 = malt extract (40 g/L) + yeast extract (6.35 g/L)

$^{a–d}$Mean ± standard deviation (n = 3) in the same row not sharing the same superscripts were significantly different at 5% level according to Duncan's multiple range test.

Table 4. Effect of phosphate content on mycelia growth of *Agaricus blazei* Murrill M21 (g/L)

Component	MRS	Po	P-1	P-2	P-3	P-4	Po	P-1	P-2	P-3	P-4
	Cultivated at 90 rpm and 27°C for 8 days						Cultivated at 90 rpm and at 27°C for 3 days then shifted to 105 rpm for another 5 days				
Malt extract	40	50	50	50	50	50	50	50	50	50	50
Peptone	5	5	5	5	5	5	5	5	5	5	5
Yeast extract	1	1	1	1	1	1	1	1	1	1	1
Potassium phosphate monobasic	-	-	0.125	0.25	0.50	0.75	-	0.125	0.25	0.50	0.75
Dried mycelia weight	6.25 ± 0.27[a]	6.45 ± 0.2[a,b]	6.57 ± 0.09[b]	7.81 ± 0.60[c]	6.60 ± 0.30[b]	5.59 ± 0.28[a]	11.38 ± 0.10[f]	10.76 ± 0.72[e]	9.95 ± 0.62[d]	9.64 ± 0.65[d]	9.72 ± 0.93[d]

Abbreviations: MRS = de Man-Rogosa-Sharpe medium; Po = potassium phosphate monobasic (0 g/L); P-1 = potassium phosphate monobasic (0.125 g/L); P-2 = potassium phosphate monobasic (0.25 g/L); P-3 = potassium phosphate monobasic (0.50 g/L); P-4 = potassium phosphate monobasic (0.75 g/L).

[a–f]Mean ± standard deviation (n = 3) in the same row not sharing the same superscripts were significantly different at 5% level according to Duncan's multiple range test.

growth of *A. blazei* Murrill in submerged cultivation. In this study, KH_2PO_4 0.25% was the optimal concentration for the mycelia growth of *A. blazei* Murrill M21, and achieving a loose mycelia pellet. In the 2-step shaking test, it was found that the mycelia yield increased when the shaking speed was shifted to 105 rpm at the third day. However, phosphate supplementation had a slight inhibitory effect on the mycelia yield. There were also significant effects of varying the amount of phosphate supplementation, and between the 1-step and 2-step shaking tests.

Effect of inoculum density on mycelia growth

Inoculum density is very important in fungal submerged cultivation and affects fungal physiology as well as growth. Too high or too low an inoculum density reduced the mycelia yield [19,31,33-35]. Since *A. blazei* Murrill has no conidiospore, the inoculum was performed by mycelia suspension with Waring blender method. Dried mycelia weight increased from 4.83 ± 0.16 g/L in 3.80 mg/L of inoculum to 7.88 ± 0.08 g/L in 11.40 mg/L of inoculum. There is a linear correlation between inoculum density and mycelia yield. However, it is very difficult to control the exact inoculum density in different batches and different tested strains. Mycelia growth was not significantly affected when the inoculum density was higher than 40 mg/L.

Effect of tested strains on mycelia growth

The growth rate of *A. blazei* Murrill M162 was slow in submerged cultivation; therefore, a further 15 *A. blazei* Murrill isolated strains were used for mycelium production with optimal culture media and conditions at 90 rpm and at 27°C for 3 days and then shifted to 105 rpm for another 5 days, and the results are shown in Table 5. It was found that *A. blazei* Murrill M72 had the highest dried mycelia weight (each liter produced 10.83 ± 0.24 g), followed by *A. blazei* Murrill M121, M21, M111, M192, M33, M101 and M171, the dried mycelia weights for which were between 9.71 ± 0.72 and 10.58 ± 0.40 g/L. There were no significant differences among these tested organisms. The growth rates of *A. blazei* Murrill M152, M62 and M22 were in the second rank, and each liter produced dried mycelia weight from 8.04 ± 0.91 to 8.28 ± 1.36 g, that was significant lower than the above tested organisms. *A. blazei* Murrill M52, BCRC 36814, M23 and A321 had low dried mycelia weight yield (4.26 ± 0.04 to 6.94 ± 0.05 g/L). Yang et al [5] reported a yield of 17.7 g/L dried mycelium weight in submerged cultivation of *A. blazei* Murrill. In this study, the mycelia yields of *A. blazei* Murrill isolates were less than those of Yang et al [5], but higher than those of Liu and Shu (7.3 g/L) [8] and Yoneyama et al (3.1 g/L) [6,7].

Crude polysaccharide yield

Crude polysaccharide yields of the mycelia of *A. blazei* Murrill isolated strains and fruiting bodies cultivated in Taiwan and Brazil are shown in Table 5. Dried fruiting bodies of *A. blazei* Murrill had 3.12 ± 0.03 to 3.12 ± 0.04% of crude polysaccharide, and fresh fruiting bodies had 0.50 ± 0.01%. Mycelia of *A. blazei* Murrill M152 had the highest crude polysaccharide yield (3.03 ± 0.05%); *A. blazei* Murrill M21, M72, and M101 had the next highest yield (1.50 ± 0.15 to 1.93 ± 0.12%); with *A. blazei* Murrill M62, M22, M52, BCRC 36814,

Table 5. Mycelia dry weight, crude polysaccharide extract, protein and total carbohydrate contents of crude polysaccharide extract of *Agaricus blazei* Murrill

Tested strains	Inoculum density (mg/L)	Dried mycelia weight (g/L)	Crude polysaccharide extract		Protein (%, w/w)	Total carbohydrate (as glucose) [%, w/w]
			(%, w/w)	(g/L)		
M21	8.80	10.33 ± 0.08^{e}	1.93 ± 0.12^{g}	0.199 ± 0.010	ND	92.10 ± 0.15^{c,d}
M22	13.50	8.04 ± 0.91^{d}	0.65 ± 0.04^{a}	0.052 ± 0.003	1.34 ± 0.05^{d}	90.94 ± 0.89^{c}
M23	6.30	5.39 ± 0.18^{b}	0.91 ± 0.04^{b,c}	0.049 ± 0.003	0.27 ± 0.05^{a}	91.36 ± 0.87^{c,d}
M33	10.50	9.81 ± 0.65^{e}	1.31 ± 0.07^{d,e}	0.128 ± 0.006	ND	89.87 ± 0.53^{c}
M52	6.80	6.94 ± 0.05^{c}	0.68 ± 0.01^{a}	0.047 ± 0.001	ND	82.27 ± 0.99^{a}
M62	15.40	8.12 ± 0.55^{d}	0.62 ± 0.02^{a}	0.050 ± 0.001	ND	93.35 ± 1.0^{c,d}
M72	8.70	10.83 ± 0.24^{e}	1.68 ± 0.02^{f}	0.182 ± 0.001	0.40 ± 0.11^{b}	97.52 ± 0.32^{d}
M101	9.60	9.76 ± 1.00^{e}	1.50 ± 0.15^{e,f}	0.146 ± 0.013	0.87 ± 0.07^{c}	92.06 ± 0.45^{c,d}
M111	10.20	10.19 ± 0.53^{e}	0.91 ± 0.11^{b,c}	0.093 ± 0.009	0.40 ± 0.01^{b}	97.79 ± 0.77^{d}
M121	4.20	10.58 ± 0.40^{e}	1.11 ± 0.02^{c,d}	0.117 ± 0.002	0.22 ± 0.03^{a}	96.19 ± 0.59^{d}
M152	9.50	8.28 ± 1.36^{d}	3.03 ± 0.05^{h}	0.251 ± 0.004	1.27 ± 0.03^{d}	86.69 ± 0.67^{b}
M171	5.70	9.71 ± 0.72^{e}	1.17 ± 0.09^{d}	0.114 ± 0.008	ND	99.14 ± 0.74^{d}
M192	11.30	9.87 ± 0.15^{e}	0.71 ± 0.13^{a}	0.071 ± 0.001	1.63 ± 0.05^{e}	85.33 ± 0.25^{b}
BCRC 36814	8.90	6.64 ± 0.49^{c}	0.68 ± 0.07^{a}	0.084 ± 0.006	ND	83.56 ± 0.61^{a}
A 321	5.10	4.26 ± 0.04^{a}	1.26 ± 0.41^{d}	0.029 ± 0.034	0.24 ± 0.08^{a}	84.89 ± 0.73^{a}
Fresh fruiting bodies, Taiwan		-	0.50 ± 0.01^{a}	-	0.16 ± 0.02^{a}	89.40 ± 0.53^{c}
Dried fruiting bodies, Taiwan		-	3.12 ± 0.04^{h}	-	0.20 ± 0.06^{a}	84.21 ± 0.91^{a}
Dried fruiting bodies, Brazil		-	3.12 ± 0.03^{h}	-	ND	92.36± 0.82^{c,d}

Abbreviation: ND = not determinable (below 0.1 mg/mL).

$^{a–h}$Mean ± standard deviation (n = 3) in the same row not sharing the same superscripts were significantly different at 5% level according to Duncan's multiple range test.

and M192 the lowest (0.62 ± 0.02 to 0.71 ± 0.13%). Crude polysaccharide yield differed significantly among these tested strains. Crude polysaccharide yields of this study were lower than those of Mizuno et al (6.5%) [3] and Dong et al (5.6%) [36] from the dried fruiting bodies. However, in this study, crude polysaccharide yields were eliminated from protein by SSA precipitation. Therefore, the protein contents of crude polysaccharide extract were between nil and 1.63 ± 0.05%, and the total carbohydrate content (as glucose) ranged from 82.27 ± 0.99 to 99.14 ± 0.74%. The protein yield in this study was less than that of Dong et al [36] (protein content 21.6%) and the total carbohydrate content (57.5%) was higher.

Molecular weight distribution of crude polysaccharide

Molecular weight distribution of crude polysaccharide extract of *A. blazei* Murrill M21 mycelia was determined by HPGPC and the results are shown in Fig. 6. Four peaks were found for the crude polysaccharide extract and the approximate molecular weights of the peaks were 274.1, 32.7, 7.5, and 2.1 kDa using the interpolate method of molecular weight standard curve. When the crude polysaccharide extract solution was neutralized with 0.01 N hydrochloric acid and filtered through 0.45 μm membrane filter, and the solid material redissolved in 0.1 N sodium hydroxide and analyzed with HPGPC again, only a single peak area was obtained, at molecular weight 2.1 kDa. Both the fluorescence wavelength and the light intensity were the same as the crude polysaccharide extract of molecular weight 2.1 kDa. Therefore, the 2.1 kDa fraction of the crude polysaccharide extract of *A. blazei* Murrill M21 might be β-(1,3)-D-glucan. The polysaccharide components of *A. blazei* Murrill depended on extraction methods and molecular weight determination methods. The molecular weights of active anti-tumor soluble polysaccharides were 2000 kDa [3], 380 kDa [37] and 170 kDa [36]. Therefore, further confirmation of the composition and active fraction of crude polysaccharide of *A. blazei* Murrill is necessary.

In conclusion, 16 isolates of *A. blazei* Murrill from 22 fruiting bodies produced in Taiwan were high-identity and matched the ITS1 sequences of *A. blazei* Murrill. The phylogenic tree of *A. blazei* Murrill isolates generated from RAPDs arranged samples into 3 groups and 2 independent cases. With the optimization of culture media and conditions, each liter of *A. blazei* Murrill M72 yielded 10.83 g of dried mycelia weight, and each liter of *A. blazei* Murrill M152 produced 0.251 g crude

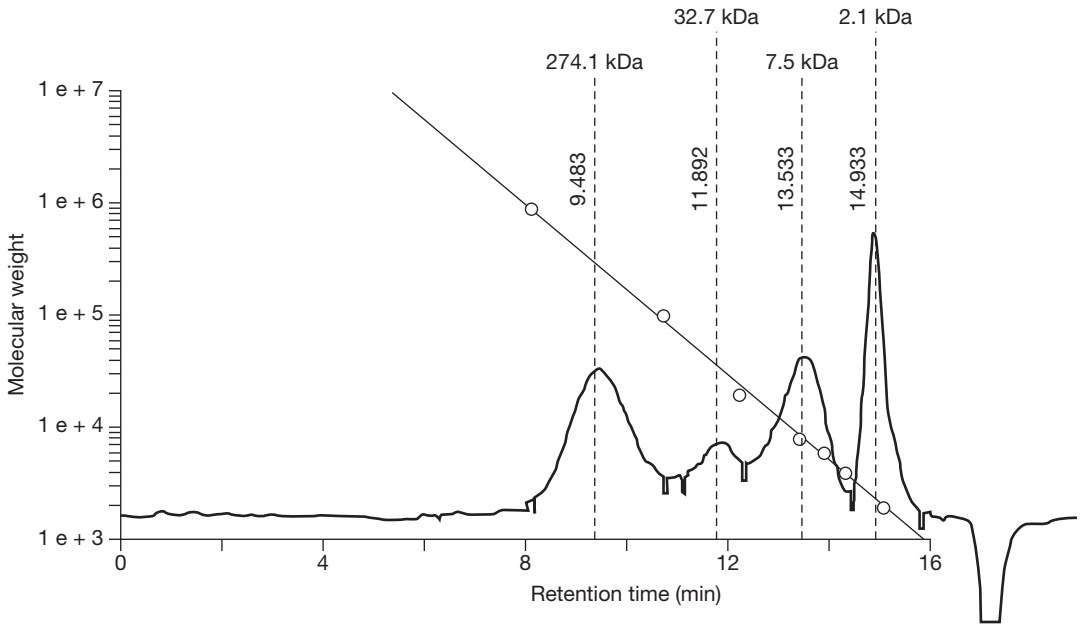

Fig. 6. High-performance gel permeation chromatography analysis of crude polysaccharide extract of *Agaricus blazei* Murrill M21.

polysaccharide (3.03% of dried mycelia weight). Mycelia and polysaccharide production of *A. blazei* Murrill with submerged fermentation is a potential feasible prospect in the future.

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