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Optimization of Medium pH, Growth Media Compositions and Analysis of Nutritional Components of *Ganoderma lucidum* in Submerged Culture Fermentation

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LQG and JFL designed the study and were the supervisors of authors QZ and WY. Authors QZ and WY conducted the experiments. Author QZ drafted the manuscript and performed the analyses. Authors LQG and JFL reviewed and revised the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was undertaken to identify the optimal submerged culture conditions for four strains of *Ganoderma lucidum*: *Ganoderma lucidum-8* (Ga-8), *Ganoderma sinense* (Ga-Sin), *Ganoderma lucidum-0201* (Ga-0201) and *Ganoderma atrum* (Ga-Atr) and analyze their nutritional components.
Study Design: Orthogonal experiment to identify optimal submerged culture and nutritional components analyses.
Place and Duration of Study: The experiments were conducted in the laboratories of College of Food Science, South China Agricultural University between January and July 2005.

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Results: Orthogonal experiment showed that all the strains grew well in M1 medium, with initial pH of 5.5. Ga-0201 grew best and had the largest mycelial biomass among four studied strains. Medium played the most important role in influencing the cultivation outcomes, followed by pH and then strain. Four strains of *G. lucidum* were subsequently submerged cultivated in the optimal medium and pH, and the nutritional compositions of their mycelial biomass were analyzed. Ga-Sin, high in ash, intracellular polysaccharide (IPS), extracellular polysaccharide (EPS), crude protein and crude fat; and Ga-0201, high in crude protein and crude fiber, had greater potential for research and development than the other two strains.
Conclusion: These results can be widely applied to *G. lucidum* fermentations on a large scale and selection of species for functional food use.

Keywords: Ganoderma lucidum; Ganoderma sinense; Ganoderma atrum; submerged culture; nutritional components.

1. INTRODUCTION

Ganoderma lucidum (Fr.) Karst is a species of basidiomycetes that belongs to polyporaceae (or *Ganodermataceae*) of Aphyllophorales [1,2]. Its fruit body is called “Lingzhi” in Chinese and “Reishi” in Japanese [3]. For more than 2000 years, this mushroom has been regarded in the Orient as popular or effective medicine, and used to treat various human diseases, such as hepatitis, arthritis, nephritis, bronchitis, asthma, arteriosclerosis, hypertension, cancers and gastric ulcer [1,4-6]. Recent studies on *G. lucidum* have also demonstrated effective antioxidant properties [7] and numerous biological activities, including antitumor, anti-inflammatory effects and cytotoxicity to hepatomacells [3,6,8,9].

With the increased concern of nutrition and healthy eating worldwide, *G. lucidum*, which has potential health benefits, has gained wide popularity as a healthy food [10]. Supplying the market with high quality and quantity of *G. lucidum* products is needed. *G. lucidum* has been successfully produced in China, Japan, Korea, and Taiwan via solid cultivation, using substrates such as grain, sawdust or wood [1]. However, this traditional method is time consuming, usually takes several months to complete the cultivation. Also, in the course of cultivation, it is difficult to control the product quality. Submerged fermentation is seen to become a promising alternative for solid cultivation, because of its ability to yield a large amount of biomass in a compact space, in a shorter time and with lower risk of contamination [11-14]. In addition, extracellular polysaccharide (EPS) which has synergistic effect with mycelia on biological activities can be simultaneously produced [14].

Optimal submerged culture condition can ensure the quantity and quality of the mycelia production of *G. lucidum*, however such information is scare in the literature. Therefore, this study on one hand aimed at determining the optimal submerged culture conditions for *G. lucidum*by orthogonal experiment, and in turn improving the fermentation process of the *G. lucidum*. On the other hand, since the nutritional compositions vary from strain to strain and were influenced by the culture conditions [15], this study also examined the nutritional components of four strains submerged cultivated in the optimal conditions, including moisture, ash, intracellular polysaccharide (IPS), EPS, crude protein, crude fiber and crude fat.

2. MATERIALS AND METHODS

2.1 Organism

There are four strains of the genus *Ganoderma:Ganoderma lucidum-8* (Ga-8), *Ganodermasinense* (Ga-Sin), *Ganoderma lucidum-0201* (Ga-0201), and *Ganoderma atrum* (Ga-Atr). They are routinely maintained on the PDA slopes at 4°C. Ga-8 is a commercial strain widely cultivated in China. Ga-SinandGa-Atr are well commonly used as medicinal plants. Ga-0201 is a novel wild strain of *G. lucidum* collected from a forest in Southeast China. Its fruiting body has a shinning surface.

2.2 Experimental Procedure

The mycelia were transferred from the PDA slopes to the Petri-dish containing PDA medium, and cultivated at 28°C for 10 days. Mycelial agar discs (similar size as a read bean) were obtained by a self-designed cutter and used as the inoculum in a shake flask culture.

The optimization on culture conditions was conducted by the orthogonal experiment design $L_{16}(4^3)$ [16]. Orthogonal experiment adopteda three-factors (strain, medium and pH) by four-levels design (Table 1). Strains had four levels: Ga-8, Ga-Sin, Ga-0201 and Ga-Atr. Media had four levels. M1 (g / l): 200 potato, 20 glucose, 3 KH_2PO_4, 1.5 $MgSO_4$, 5-10 mg/l Vitamin B1; M2 (g / l): 20 maize flour, 20 sucrose, 2 KH_2PO_4 , 1 $MgSO_4$; M3 (g / l): 20 maize flour, 20 glucose, 2 KH_2PO_4, 1 $MgSO_4$; M4 (g / l): 20 maize flour, 20 soybean flour, 20 glucose, 2 KH_2PO_4 , 1 $MgSO_4$).pH values had four levels: 5.5, 6.0, 6.5, 7.0. Two sets of shake flasks were prepared at the same time for each group. The initial pH was adjusted to the desired value (level) by adding either NaOH or HCl. Media were sterilized at 121ºC, 0.1MPa for 30 min. The 250 ml flasks containing 100ml of the medium were inoculated with 5% (v/v) of the inoculum and incubated at 27ºC on a rotary shaker (HQL150C) under 150 r/min for 8 days. The mycelial biomass, the growth status and appearance of mycelium, final pH and the ability of resisting contamination were examined during this period.

Table 1. L_{16} (4^3) orthogonal experimental design

Run no.	A (Strain)	B (Medium)	C (Initial pH)
1	Ga-8	1	7.0
2	Ga-8	2	6.5
3	Ga-8	3	6.0
4	Ga-8	4	5.5
5	Ga-Sin	1	6.5
6	Ga-Sin	2	7.0
7	Ga-Sin	3	5.5
8	Ga-Sin	4	6.0
9	Ga-0201	1	6.0
10	Ga-0201	2	5.5
11	Ga-0201	3	7.0
12	Ga-0201	4	6.5
13	Ga-Atr	1	5.5
14	Ga-Atr	2	6.0
15	Ga-Atr	3	6.5
16	Ga-Atr	4	7.0

For nutritional component analyses, the magnified fermentations were carried out under the optimal conditions in 500 ml flasks containing 300 ml of medium with 7% (v/v) of the inoculum and incubated at 27ºC on a rotary shaker (HZQ-F160) under 170 r/min for 6 days.

2.3 Component Analysis Methods

2.3.1 Biomass determination and pretreatment

Mycelium was separated from the cultivation broth by centrifugation at 3000 r/min for 15 min. Then the biomass was weighted and the fermentation supernatants were stored at 4ºC, and later prepared for analyses of extracellular polysaccharide (EPS). Subsequently, mycelium was washed with distilled water, and then collected by filter cloth, dried at 60°c for sufficient time until a constant dry weight was obtained and finally ground to powder.

2.3.2 Analyses of moisture, ash, crude protein, crude fiber and crude fat

Moisture was analyzed by a Moisture Determination Balance (AD-4714A). Contents of ash, crude protein, crude fiber and crude fat were determined by the Loss of Weight Method [17], Kjeldahl Method [17], Loss of Weight Method [18] and Soxhlet Extractor Method [17], respectively.

2.3.3 Analyses of polysaccharides

For the analysis of intracellular polysaccharides (IPS), about 1 g of dried mycelia was decolored by using 95% ethanol. Then the mycelia were dried. Polysaccharides were extracted with 70ml stilled water at 90ºC for 2 h, and further extracted by ultrasonic wave for 0.5 h. The supernatant was diluted in 100 ml and assayed by Phenol-Sulfuric Acid Method [19]. For the determination of EPS, 100 ml of fermentation supernatant was concentrated to 20ml at the temperature of no higher than 90ºC, and then precipitated by adding 60ml of 95% ethanol at 4ºC for 18 h. The precipitation was washed with 75% ethanol and then absolute ethanol for five times. Crude EPS was obtained after being dried by vacuum. After that, crude EPS was dissolved in 50 ml 80ºC hot water for 2 h. The supernatant was diluted in 100 ml and measured by Phenol-Sulfuric Acid Method [19]. For the purification of polysaccharides, Sevag Method was used. Then crude polysaccharides were precipitated by adding 3 volumes of 95% ethanol at 4ºC for 24 h. Purified polysaccharides were obtained after being washed with absolute ethanol and absolute ether for five times.

2.4 Statistical Analysis

Analysis of Variance (by using software Microsoft Excel) and Duncan's New Multiple-range Test

with α＝0.05 were performed [20,21]. In the bar graphs, values were plotted as mean ± S.E. After multiple comparisons, the means in the bar graphs were followed with different small letters "a-d" based on their values and statistical differences. In the case that a mean was followed with "ab", this mean was not significantly different from a mean with "a", and was not significantly different from another mean with "b". However, means with different letters were significantly different at the level of 0.05.

3. RESULTS AND DISCUSSION

3.1 Optimization of Culturing Conditions

Major indicator for optimal culture conditions was mycelial biomass. Secondary indicators included appearance, final pH, and resistance to contamination by bacteria.

3.1.1 Identification of the group with the largest amount of biomass

In the orthogonal experiment, group $A_3B_1C_3$ (group 9) had the largest production of biomass (159.20 mg/ml) (Table 2). However theoretically Fig. 1 suggested that group $A_3B_1C_4$ had largest amount of biomass. Due to the absence of this group in actual experiment, a supplementary experiment (SuppE) was conducted. The result of SuppE showed that the biomass of group $A_3B_1C_4$ (243.08 mg/ml, Table 2) was larger than that of $A_3B_1C_3$, which safely drew a conclusion that group $A_3B_1C_4$ (M1, pH5.5, Ga-0201) had the largest production of mycelial biomass.

3.1.2 Comparison by the growth status of mycelia in the orthogonal experiment

Strain of high quality had common characters: large quantity of mycelial balls, uniform in size, long and thick mycelia, slightly ropiness fermentation supernatants and strong resistance to contamination by bacteria. According to Table 2, the appearance of group 9, 4, 12 and 5 had the above characters. Group 9 (Ga-0201, M1, pH6.0) was the best among 16 groups, followed by group 4, 12 and 5. Ga-0201 grew better than other three strains, while Ga-Atr was the worst in growth because of being easily contaminatedby bacteria and its heterogeneity of the size of mycelial balls. M1 was the most suitable medium as most of long mycelia and ropiness fermentation supernatants appeared in it. The initial pH between 5.5 and 6.5 was suitable for cultivation. All strains did not grow well in supernatants having a pH value of 7. Comparisons using major and secondary indicators reached the same conclusion that M1 Medium and pH5.5 were the best cultural conditions. The SuppE group ($A_3B_1C_4$) was the best design.

3.2 Effect of Culturing Conditions on the Growth of Mycelia

Ranges (R) in Table 2 were compared. The result $R_B>R_C>R_A$ indicated that among three factors in the orthogonal experiment, medium played the most crucial role; then next was pH value and finally came strain. The effects of medium and pH on the growth of mycelia were discussed as follows respectively.

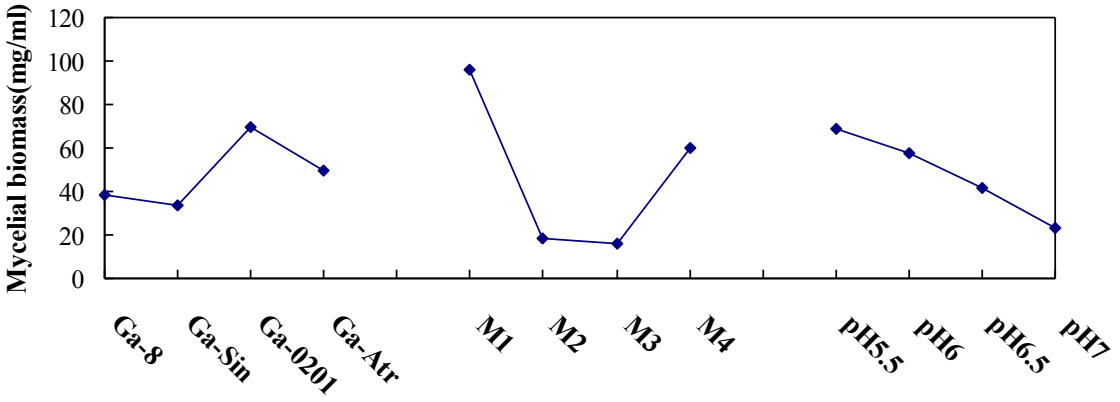

Fig. 1. Influences of strain, medium and initial pH on the mycelial biomass in the orthogonal experiment

3.2.1 Effect of medium to the growth of mycelia

Fig. 2. illustrated that M1 was the best and M4 ranked second (except for Ga-8). Four strains did not grow well in M2 or M3. This meant that potato, which contains 18.8% carbohydrate, 4.4% protein and 7.1% fiber [22] was appropriate for the growth of *G. lucidum* since it could be used as both carbon and nitrogen source. The C/N ratio of either M2 or M3 was improper, because maize flour is high in starch and low in nitrogen. The amount of biomass in M4 was larger than that in M3. This was because M4 contained 2% of soybean (whose content of nitrogen was 8.0% [23]) while M3 did not. In addition, glucose was the best carbon source and appropriate content of K^+, PO_4^{2-}, Mg^{2+} and Vitamin B were optimal for the growth of *G. lucidum* [24].

3.2.2 Relationship between pH and biomass

In general, mycelia can only grow within a certain pH range, and metabolite formation is often affected by pH [25]. In this study, pH showed a decrease during the cultivation in all cases. Largest amount of biomass was obtained when initial pH from 5.5 to 6.0 and final pH below 4.0. Smallest amount of biomass was obtained when initial pH 7.0 or final pH above 4.5. Final pH values of all the groups of Ga-0201, whose amount of biomass was the largest, was below 4.0; while final pH of all the groups of Ga-Sin, whose amount of biomass was the smallest, was above 4.0 (Fig. 3).

3.3 Analysis of Nutritional Components Belonging to Four Strains of *Ganoderma* genus

Moisture contents of Ga-0201 (93.2%) and Ga-Atr (91.2%) were significant higher than that of Ga-8 (84.0%) (Table 3). Moisture content is probably related to the volume of mycelial ball. Ga-Atr, whose mycelial balls were big, contained more water while Ga-8, whose mycelial balls were small, contained less water.

Ash content of Ga-Sin (13.88%) was significantly higher than that of Ga-Atr (10.68%), Ga-8 (10.07%) and Ga-0201 (9.68%) (Table 3). It has been reported that ash content of edible and medicinal fungi was less than 9% [26]; thereby it is probable that strains studied here were high in mineral and microelement.

There were considerable differences in the polysaccharide contents belonging to four strains of three species of *Ganoderma* genus. For ISP, Ga-8, which contained 23.88% ranked first; then next was Ga-Sin with 20.33%, followed by Ga-Atr, containing 14.89%; and finally came Ga-0201 with 10.67%. As for EPS, Ga-Sin contained 34.18%, which was considerably higher than that of Ga-Atr, Ga-8 and Ga-0201, at 14.36%,13.17% and 11.91%, respectively. Ga-8 contained more IPS than EPS whereas Ga-Sin contained less IPS than EPS. As for Ga-Atr and Ga-0201, the EPS content was close to the IPS (Table 3). Polysaccharide produced by *G. ucidum* is a type of carcinostatic agent, which has antitumor [8] and hypoglycemic activities [27]. Ga-Sin, high in both IPS and EPS, was the ideal strain for polysaccharides production.

Protein contents of Ga-Sin (25.32%) and Ga-0201 (24.74%) were higher than that of Ga-Atr (22.88%) and Ga-8 (22.88%) (Table 3). Generally, mushrooms are a good source of protein, and their protein contents range from 19% to 35% of dry weight [28]. Protein contents of these four strains were within this range.

Ga-0201 contained 7.114% of crude fiber, significantly higher than those of Ga-8 (5.660%) and Ga-Atr (5.430%). Ga-Sin contained just half of the crude fiber of Ga-0201 (Table 3). Except from Ga-0201, three strains studied were considered to be low in fiber, as it has been reported that the amount of crude fiber of edible and medicinal fungi was generally greater than 7% [26].

Crude fat contents, which differed significantly, were in order of Ga-Sin (0.473%), Ga-Atr (0.428%), Ga-0201 (0.353%) and Ga-8 (0.307%) (Table 3). The crude fat contents of four strains were noticeably lower than most mushrooms (range between 1.1% and 8.3%) [28].

Mycelia of *G. lucidum* are high in moisture, ash, polysaccharides and crude protein; and low in crude fiber and crude fat. Ga-Sin was relatively higher in ash, EPS, ISP, crude protein and crude fat than other three strains studied; Ga-0201 had higher amounts of moisture, crude protein and crude fiber than other three strains studied; and Ga-8 has the highest level of IPS among all strains in this study.

Table 2. The results of orthogonal experiment, fermentation on a rotary shaker at 27°c for 8 days

Run no.	Biomass (mg/ml)	Mycelial ball		Length of mycelium	Physical characteristics of the culture media	Contami-nationby bacteria
		Quantity	Size			
1	25.98	many	small	short	middle	no
2	13.8	least	very small	very short	notropiness, little turbidity	no
3	13.78	few	hetero-geneous	very short	notropiness , clear	no
4	99.8	many	big	very long	ropiness , turbidity	no
5	67.85	many	big	short	middle	no
6	12.92	least	hetero-geneous	very short	notropiness , turbidity	no
7	13.64	few	small	very short	notropiness , turbidity	no
8	40.82	many	hetero-geneous	very short	notropiness , turbidity	no
9	159.20	most	small	long	ropiness	no
10	29.17	least	hetero-geneous	long	notropiness , clear	no
11	21.88	few	hetero-geneous	long	notropiness , clear	no
12	68.65	many	small	long	notropiness , clear	no
13	132.14	many	hetero-geneous	very short	notropiness , clear	no
14	17.91	few	big	long	notropiness , turbidity	yes
15	16.26	few	hetero-geneous	long	notropiness , clear	no
16	31.97	many	hetero-geneous	long	notropiness , clear	yes
SuppE	243.08	many	big	long	notropiness , clear, fragrance	no

Table 3. Nutritional compositions (%) of four strains of *Ganoderma* genus

	Moisture	Ash	EPS	IPS	Crude protein	Crude fiber	Crude fat
Ga-8	84.0 ± 1.8^{b}	10.07 ± 0.16^{b}	13.17 ± 0.07^{bc}	23.88 ± 0.47^{a}	22.56 ± 0.10^{b}	5.660 ± 0.071^{b}	0.3072 ± 0.0072^{d}
Ga-Sin	87.9 ± 1.6^{ab}	13.88 ± 1.00^{a}	34.18 ± 0.11^{a}	20.33 ± 0.03^{b}	25.32 ± 0.49^{a}	3.516 ± 0.103^{c}	0.4731 ± 0.0072^{a}
Ga-0201	93.2 ± 0.2^{a}	9.68 ± 0.14^{b}	11.91 ± 0.91^{c}	10.67 ± 0.25^{d}	24.74 ± 0.69^{a}	7.114 ± 0.113^{a}	0.3533 ± 0.0081^{c}
Ga-Atr	91.2 ± 1.3^{a}	10.68 ± 0.31^{b}	14.36 ± 0.15^{b}	14.89 ± 0.64^{c}	22.88 ± 0.33^{b}	5.430 ± 0.196^{b}	0.4279 ± 0.0100^{b}

^{ab} different subscripts denote significant difference within groups using New Multiple-range Test with $\alpha=0.05$

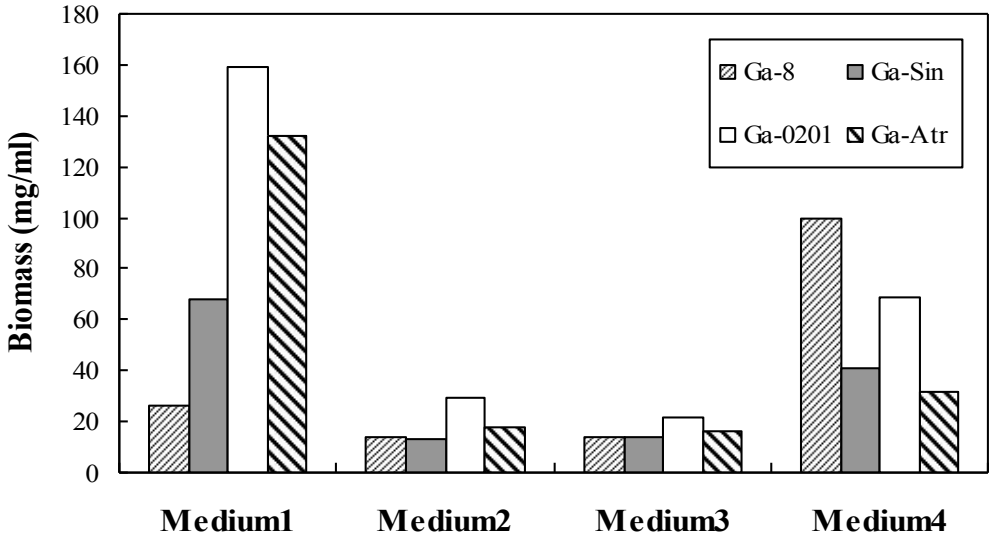

Fig. 2. Effect of medium on the biomass of four strains of *Ganoderma* genus in the orthogonal experiment

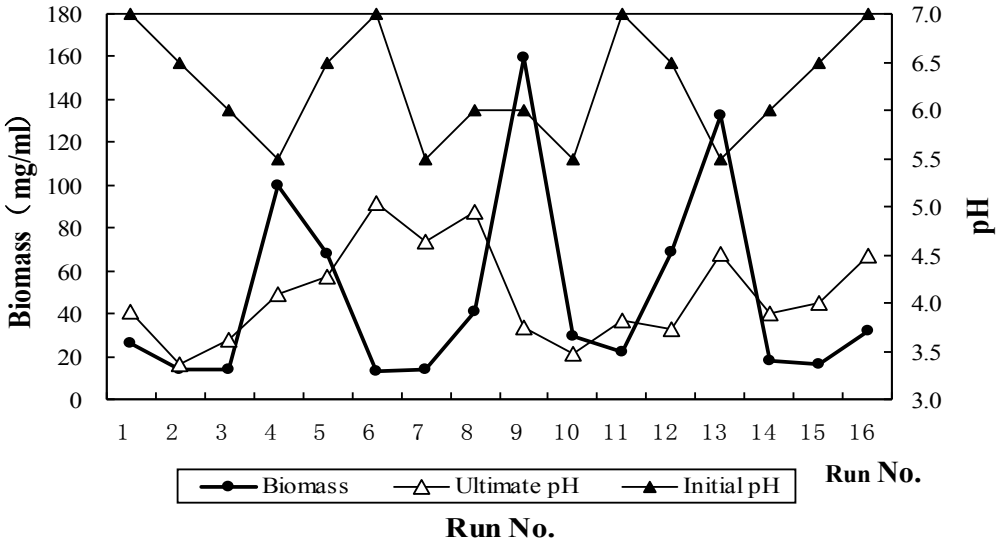

Fig. 3. The evolution of biomass amount and pH in orthogonal experiment, the pH was measured with a digital pH meter (Delta 320)

4. CONCLUSION

This study demonstrated that the optimal initial pH for four strains of *Ganoderma* genus was 5.5.M1 is just the best medium among the others (M2, M3, M4). Among three factors affecting mycelial biomass, medium played the most crucial role; then next was initial pH and finally came strain. These findings can be useful as a basis in a large scale fermentation of *G. lucidum*. Among four species, Ga-0201 had the highest amount of biomass in submerged fermentation and its mycelia were higher in crude protein and fiber than other three strains. Ga-Sin was valuable in its ash, EPS, ISP, crude protein and crude fat contents. This information is useful for choosing the ideal strains for further investigation or for large scale cultivation. Ga-Sin can be used in polysaccharides production and the novel wild strain Ga-0201 might be developed as prebiotic products.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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