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THE DRIED MYCELIUM OF *GANODERMA LUCIDUM* EXHIBITING IMPROVING INTRACELLULAR POLYSACCHARIDE CONTENT BY SUBMERGED FERMENTATION OPTIMIZATION IN LARGE-SCALE FERMENTATION PROCESSES AND ITS FOOD SAFETY

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ABSTRACT

This study mainly focused on increasing intracellular polysaccharide (IPS) content in the medicinal fungus *Ganoderma lucidum* by optimized cultured conditions (containing carbon sources, initial pH values, temperatures and carrier-to-noise ratios) in large-scale fermentor. The maximum IPS content of 6.68% in a 1500-L fermentor was achieved in a medium containing 30 g/L glucose, 5 g/L soybean meal, and 10 g/L corn flour at an initial pH 6.0 and temperature 28°C and was found to be 2.27 times higher than that of unoptimized conditons (2.93%). When expanding culture in a 10000-L fermentor was performed under optimum conditions, the highest IPS content (6.30%) and dry mycelium weight (about 17.85 g/L) were obtained at only 52h. According to the analysis of AS contents and Pb contents from fermentation raw materials by the determination of graphite furnace atomic absorption spectrophotometer, As content and Pb content of the dried mycelium were decreased to food safe range by adjusting fermentation raw materials. The results can promote its industrial-level production.

1.Introduction

Ganoderma lucidum (Leyss.:Fr.) Karst, a basidiomycete belonging to the polyporaceae, is one of very famous medicinal herbs in worldwide, especially in China, Korea and Japan. Because of its high medicinal value, *G. lucidum* has received wide popularity as a health food and medicine. Nowadays, the yield of fruiting bodies has not satisfied its consumer demand, so submerged culture for producing mycelium of *G. lucidum*, which has many advantages for industrial-level production, such as short production cycle, stable food safe (such as low heavy metal content and not pesticide contamination) and so on (Chen and Gu, 2008), has a good prospects for application. The polysaccharides isolated from fruiting bodies and cultured mycelium of *G. lucidum* have antitumor activity, immunomodulation and antioxidation (Hsiao et al., 2004; Mojadadi et al., 2006; Sudheesh et al., 2009), and its content is one of the key indexes for evaluating medicinal value of *G. lucidum* products. Improving intracellular polysaccharide (IPS) contents of mycelium by submerged cultured optimization have been studied in several reports (Babitskay et al., 2005; Simonić et al., 2008; Tang et al., 2011; Yang et al., 2013). However, their processes in all studies don't suit industrial-level production, such as long fermentation time, mall fermentation scale and unstable food safety. In the present study, submerged fermentation optimization for producing mycelium of *G. lucidum* exhibiting

high IPS content was performed in large-scale fermentor during short cultured time.

Furthermore, those mycelium with safe heavy metal contents were obtained by the adjusting of fermentation raw materials.

2. Materials and methods

2.1. Maintenance of *G lucidum*

The strain of *G.lucidum* used in this study was maintained on potato dextrose agar (PDA) slants. The slant with mycelium was incubated at 28°C for 6 days, and then stored at 4°C for the following experiment.

2.2. Cultured medium of *G lucidum*

Preculture medium included the following components (g/L): glucose 20, soybean meal (AS content of 0.24ppm, Pb content of 0.43ppm) 15, corn flour 10, corn steep powder 15, inorganic salt 2.0, vitamin 0.004, bean oil 1, antifoam 0.5. The initial pH value was adjusted to 6.0 by adding industrial grade solid caustic sodaor analytical grade solid caustic soda.

Cultured medium consisted of the following components (g/L): sucrose or glucose 30, soybean meal 5, corn flour 5, corn steep powder 10, inorganic salt 2.0, vitamin 0.004, bean oil 1, antifoam 0.5. The initial pH value was adjusted to 6.0 by adding industrial grade solid caustic soda or analytical grade solid caustic soda.

2.3. Preculture of *G lucidum* in flasks

For the first preculture in flasks, 500-mL flasks containing 200 mL of preculture medium were sterilized at 120°C for 30 min, and then cooled to room temperature. The strain from PDA slant was inoculated into 500-mL flasks and followed by 4-day incubation at 28°C on a rotary shaker (150 rpm).

For the second preculture in flasks, 1-L flasks containing 400 mL of preculture media were sterilized at 120°C for 30 min, and then cooled to room temperature. Preculture medium with *G lucidum* prepared in a 500-mL flask was inoculated into 1-L flasks with volume ratio of 10%, and then followed by 3-day incubation at 28°C on a rotary shaker (150 rpm).

For preculture in fermentor, 200-L fermentor with a working volume of 120 L of preculture medium was sterilized by steam at 120°C for 60 min, and then cooled to room temperature. 800 mL of preculture medium with *G lucidum* prepared in a 1-L flask was inoculated into 200-L fermentor, and then culture was performed with aeration rate (1.11 $V \cdot V^{-1} \cdot min^{-1}$) at 28°C for 3 d.

2.4. Fermentation culture of *G lucidum* in large-scale fermentors

For fermentation culture, 1500-L fermentor with a working volume of 800L of cultivation medium were sterilized by steam at 120°C for 60 min, and then cooled to room temperature. 120 L of preculture medium with *G lucidum* prepared in 200-L fermentor was translated into 1500-L fermentor with a working volume of 800 L of cultured medium, and then fermentation was performed with agitation speed (0 rpm) and aeration rate (0.93 $V \cdot V^{-1} \cdot min^{-1}$) at 28°C for 72h. For amplication culture, 10000-L fermentor with a working volume of 7000 L of cultured medium was sterilized by steam at 120°C for 60 min, and then cooled to room temperature. 120 L of medium with *G lucidum* prepared in 200-L fermentor was translated into 10000-L fermentor with a working volume of 800L of cultured medium, and then fermentation was performed with aeration rate (0.93 $V \cdot V^{-1} \cdot min^{-1}$) at 28°C for 60h.

2.5. Experimental design

Two different carbon sources at the concentrations of 30g/L (glucose or sucrose) were tested in order to determine the effect on IPS content, pH value and mycelium concentration. In optimum carbon source condition, the influences of two different initial pH value (6.0 and 4.5) and two different temperatures (28°C and 32 °C) were evaluated, respectively. In the optimum conditions, the variation of IPS content, pH value and mycelium concentration was tested by adjusting corn powder content and soybean meal content. Subsequently, the culture in 10000-L fermentor was performed in optimal

conditions. Finally, the experiment for reducing AS content and Pb content in mycelium of *G. lucidum* was carried out.

2.6 Analytical methods

For the analysis of IPS content, wet mycelium was dried at 85°C for 24h, then the dry mycelium (1.0 g) grinded in mortar was sieved by using 2μm sieve. Mycelium powder was extracted by boiling water for 2h, and extracted liquid followed a precipitation with absolute ethanol at 4°C for 12h.

The precipitated polysaccharide was collected by centrifugation at 3000 rpm for 10 min and was dissolved by distilled water. Polysaccharide content of the final solution was determined by anthrone-sulfuric acid method according to Chinese pharmacopoeia (Anonymous, 2005). IPS content was present as g/g (dry mycelium weight) 100%.

For the analysis of growth state of *G. lucidum*, mycelium concentration was determined in real-time. Fermentation broth (100 mL) was centrifuged at 3000 rpm for 30min, and then wet mycelium weight was determined by electronic balance. Mycelium concentration was describe as g (wet mycelium weight in 100 mL broth) /100mL·100%.

The pH value of cultured liquid was measured with a digital pH meter.

AS content and Pb content in dry mycelium were determined by graphite furnace atomic absorption spectrophotometer according to Xing et al., method (Xing et al., 2002).

3. Results and discussion

3.1. The effect of different carbon sources, temperatures and initial pH values on IPS content, pH value and mycelium concentration from *G. lucidum*

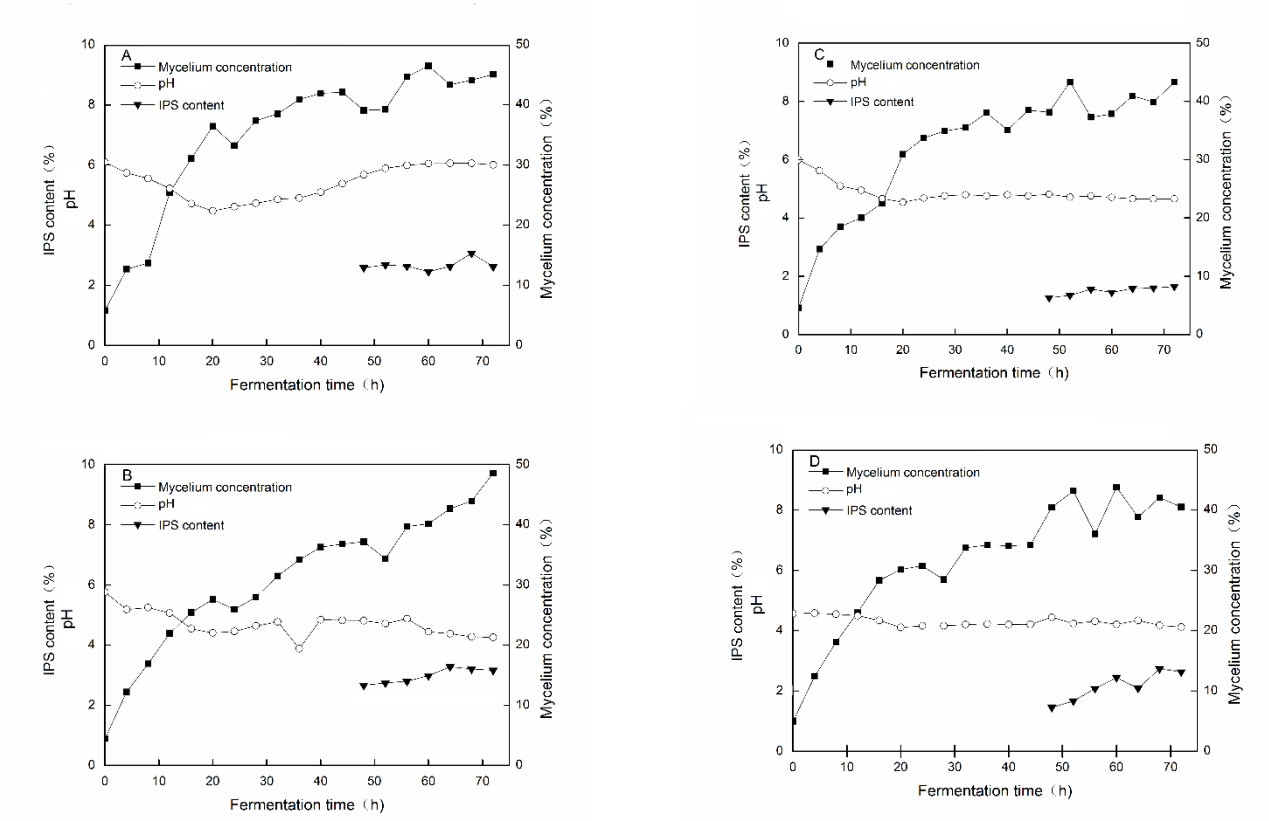

Figure 1. The time course of IPS content, pH and mycelium concentration by using *G. lucidum* under different cultured conditions in 1500-L fermentor. A, Sucrose as main carbon source; B, Glucose as main carbon source; C, Cultured temperature of 32°C; D, Initial pH value of 4.5

Fig. 1A and Fig.1B revealed that the maximum IPS content of 2.93% reached in the presence of sucrose was lower than the highest IPS content of 3.41% obtained in the presence of glucose.

The results weren't inconsistent to the previous report, which sucrose was the better substrate for IPS content compared to glucose (Tang and Zhong, 2002). The variations of pH value between Fig. 1A and Fig. 1B were different: pH value in the presence of sucrose sharply increased from 4.47 to 6.01 between 20 h to 72h, whereas pH value in the presence of glucose slightly decreased from 4.4 to 4.26 between 20 h to 72h.

Possible explain is that the pathways of utilization of *G lucidum* were different between glucose and sucrose, and lead its different secretion level of acid material and alkaline material. Mycelium concentration in the presence of sucrose rapidly increased during exponential phase (4h-20h), and then slowly rose to 46.45% at 60h (Fig.1A). Mycelium concentration in the presence of glucose gradually increased all the time, and the highest mycelium concentration of 48.6% was achieved at 72h (Fig.1B). The result indicated that glucose was a more suitable growth substrate than sucrose. Temperature has significant effect on synthesis of polysaccharides of *G. lucidum* (Babitskaya et al., 2005; Kim et al., 2006; Lee etal., 2007; Yang and Liau, 1998). When cultured temperature was increased from 28 to 32°C, the maximum IPS content was decreased from 3.41% to 1.71% (Fig. 1B and Fig. 1-C). The result is similar to the report of Babitskaya et al, which maximum end polysaccharides was obtained at 25-30°C (Babitskaya et al., 2005). Variation trend of pH value was similar between Fig. 1B and Fig. 1-C, pH values rapidly decreased from 0 h to 24h, and then almost no reduction of pH value was showed after 24h. The highest mycelium concentration of 43.4% at 32°C was lower than 48.6% at 28°C (Fig. 1B and Fig. 1C). The result is agreement to a previous report, which the optimum temperature for the culture mycelium growth was obtained at 28°C (Kim et al., 2006) .

The initial medium pH can greatly affect cell membrane function, cell growth, morphology and structure, salt solubility, the ionic state of substrates, the uptake of various nutrients, and product biosynthesis (Fang and Zhong, 2002a). When initial pH value was adjusted from 6.0 to 4.5, the highest IPS content was reduced from 3.41% to 2.74% (Fig. 1B and Fig. 1D). However, Fang et al found that lowering the initial pH from 6.5 to 3.5 gradually led to a higher IPS content (7.75%) (Lee et. al., 2007). Likewise, Simonić et al showed that IPS production in medium with initial pH 4.5 was the highest value among that of initial pH values (4.0, 5.0, 5.5 and 6.0) (Simonić et. al., 2008). Although the initial pH value was set at 4.5 or 6.0, their final pH value was about 4.0 at incubation of 72h and slightly higher than the pH value (3.54) in the literature (Yang and Liau, 1998). Fang and Zhong reported that at an initial pH of 6.5, a maximum in biomass of 17.3 g/L by dry weight was achieved (Fang and Zhong , 2002a).In *Ganoderma resinaceum* DG-6556, the maximum mycelium growth was obtained at an initial pH of 7.0(Kim et. al., 2006). Simonić et al found that biomass production increased gradually by increasing the initial pH and reached the peak at an initial pH of 5.5 (Simonić et. al., 2008). These authors confirmed the range of initial pH value (5.5-7.0) was the most suitable for growth. In the study, the highest mycelium concentration of 43.82% at initial pH value of 4.5 was lower than that at initial pH value of 6.0 (48.6%). This result was in the range mentioned above.

3.2. The effect of the regulation of carrier-to-noise ratios (C/Ns) on IPS content, pH value and mycelium concentration from *G. lucidum*

Previous studies indicated that C/N is thought as the important factor for the polysaccharide biosynthesis of *G.lucidum*, and suitable C/N can obviously enhance polysaccharide production (Babitskaya et. al., 2005; Lee et. al., 2007; Yuan et. al.,2012). In the study, corn flour and soybean meal was generally regarded as carbon source (a

general C/N ratio of 97.3) and nitrogen source (a general C/N ratio of 16.76). The highest IPS content of 3.47%, 2.77%, 5.45%, 6.68%, and 3.41% were shown in Fig. 2 A, B, C, D, and Fig. 1B, respectively. The maximum IPS content of 6.68% was revealed in the medium containing soybean meal 5 g/L and corn flour 10 g/L (Fig. 2D). We found that increasing nitrogen source content decreased IPS content (Fig. 1B, Fig. 2 A, and Fig. 2B), whereas increasing carbon source content enhanced IPS content (Fig. 1B and Fig. 2D).

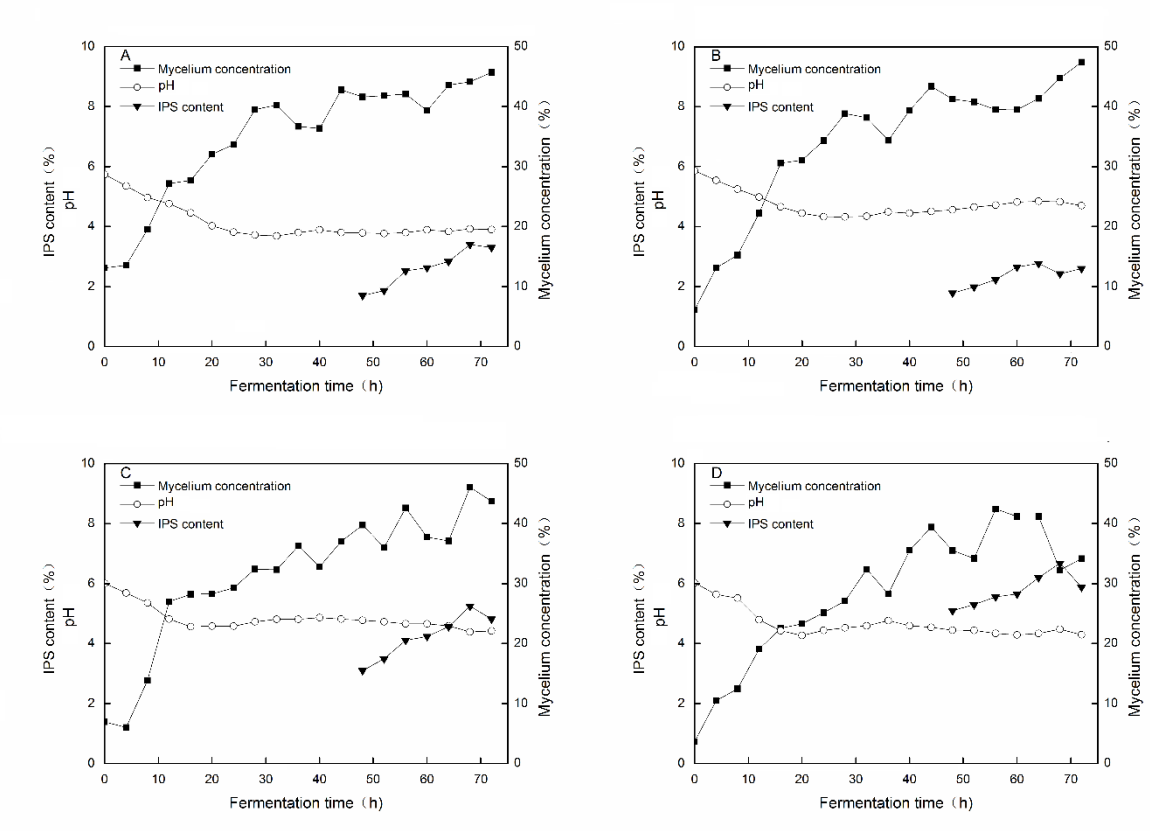

Figure 2. The time course of IPS content, pH and mycelium concentration by using G. lucidum under differnent C/Ns in 1500-L fermentor. A, soybean meal 10g/L and corn flour 5 g/L; B, soybean meal 15 g/L and corn flour 5 g/L; C, soybean meal 10 g/L and corn flour 10 g/L; D, soybean meal 5 g/L and corn flour 10 g/L

However, the result was converse to previous report which the endo polysaccharides reduced with increasing C/Ns in *G. applanatum* (Babitskaya et. al., 2005). This converse variance of IPS content from the same kind of fungus may differ due to strains and their culture conditions. Variety trend of pH value by regulation of C/Ns among Fig. 2A, B, C, D, and Fig. 1B showed similarly that pH value quickly decreased before 20h and then almost not changes was observed after 20 h.

Mycelium concentration sharply increased at exponential phase, and then slightly changes at stationary phase (Fig. 2A, B, C, D, and Fig. 1B), the maximum mycelium concentration was 45.9%, 47.45%, 46.1%, 42.45%, and 48.6% in Fig. 2A, B, C, D, and Fig. 1B, respectively.

3.3. Fermentation amplification in 10000-L fermentor under optimum conditions and reducing Pb content and As content of mycelium from *G. lucidum*

The profiles of IPS content, mycelium concentration and pH value in fermentation process was shown in Fig. 3A. IPS content firstly increased from 48h to 52h, and then decreased. The maximum IPS content of 6.3% was obtained at 52h. Although previous

reports showed the maximal IPS content of 23%(Tang et. al., 2011), their measure method of phenol-sulfuric acid were different from the measure method in present study.

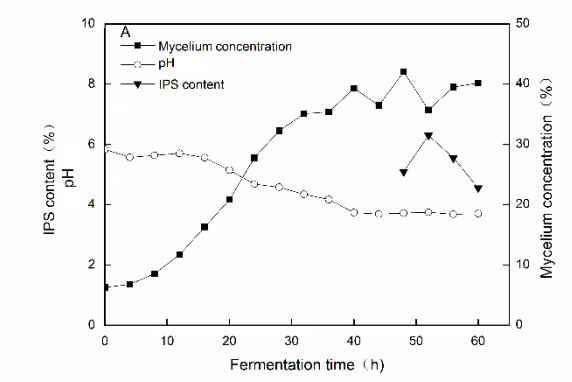

Figure 3 A. The time course of IPS content, pH and mycelium concentration by using *G. lucidum* under optimum conditions in 10000-L fermentor.

In our pervious research, we noticed that IPS content with phenol-sulfuric acid method was obviously higher than that with anthrone-sulfuric acid method, so the comparison did not make among those results. The authors found that the highest IPS content was only 2.74% among those fruiting bodies from 18 varieties of *Ganoderma Lucidum* Karst (Xu and Xu, 2004).

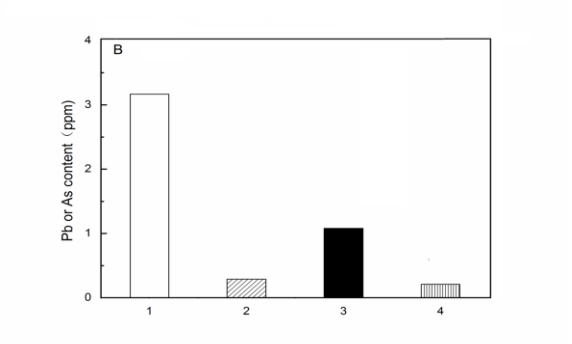

Figure 3B. The comparison of AS content or Pb content in *G. lucidum* of before and after treatment. 1, Pb content using industrial grade solid caustic soda ; 2, Pb content using analytical grade solid caustic soda ; 3, As content using industrial grade solid caustic soda ; 4, As content using analytical grade solid caustic soda

Mycelium concentration sharply increased from 0 h to 32h and then slightly increased after 32h, the concentration of 35.7% was achieved at 52h (Fig. 3A). According to the calculation of water content of 95%, dry mycelium weight was about 17.85 g/L at 52h and was moderate level among that of other study (14.7 g/L, 29.2 g/L, and 12.4 g/L) (Fang and Zhong , 2002a; Habijanic et. al.,2013; Simonić et. al., 2008). The pH value sharply decreased from 0 h to 40 h, and the final pH value was 3.71 (Fig. 3A). Short cultivation time is a very important element for industrial application. The cultivation time of 52h in present study was the shortest compared to those values in the above reports (168h and 336h) (Simonić et. al., 2008; Tang et. al., 2011).

Excessive heavy met. al., content in mycelium from *G. lucidum* do harm to personal health, so it must be controlled in safe range. In previous study, the contents of Pb (3.17 ppm) and As (1.08 ppm) in dried fermented mycelium was obtained using industrial grade solid caustic soda and far exceed food safety standard. The Pb and As contents in those fermentation raw materials were determined to solving the solution, and the results showed in table 1.

Table 1. The contents of Pb and As in different fermentation raw materials(ppm)

Fermentation raw materials	As	Pb
Glucose	0.02	0.16
Sucrose	0.03	0.13
Soybean meal	0.24	0.43
Corn flour	0.04	0.56
Corn steep powder	0.11	0.60
Bean oil	0.03	0.12
Antifoam	0.04	0.31
Industrial grade solid caustic soda	5.12	8.11
Analytical grade solid caustic soda	0.11	0.08

All materials, except for industrial grade solid caustic soda, contained low contents of Pb and As (below 1ppm). Industrial grade solid caustic soda has high contents of Pb (5.12ppm) and As (8.11ppm). The Fig. 3B showed Pb content (3.17 ppm) and As content

(1.08 ppm) in the dried fermented mycelium using industrial grade solid caustic soda was 10.93 and 5.14 folds higher than Pb content (0.29 ppm) and As content (0.21 ppm) using analytical grade solid caustic soda which did not exceed the limits of Chinese national standards (Pb content below 1ppm, As content below 2 ppm).

4. Conclusions

In the work, the different effects of carbon sources, initial pH values and regulation of C/Ns on IPS content, pH value and mycelium concentration were investigated under submerged fermentation of *G. lucidum* in 1500-L fermentor, and then the optimal cultured conditions for IPS content were identified. Subsequently, the highest IPS content of 6.3%, dry weight of 17.85g/L and safe heavy met. al., contents was achieved during incubation of only 52h by amplification fermentation in a 10000-L fermentor under the optimum conditions. The result can promote industrial-level production for mycelium of *G. lucidum*.

5.References

Anonymous. (2005). Pharmacopoeia of People's Republic of China. Committee of Chinese Pharmacopoeia, Chemical Industry Press, Beijing.

Babitskaya, V. G., Shcherba, V. V., Puchkova, T. A., et al. (2005). Polysaccharides of *Ganoderma lucidum*: factors affecting their production. *Applied Biochemistry and Microbiology*, 41(2): 169-173

Chen, Z.J., Gu, Z.X. (2008). Progress of studies on the main active substances and submerged fermentation technology of *Ganoderma lucidum*. *Food Researh and Development*,29(3):186-190

Fang, Q. H., Zhong, J. J. (2002) Effect of initial pH on production of ganoderic acid and polysaccharide by submerged fermentation of *Ganoderma lucidum*. *Process Biochemistry* , 37(7): 769-774.

Habijanic, J., Berovic, M., Boh, B., et al. (2013). Production of biomass and polysaccharides of Lingzhi or Reishi medicinal mushroom, *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst.(higher Basidiomycetes), by submerged cultivation. *International journal of medicinal mushrooms*, 15(1):81-89

Hsiao,W. L.W., Li, Y. Q., Lee, T. L., et al. (2004). Medicinal mushroom extracts inhibit ras-induced cell transformation and the inhibitory effect requires the presence of normal cells. *Carcinogenesis*, 25(7): 1177-1183.

Kim, H. M., Paik, S., Ra, K. S., et al.(2006). Enhanced production of exopolysaccharides by fed-batch culture of *Ganoderma resinaceum* DG-6556. *Journal of microbiology*, 44(2): 233-242.

Lee, W. Y., Park, Y., Ahn, J. K., et al. (2007) Factors influencing the production of endopolysaccharide and exopolysaccharide from *Ganoderma applanatum*. *Enzyme and Microbial Technology*, 40(2): 249-254.

Mojadadi, S., Ebtekar, M., Hassan, Z. M. (2006). Immunomodulatory effects of Ganoderma lucidum (W. Curt.: Fr.) P. Karst.(aphyllophoromycetideae) on CD4+/CD8+ tumor infiltrating lymphocytes in breast-cancer-bearing mice. *International Journal of Medicinal Mushrooms*,8 (4): 315-320

Simonić, J., Stajić, M., Glamočlija, J.,et. al.,. (2008). Optimization of submerged cultivation conditions for extra- and intracellular polysaccharide production by medicinal Ling Zhi or Reishi mushroom *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Aphyllophoromycetideae). *International Journal of Medicinal Mushrooms*, 10 (4): 351-360

Sudheesh, N.P., Ajith, T.A., Janardhanan, K.K. (2009). *Ganoderma lucidum* (Fr.) P. Karst enhances activities of heart mitochondrial enzymes and respiratory chain complexes in the aged rat. *Biogerontology*.10(5):627-636

Tang, Y. J., Zhong, J. J. (2002) Fed-batch fermentation of *Ganoderma lucidum* for hyperproduction of polysaccharide and ganoderic acid. *Enzyme and Microbial Technology* , 31(1): 20-28.

Tang, Y. J., Zhang, W., Liu, R. S., et al. (2011). Scale-up study on the fed-batch fermentation of *Ganoderma lucidum* for the hyperproduction of ganoderic acid and *Ganoderma* polysaccharides. *Process Biochemistry*, 46(1): 404-408.

Xing, Z., Tang, Q., Zhou, C., et al. (2001) Analysis of trace elements and their safety in different *ganoderma* fruit bodies and coarse polysaccharide. *Mycosystema*, 21(1): 107-111.

Xu, L.C., Xu, S.C.(2004) Determination of content of polysaccharides of 18 varieties of *Ganoderma lucidum* karst with different origins. *World Science Technology-Modernization of Traditional Chinese Medicine*,6(2):57-60.

Yang, F. C., Liau,C.B.(1998). The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged cultures. *Process Biochemistry*, 33(5): 547-553.

Yang, H., Min, W., Bi, P., et al. (2013). Stimulatory effects of Coix lacryma-jobi oil on the myceliuml growth and metabolites biosynthesis by the submerged culture of *Ganoderma lucidum*. *Biochemical Engineering Journal*, 76: 77-82.

Yuan, B., Chi, X., Zhang, R (2012). Optimization of exopolysaccharides production from a novel strain of Ganoderma lucidum CAU5501 in submerged culture. *Brazilian Journal of Microbiology*, 43(2): 490-497.

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