INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
16–0095/2016/18–4–773–779
DOI: 10.17957/IJAB/15.0165
http://www.fspublishers.org

Full Length Article

Sonoporation of *Ganoderma lucidum* Mycelium for High Biomass and Exopolysaccharide Productivity in Submerged Culture

Shengrong Liu*, Weirui Zhang, Yunbo Kuang and Shengyang Zheng
Department of Biology, Ningde Normal University, Ningde 352100, China
*For correspondence: fjhost@163.com

Abstract

Ganoderma lucidum, known as Lingzhi in China and Reishi in Japan, is a famous medicinal mushroom that is commonly produced by solid-state fermentation. Recently, submerged culture has been considered as a promising alternative for production of *G. lucidum*. Herein, to provide a theoretical base for preparation of a homogenous inoculum for *G. lucidum* submerged culture we investigated the effect of sonoporation on *G. lucidum* mycelium. Submerged culture was also evaluated using the sonoporated mycelium. Microscopic investigation indicated that the mycelial pellets (0.55 g, dry weight) contained in 60 mL solution were almost completely disappeared within 20 s by sonoporation (20 kHz and 125 w). The homogenate obtained after sonoporation had a 3.75×10^6 cfu/mL cell concentration. The sonoporation caused rapid leakage of intracellular protein that increased linearly with time. The metabolic activity of the cells decreased rapidly after sonoporation. Sonoporation also negatively affected laccase activity. When the homogenate was used at 4.76% (v/v) inoculum level, the cultures produced biomass and exopolysaccharide of 15.75 and 0.54 g/L, respectively; these values were 129.26 and 45.95% higher than that of the control culture using a pellet-type inoculum without sonoporation. Optimal inoculum size was 5.88–6.97% (v/v). We conclude that sonoporation is an effective method for homogenization of *G. lucidum* mycelium, and sonoporated mycelium can improve productivity significantly in *G. lucidum* submerged culture and requires lower levels of starting inoculum. © 2016 Friends Science Publishers

Keywords: *Ganoderma lucidum*; Sonoporation; Homogenous inoculum; Submerged culture; Polysaccharide

Introduction

Ganoderma lucidum (Fr.) Karst is a species of basidiomycetes that belongs to polyporacceae (or Ganodermaceae) of Aphyllophorales. Its fan-like fruiting bodies with wood texture is commonly called "Lingzhi" in Chinese and "Reishi" in Japanese. For centuries in the Far East, the fruiting body of *G. lucidum* was regarded as a popular folk medicine for treating various human diseases, such as hepatitis, arthritis, nephritis, bronchitis, asthma, arteriosclerosis, hypertension, hypercholesterolemia, and gastric cancer (Jong and Birmingham, 1992; Paterson, 2006). The molecular mechanisms underlying the health and medical benefits of *G. lucidum* have been investigated extensively (Sone *et al*., 1985; Kimura *et al*., 2002; Chien *et al*., 2004). The major active components of *G. lucidum* include it's polysaccharide and ganoderic acid (Xu *et al*., 2010).

As the beneficial effect of *G. lucidum* on human health has been well-recognized, its demand is increasing rapidly in Asian countries, including China, Korea, Japan, and Thailand. However, *G. lucidum* occurs rarely in nature and hence, sufficient amounts of wild mushrooms cannot be collected for commercial exploitation. To meet the great demand for *G. lucidum*, artificial cultivation on solid substrates such as lignocellulosic materials, especially sawdust, supplemented with wheat or rice bran has been developed over the past several decades (Berovič *et al*., 2003), and is now a major method for producing *G. lucidum* products. However, the solid-state bioprocess has several significant disadvantages, including the requirement of a long production cycle and the continuous fluctuation of product quality (Tang and Zhong, 2002).

In recent years, submerged culture, a microbial bioprocess commonly used for producing various primary and secondary metabolites, has been utilized to cultivate higher basidiomycetes mushrooms for the production of biomass and various bioactive substances (Lee *et al*., 2003; Shih *et al*., 2006). The significant advantages of submerged culture over solid-state fermentation include shorter production period, lower production cost and easy regulation of environmental parameters (Bae *et al*., 2000; Chang *et al*., 2006). Consequently, submerged culture of *G. lucidum* as a promising alternative to solid-state fermentation has received special attention for the production of its biomass and bioactive substances (Xu *et al*., 2008; Zhang and Zhong, 2013).

As *G. lucidum* mainly grows in the form of mycelial

To cite this paper: Liu, S., W. Zhang, Y. Kuang and S. Zheng, 2016. Sonoporation of *Ganoderma lucidum* mycelium for high biomass and exopolysaccharide productivity in submerged culture. *Int. J. Agric. Biol*., 18: 773–779

pellets in liquid culture, its homogenization becomes essential before its inoculation into the fermentation medium. Currently, several techniques have been frequently used to prepare homogenous inocula of edible mushrooms, including the use of a blender (Jäger *et al*., 1985), a homogenizer (Lee *et al*., 2007), a pestle-mortar (Upadhyay *et al*., 2014), agitation in shake flasks containing glass beads (Emelyanova, 2005), and simply washed mat cultures (Zhu *et al*., 2008). Among these, the homogenizer method has been most frequently used.

In the present study, to provide a theoretical base for the preparation of an inoculum for *G. lucidum* submerged culture, the homogenization efficiency of the cells by sonoporation was examined. Its effects on cellular constituents leakage, metabolic activity, and laccase activity was also determined. Moreover, submerged culture using sonoporated mycelium as inoculum was examined for biomass and exopolysaccharide (EPS) production, followed by optimization of inoculum level. The present study demonstrated that sonoporation can immediately and efficiently homogenize the mycelium of *G. lucidum*, and the sonoporated mycelium as inoculum showed high productivity of biomass and EPS in submerged culture.

Materials and Methods

Strains and Media

G. lucidum, registered as G10016, was obtained from the Mycological Research Centre, Fujian Agriculture and Forestry University, Fuzhou, China. It was maintained on potato dextrose slants at 4°C and subcultured every four weeks. Growth was maintained at 25°C for 7 days.

The medium used for both seed culture and submerged culture consisted of the following components (per liter): glucose, 35 g; peptone, 5 g; yeast extract, 4 g; KH_2PO_4, 1.5 g; $MgSO_4$, 1 g; and vitamin B_1, 10 mg. It was autoclaved at 121°C for 20 min.

Culture Conditions

Slants were inoculated with *G. lucidum* mycelia in agar medium and incubated at 25℃ for 7 days. For the first pre-culture, an entire slant was squashed into approximately soybean-sized agar blocks with a sterile inoculated rake, and a half of the agar blocks were transferred into a 250 mL shake flask containing 80 mL medium. The inoculated flask was grown at 28°C for 4 days with shaking at 170 rpm. For the second pre-culture, the first pre-culture (5 mL) was inoculated into 80 mL medium in a 250 mL flask, and cultivated at 28°C for 3 days with shaking at 170 rpm.

For submerged culture, a 250 mL flask contained 80 mL medium was inoculated with 4 mL of the homogenate prepared by sonoporation (as described in the "Sonoporation" section) of the second pre-culture. Culture was carried out at 170 rpm and 28°C for 6 days. As a control, the second pre-culture (4 mL) without sonoporation in the pellet-type form was used. For the optimization of inoculum size, 80 mL of medium was inoculated with the homogenate varying from 1 mL to 7 mL. At the end of cultivation, samples were analyzed for biomass, EPS, and intracellular polysaccharide (IPS).

Sample Preparation

To prepare samples for microscopic examination, plate count, and investigation of the effects of sonoporation on the leakage of cellular constituents, laccase activity, and metabolic activity, 60 mL of the second pre-culture broth was used to harvest the mycelia (about 0.55 g, dry weight) by filtration on a sterile nylon cloth with 60 μm pore size that was then washed thoroughly with sterilized distilled water. The cells obtained were re-suspended in 60 mL sterile 0.75% saline in a 100 mL flask, and were employed for sonoporation for a total of 12 cycles (each cycle consisted of 5 s burst and 5 s break), and every two cycles samples were withdrawn. To prepare a homogenous inoculum for use in submerged culture, the second pre-culture broth of 60 mL was directly transferred into a 100 mL sterilized flask and sonoporated for 12 cycles.

Sonoporation

An ultrasonicator (JY96-IIN, Ningbo Xingzhi Bioscience and Biotechnology Co., Ltd, Ningbo, China) was used, which provided a frequency of 20 kHz and a horn tip of 6 mm. The operation was as follows: the tip after disinfected with 75% ethanol was immersed into the samples contained in 100 mL flasks, with a distance of approximately 1 cm above the bottom of the flasks. Energy at a power of 125 w was applied in pulse mode with a 5 s burst and 5 s break. To prevent overheating, the flasks were placed in an ice bath during the process.

Microscopic Investigation

Sonoporated samples were initially diluted 10-fold. Aliquots of 50 μL were pipetted onto a clean slide and covered with a coverlip. A microscope with a 10X objective (DM750, Leica, Germany) and equipped with a camera (Leica DMC2900, Leica, Germany) was used for analysis.

Plate Count

Sonoporated samples were diluted 10-fold serially up to 10000-fold. After vortexing, aliquots of 0.2 mL were uniformly spread on PDA agar medium in a Petri dish (9 cm diameter) and sealed with a Parafilm membrane (Polisciences Inc., Warrington, USA). After incubation at 25°C for 3 d, the number of colonies was counted and expressed as cfu/mL. For comparison, the number of pellets in 5 mL the second pre-culture was visually counted, and averaged to obtain the starting value of cfu/mL.

Protein Leakage Assay

The protein concentration was determined using the dye binding method as described by Bradford (1976), using bovine serum albumin (BSA) as a standard.

Laccase Activity Assay

Laccase activity was assayed according to the method of Bourbonnais and Paice (1990) using 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate. The assays were conducted in a 3 mL reaction mixture consisting of 2.5 mL of 0.1 M sodium acetate buffer (pH 5.0), 0.2 mL of 1 mM ABTS solution, and 0.3 mL supernatant of the homogenate. The change in absorbance at 420 nm was recorded over 3 min and one activity unit was defined as the amount of enzyme that oxidized 1 µmol ABTS/min. The extinction coefficient of 3.6×10^4 mol^{-1} cm^{-1} was used for oxidized ABTS (Bourbonnais and Paice, 1990).

Metabolic Activity Assay

Fluorescent dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was utilized for determining metabolic activity of the cells. It was dissolved in sterilized phosphate-buffered saline (PBS) buffer at 5 mg/mL and stored at -20°C. Before use, the stock MTT solution was thawed at room temperature. Samples of 0.5 mL and 0.1 mL MTT solution were subsequently added into 0.4 mL sterile water with a total volume of 1 mL in a 5 mL vial, yielding a final MTT concentration of 0.5 mg/mL. Samples were stained for 8 h at 28°C on a rotary shaker at 120 rpm.

After staining, the mycelia were harvested and washed twice with distilled water by centrifugation at 8000 rpm for 10 min. For extraction of the MTT-formazan, the mycelial pellets were suspended in 3 mL acidified isopropyl alcohol (0.04 M/L HCl in isopropanol) and shaken at 150 rpm in a rotary incubator for 5 h. After centrifugation, the absorbance of the supernatants was measured at 570 nm with acidified isopropyl alcohol as the control. For improving accuracy, the initial metabolic activity was determined from a large volume sample (5 mL) without sonoporation, and then its initial value in 0.5 mL volume was calculated. The initial metabolic activity was considered as 100%.

Biomass Measurement

As *G. lucdium* mainly grows as a pellet form in liquid fermentation, obtaining a sample from a flask culture by a pipette was not feasible. For this reason, three flasks were taken as three replicates at the end of submerged culture. Whole culture broth in each shake flask was used to separate the mycelial biomass by centrifuging at 8000 g for 10 min. The precipitated mycelia were washed three times with distilled water, and dried at 60°C for a sufficient time to a constant weight.

Measurement of Polysaccharides

For determination of EPS, the crude EPS in supernatants was precipitated by the addition of four times the volume of 95% (v/v) ethanol and left overnight. The insoluble components were harvested by centrifugation at 8000 g for 10 min, suspended in 1 M NaOH, and kept at 60°C for 1 h. After centrifugation, the resultant supernatant was used for EPS measurement. For IPS determination, the dried mycelia (100 mg) were ground into fine powder (60 mesh), and then suspended in 5 mL of 1 M NaOH at 60°C for 1 h. The supernatant was obtained and used for analysis. Both supernatants were measured by the phenol-sulfuric acid method (Dubois *et al.*, 1956).

Statistical Analysis

All experiments were performed in triplicate and data were analyzed using SAS (version 8; SAS Institute, Inc., Cary, NC, USA). Results are expressed as the mean ± SD.

Results

Visual and Microscopic Observation of Sonoporated Mycelia and Plate Count Assay

Fig. 1 shows the morphology of pellets of *G. lucidum* before and after sonoporation. Fig. 1A shows that *G. lucidum* grew mainly as large fluffy pellets in submerged culture and also many spike-like mycelia can be observed on the surface of the pellets (Fig. 1B). Fig. 1C shows the complete homogenization of the pellets after 60 s sonoporation. Here, it should be noted that most of the pellets were disappeared within 10 s sonoporation. Fig. 1D-F shows mycelia by microscopy after different sonoporation times and from these it appears that the homogenization content of mycelia increased with sonoporation time.

As shown in Fig. 2, the cell (in the form of hyphal fragment and mycelial aggregate) concentration increased sharply from an initial value of 1.26 log cfu/mL to 5.87 log cfu/mL within 10 s sonoporation, indicating that the mycelial pellet of *G. lucidum* can be immediately and effectively homogenized. At 20 s sonoporation it increased to 6.48 log cfu/mL, and thereafter the increase was not obvious, suggesting that the homogenization content of mycelia did not significantly improve. This result was in general accordance with our microscopic examination, which showed that most of the pellets were disappeared by 10 s sonoporation. By combining the microscopic data with the plate count, it can be concluded that sonoporation can immediately and effectively homogenize the liquid-cultured pellets of *G. lucidum*.

Table 1: Results of biomass, EPS, and IPS in flask cultures using either sonoporated or unsonoporated mycelia as inoculums

Inoculum[a]	Biomass (g/L)	EPS[b] (g/L)	IPS[b] (mg/100 mg dry weight)
Sonoporated mycelia	15.75 ± 1.32	0.54 ± 0.08	1.59 ± 0.06
Unsonoporated mycelia	6.87 ± 0.74	0.37 ± 0.05	1.87 ± 0.09

[a]Inoculum size was 4 ml at 4.76% (v/v)
[b]EPS, exopolysaccharides; IPS, intracellular polysaccharides

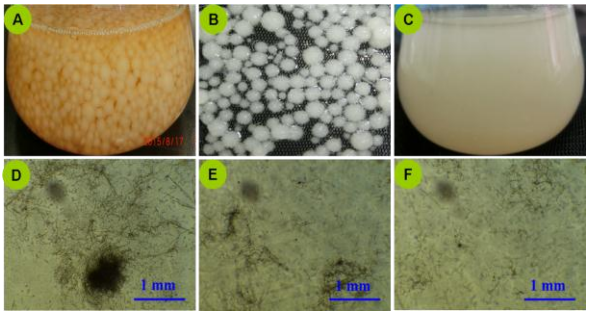

Fig. 1: Images of *G. lucidum* mycelia before and after sonoporation. *A.* Morphology of submerged mycelia as large fluffy pellets in submerged culture before sonoporation. *B.* Morphology of pellets before sonoporation showing spike-like mycelia on the surface of the pellets. *C.* Mycelial suspension after sonoporation for 60 s; *D-F*. Mycelia after sonoporation for 10 s, 20 s, and 60 s, respectively

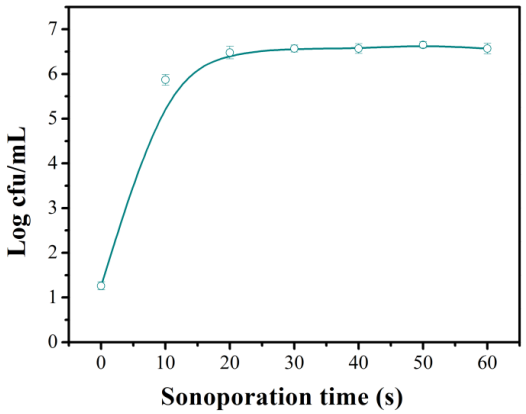

Fig. 2: Changes in the cell number after sonoporation of *G. lucidum* mycelia at the indicated time points

Effects of Sonoporation on Leakage of Cellular Constituents, Laccase Activity and Metabolic Activity

Leakage of intracellular protein is often used as an indicator for assessing cell damage caused by physical, chemical, and biological treatment (Woo *et al*., 2000). Time profile of protein leakage, leaked laccase activity and residual metabolic activity of *G. lucidum* mycelia during sonoporation is shown in Fig. 3. Substantial protein leakage from the mycelium was detected after 10 s sonoporation, and increased almost linearly with time in the period tested (0–60 s), possibly indicating that the content of fragmented mycelia steadily improved. However, the cell concentration remained almost unchanged as compared to the sample after 20 s sonoporation (see Fig. 2). This inconsistency may be due to the strong aggregation of the fragmented mycelia in the subsequent experiments, which led to the reduction of number of hyphal fragment and mycelial aggregate, since the mycelial aggregation is a common phenomenon manifested by filamentous fungi in liquid culture (Yang *et al*., 2009).

Laccase is one of the most important enzymes involved the degradation of lignocellulosic wastes, and produced by various white-rot fungi like *G. lucidum*. Fig. 3 indicates that significant laccase activity was detected after 10 s sonoporation. However, only a trend towards increase was observed after that time point. Apparently, laccase activity did not positively correlate with the amount of protein leakage, perhaps due to the inactivation of laccase that may have occurred in the regions of very high temperature and pressure during the collapse of cavitation bubbles during sonoporation (Ho *et al*., 2006).

As shown in Fig. 3, the metabolic activity of the fungus decreased considerably from 100% to 63.12% within 10 s sonoporation, and then decreased to 45.53% after a further 10 s treatment. Thereafter, it decreased at a very low rate. The sharp decrease in the initial period (0–20 s) may be mainly due to the mycelial fragmentation, since most of the pellets were fragmented in this period, as observed by microscopic examination. On the other hand, little loss of the metabolic activity post the 20 s sonoporation time point suggests that the activity of the fragmented mycelia was not significantly influenced by sonoporation.

Performance of Sonoporated Mycelia as Inoculum for Submerged Culture

The comparative results of submerged culture with the sonoporated and unsonoporated (the control) mycelia as inocula are listed in Table 1, and the corresponding morphology of pellets is shown in Fig. 4. As presented in Table 1, the production of biomass and EPS reached 15.75 g/L and 0.54 g/L, respectively in cultures with the sonoporated inoculum. These were 129.26% and 45.95% higher than those obtained in the control culture (6.87 g/L and 0.37 g/L). Significantly enhanced productivity may be closely related to the high amounts of hyphal fragment and mycelial aggregates as growing points in the culture broth,

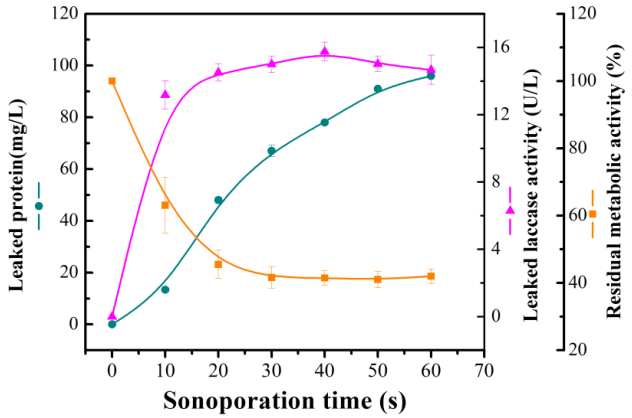

Fig. 3: Time profiles of intracellular protein leakage from *G. lucidum* mycelia, leaked laccase activity and residual metabolic activity after sonoporation

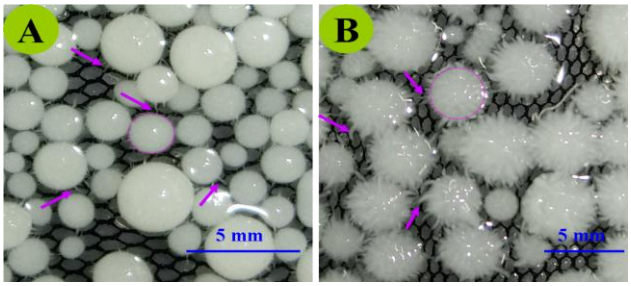

Fig. 4: The morphology of pellets of *G. lucidum* grown in submerged cultures using (A) sonoporated mycelia and (B) pellet-type mycelia (without sonoporation) as inoculums. *Circles* indicate the core regions of the pellets, and *arrows* point to the hyphae growing on the outer region of pellet

resulting in rapid growth and complete utilization of the substrate. Fig. 4 shows that small, smooth and compact pellets were formed in cultures using the sonoporated mycelium as homogenized inoculum, while large, fluffy and loose pellets were formed in the control culture (pellet-type inoculum), perhaps due to the difference in the hyphal growing points in the culture broth.

Effect of the Inoculum Size of Sonoporated Mycelium on Biomass and EPS Production

As shown in Fig. 5, the final biomass production generally increased with increasing inoculum, up to 6 mL (6.97%, v/v), and there was still a slight increase when the inoculum was increased to 7 mL (8.05%, v/v). However, compared with the gross trend of biomass increase, an increase in the inoculum level from 2 mL (2.44%, v/v) to 3 mL (3.61%, v/v) was not obvious. It can be reasonably assumed that the concentration of the hyphal fragment and mycelial aggregate at this inoculum level in culture broth favored mycelial aggregation, resulting in little increase in hyphal growing points for the culture with increased inoculum level.

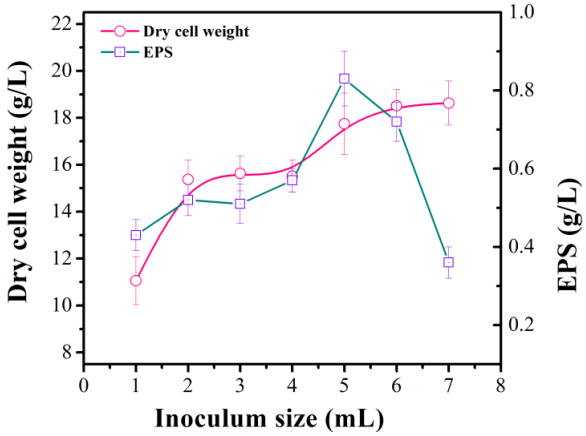

Fig. 5: Effect of inoculum size of the sonoporated mycelia of *G. lucidum* on the production of biomass and EPS

The EPS production increased with an increase in inoculum size up to 5 mL (5.88%, v/v); however, a further increase in inoculum size led to a reduction in EPS production. Several researchers have reported that relatively high sugar concentrations were beneficial for EPS production (Fang and Zhong, 2002; Kim *et al*., 2006). We, therefore, proposed that the reduction in final EPS production may be related to the low level of glucose present in culture broth during the late culture period, since high inoculum level promoted mycelial growth significantly and thereby consumed much more glucose. From these data, it can be concluded that optimized inoculum level for biomass growth and EPS production is different.

Discussion

Sonoporation that utilizes ultrasound to generate microbubbles is known to cause chemical and physical changes in biological structures (in a liquid medium), owing to the rapid formation and destruction of the generated bubbles (Koolman *et al*., 2014). Currently, in the field of biotechnology, sonoporation has a broad range of applications, including its use for inhibition of food-borne pathogens in processed food (Forghani *et al*., 2013; Koolman *et al*., 2014), disruption of various kinds of cells (Ren *et al*., 2007; Sudar *et al*., 2013; Zhou *et al*., 2014), and assistance in bioactive component extraction (Kwun *et al*., 2009; Liu *et al*., 2011). The present study demonstrated that sonoporation can immediately and effectively homogenize liquid-cultured mycelial pellets of *G. lucidum*, and highly uniform mycelia can be obtained. The fermentation potential of sonoporated mycelium of *G. lucidum* is pronounced, although the process of sonoporation negatively affected the fungus. To our knowledge, this is the first report on the use of sonoporation for preparing homogenized mycelia for use in submerged fermentation of mushrooms.

In the present study, we find that using the

sonoporated mycelia as homogenous inoculum significantly improves the production of biomass and EPS production as compared to the pellet-type inoculum. Besides this, the inoculum level can be significantly reduced, since in *G. lucidum* pellet-type submerged fermentation, it is usually required at 10% (v/v) or above (Tang and Zhong, 2002; Liu and Zhang, 2007). On the other hand, the morphology of mycelia should be considered seriously during the development of submerged culture as it significantly affects the formation of metabolites and product recovery (Du *et al*., 2003; Papagianni, 2004). As to the edible and medicinal mushrooms, the formation of pellets is generally considered as excellent. Therefore, the formation of small compact pellets as observed in this work may be an additional advantage of the homogenized inoculum prepared by sonoporation for submerged culture. Taken together, the inoculum prepared by sonoporation holds great promise for use in large scale industrial submerged culture.

In previous studies, a homogenizer-like polytron operated at a high agitation speed has been frequently used to obtain uniform inoculum for fungal liquid fermentation. In the present study, sonoporation appears to be a suitable approach for the preparation of homogenized inoculum of *G. lucidum*. Compared to the homogenizer method, a major unobvious advantage of sonoporation is that it can be easily integrated with a fermentor, since the horn tip can be separated from the ultrasonicator. Therefore, sonoporation as a means of disrupting the mycelia of edible and medicinal mushrooms for obtaining homogenized inocula on a large scale holds great potential for industrial use.

Conclusion

The present study demonstrated that sonoporation can effectively and quickly homogenize *G. lucidum* mycelia. Despite the fact that the sonoporation process negatively affected the mycelia of *G. lucidum* in terms of protein leakage, laccase activity, and metabolic activity, these mycelia as an inoculum still showed high biomass and exopolysaccharide productivity in submerged fermentation, and moreover desirable mycelia morphology was formed.

Acknowledgements

The authors would like to thank the financial support by the Program of Talent Introduction of Ningde Normal University (no. 2013Y008).

References

Bae, J.T., J. Sinha, J.P. Park, C.H. Song and J.W. Yun, 2000. Optimization of submerged culture conditions for exo-biopolymer production of *Paecilomyces japonica*. *J. Microbiol. Biotechnol.*, 10: 482−487

Berovič, M., J. Habijanič, I. Zore, B. Wraber, D. Hodžar, B. Boh and F. Pohleven, 2003. Submerged cultivation of *Ganoderma lucidum* biomass and immunostimulatory effects of fungal polysaccharides. *J. Biotechnol.*, 103: 77−86

Bourbonnais, R. and M.G. Paice, 1990. Oxidation of non-phenolic substrates, an expanded role for laccase in lignin biodegradation. *FEBS Lett*., 267: 99−102

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72: 248−254

Chang, M.Y., G.J. Tsai and J.Y. Houng, 2006. Optimization of the medium composition for the submerged culture of *Ganoderma lucidum* by Taguchi array design and steepest ascent method. *Enzyme Microb. Tech.*, 38: 407−414

Chien, C.M., J.L. Cheng, W.T. Chang, M.H. Tien, C.M. Tsao, Y.H. Chang, H.Y. Chang, J.F. Hsieh, C.H. Wong and S.T. Chen, 2004. Polysaccharides of *Ganoderma lucidum* alter cell immunophenotypic expression and enhance $CD56^+$ NK-cell cytotoxicity in cord blood. *Bioorg. Med. Chem.*, 12: 5603−5609

Du, L.X., S.J. Jia and F.P. Lu, 2003. Morphological changes of *Rhizopus chinesis* 12 in submerged culture and its relationship with antibiotic production. *Process Biochem.*, 38: 1643−1646

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, 28: 350−356

Emelyanova, E.V., 2005. Effects of cultivation conditions on the growth of the basidiomycete *Coriolus hirsutus* in a medium with pentose wood hydrolyzate. *Process Biochem.*, 40: 1119−1124

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

Forghani, F., S.M.E. Rahman, M.S. Park, J.H. Park, J. Park, K.B. Song and D.H. Oh, 2013. Ultrasonication enhanced low concentration electrolyzed water efficacy on bacteria inactivation and shelf extension on lettuce. *Food Sci. Biotechnol*., 22: 131−136

Ho, C.W., T.K. Chew, T.C. Ling, S. Kamaruddin, W.S. Tan and B.T. Tey, 2006. Efficient mechanical cell disruption of *Escherichia coli* by an ultrasonicator and recovery of intracellular hepatitis B core antigen. *Process Biochem.*, 41: 1829−1834

Jäger, A., S. Croan and T.K. Kirk, 1985. Production of ligninases and degradation of lignin in agitated submerged cultures of *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.*, 50: 1274−1278

Jong, S.C. and J.M. Birmingham, 1992. Medicinal benefits of the mushroom *Ganoderma*. *Adv. Appl. Microbiol.*, 37: 101−134

Kim, H.M., S.Y. Paik, K.S. Ra, K.B. Koo, J.W. Yun and J.W. Choi, 2006. Enhanced production of exopolysaccharides by fed-batch culture of *Ganoderma resinaceum* DG-6556. *J. Microbiol*., 44: 233−242

Kimura, Y., M. Taniguchi and K. Baba, 2002. Antitumor and antimetastatic effects on liver of triterpenoid fractions of *Ganoderma lucidum*: Mechanism of action and isolation of an active substance. *Anticancer Res.*, 22: 3309−3318

Koolman, L., P. Whyte, J. Meade, J. Lyng and D. Bolton, 2014. A combination of chemical and ultrasonication treatments to reduce *Campylobacter jejuni* on raw poultry. *Food Bioprocess Technol*., 7: 3602−3607

Kwun, K.H., G.J. Kim and H.J. Shin, 2009. Ultrasonication assistance increases the efficiency of isoflavones extraction from Kudzu (*Pueraria lobata* Ohwi) roots waste. *Biotechnol. Bioprocess Eng*., 14: 345−348

Lee, B.C., J.T. Bae, H.B. Pyo, T.B. Choe, S.W. Kim, H.J. Hwang and Y.W. Yun, 2003. Biological activities of the polysaccharides produced from submerged culture of the edible Basidiomycete *Grifola frondosa*. *Enzyme Microb. Tech.*, 32: 574−581

Lee, W.Y., Y. Park, J.W. Ahn, K.H. Ka and S.Y. Park, 2007. Factors influencing the production of endopolysaccharide and exopolysaccharide from *Ganoderma applanatum*. *Enzyme Microb. Tech.*, 40: 249−254

Liu, G.Q. and K.C. Zhang, 2007. Enhancement of polysaccharides production in *Ganoderma lucidum* by the addition of ethyl acetate extracts from *Eupolyphaga sinensis* and *Catharsius molossus*. *Appl. Microbiol. Biotechnol*., 74: 572−577

Liu, Y.M., T.N. Chen, Y.P. Qiu, Y. Cheng, Y. Cao, A.H. Zhao and W. Jia, 2011. An ultrasonication-assisted extraction and derivatization protocol for GC/TOFMS-based metabolite profiling. *Anal. Bioanal. Chem.*, 400: 1405−1417

Papagianni, M., 2004. Fungal morphology and metabolite production in submerged mycelial process. *Biotechnol. Adv.*, 22: 189−259

Paterson, R.R.M., 2006. *Ganoderma*–A therapeutic fungal biofactory. *Phytochemistry*, 67: 1985−2001

Ren, X.D., D.W. Yu, L.Yu, G. Gao, S.P. Han and Y. Feng, 2007. A new study of cell disruption to release recombinant thermostable enzyme from *Escherichia coli* by thermolysis. *J. Biotechnol*., 129: 668−673

Shih, I.L., K. Pan and C. Hsieh, 2006. Influence of nutritional components and oxygen supply on the mycelial growth and bioactive metabolites production in submerged culture of *Antrodia cinnamomea*. *Process Biochem.*, 41: 1129−1135

Sone, Y., R. Okuda, N. Wada, E. Kishida and A. Misaki, 1985. Structures and antitumor activities of the polysaccharides isolated from fruiting body and the growing fermentation of mycelium of *Ganoderma lucidum*. *Agric. Biol. Chem.*, 49: 2641−2653

Sudar, M., D. Valinger, Z. Findrik, D. Vasić-Rački and Ž. Kurtanjek, 2013. Effect of different variables on the efficiency of the Baker's yeast cell disruption process to obtain alcohol dehydrogenase activity. *Appl. Biochem. Biotechnol.*, 169: 1039−1055

Tang, Y.J. and J.J. Zhong, 2002. Fed-batch fermentation of *Ganoderma lucidum* for hyperproduction of polysaccharide and ganoderic acid. *Enzyme Microb. Tech.*, 31: 20−28

Upadhyay, M., B. Shrivastava, A. Jain, M. Kidwai, S. Kumar, J. Gomes, D.G. Goswami, A.K. Panda and R.C. Kuhad, 2014. Production of ganoderic acid by *Ganoderma lucidum* RCKB-2010 and its therapeutic potential. *Ann. Microbiol*., 64: 839−846

Woo, I.S., I.K. Rhee and H.D. Park, 2000. Differential damage in bacterial cells by microwave radiation on the basis of cell wall structure. *Appl. Environ. Microbiol*., 66: 2243−2247

Xu, J.W., W. Zhao and J.J. Zhong, 2010. Biotechnological production and application of ganoderic acids. *Appl. Microbiol. Biotechnol*., 87: 457−466

Xu, P., Z.Y. Ding, Z. Qian, C.X. Zhao and K.C. Zhang, 2008. Improved production of mycelial biomass and ganoderic acid by submerged culture of *Ganoderma lucidum* SB97 using complex media. *Enzyme Microb Tech.*, 42: 325−331

Yang, F.C., M.J. Yang and S.H. Cheng, 2009. A novel method to enhance the mycelia production of *Ganoderma lucidum* in submerged cultures by polymer additives and agitation strategies. *J. Taiwan Inst. Chem. Eng.*, 40: 148−154

Zhang, W.X. and J.J. Zhong, 2013. Oxygen limitation improves Ganoderic acid biosynthesis in submerged cultivation of *Ganoderma lucidum*. *Biotechnol. Bioprocess Eng.*, 18: 972−980

Zhou, N.D., X.L. Gu, X.H. Zha and Y.P. Tian, 2014. Purification and characterization of a urethanase from *Penicillium variabile*. *Appl. Biochem. Biotechnol.*, 172: 351−360

Zhu, L.W., J.J. Zhong and Y.J. Tang, 2008. Significance of fungal elicitors on the production of ganoderic acid and *Ganoderma* polysaccharides by the submerged culture of medicinal mushroom *Ganoderma lucidum*. *Process Biochem.*, 43: 1359−1370

(Received 20 February 2016; Accepted 10 March 2016)