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Breaking the spores of the fungus *Ganoderma lucidum* by supercritical CO_2

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ABSTRACT

The hard sporoderm of *Ganoderma lucidum* spores prevents the release of bioactive components such as polysaccharides which have significant anti-tumour activity. In the present study, supercritical carbon dioxide (SC–CO_2) was used for the sporoderm breaking of *G. lucidum* spores, and polysaccharides were subsequently extracted and determined for evaluating the performances of SC–CO_2. The operating parameters were optimized by orthogonal array design (OAD), and the morphological status of sporoderm was observed by scanning electron microscope (SEM). The optimum operating conditions for SC–CO_2 breaking of sporoderm were as follows: pressure 35 MPa, temperature 25 °C, time 4 h, and CO_2 flow rate 10 kg/h. After SC–CO_2 processing, the extraction yield of polysaccharides reached 2.98%, which was 3-fold to that of the intact ones (0.94%). This method is fast, efficient and advanced enough to break the hard sporoderm of *G. lucidum*, which may provide a scientific reference for the large-scale processing of spores in the pharmaceutical and food industries.

1. Introduction

Ganoderma lucidum (Fr.) Karst. is a species of basidiomycetes which belongs to Ganodermataceae of Aphyllophorales (Yang, Ke, & Kuo, 2000). Its fruiting body is called "Lingzhi" in China and "Reishi" in Japan. For thousands of years, this fungus has been regarded as a traditional Chinese medicine (TCM) or a folk medicine for its medicinal properties. *G. lucidum* has been used for promotion of vitality and longevity (Lu et al., 2004) and prevention and treatment of various human diseases in China and other Asian countries. It is used for the treatment of asthma, diabetes, altitude sickness, cardiovascular disease, AIDS and cancer (Lin, 2001; Shiao, 2003; Wasser & Weis, 1999). *G. lucidum* appears to be very safe since oral administration of the extracts does not display any toxicity (Eo, Kim, Lee, & Han, 1999a, 1999b), and its merits have been investigated as a potential prophylactic agent for human health (Kim & Kim, 1999).

Though the fruiting body of G. *lucidum* has been utilised as medicine for several thousand years in China, its spores were recognized and utilised only in the 20th century. *G. lucidum* spores are tiny and mistlike particles of about 6.5–8.0 × 9.6–12.6 μm enwrapped with outer bilayers of sporoderm. The extremely hard and resilient sporoderm is a barrier for the release of components inside the spores. Previous studies suggest the spores contain a variety of bioactive components, including polysaccharides, triterpenoids, sterols, proteins, nucleosides, fatty acids and cerebrosides, as in the fruiting body (Yeung, Lu, Zhang, & Go, 2004). However, the bioactivity of the spores may be much higher than that of the fruiting body (Min, Nakamura, Miyashiro, Bae, & Hattori, 1998). Recent studies on Ganodermataceae fungi have demonstrated that the spores show significant anti-tumour (Zhu, Yang, Wang, Zhao, & Chen, 2000), anti-aging (Gan, Fann, Hsu, Kuo, & Lin, 1998), free radical-scavenging and anti-hepatotoxic (Lin, Lin, Chen, Ujiie, & Takada, 1995; Yen & Wu, 1999), as well as anti-human immunodeficiency virus-1 protease activities (Min et al., 1998). However, the activities of spores are closely related to the status of sporoderm. When the sporoderm is not broken, there are fewer pharmacological effects observed and, on the contrary, the breaking of the sporoderm can improve the release of active components and exertion of their effects (Min et al., 1998).

Although the sporoderm-broken spores are more effective than the intact spores, it is difficult to break the sporoderm because of the limitation of general breaking technology. In previous studies, soaking, physical smashing, ultrasonic, high pressure and enzyme hydrolysis methods have been used to break the sporoderm (Li, Li, Zhang, Xing, & Kang, 2004; Ni, Song, Li, & Xie, 2002; Wu, Zhang, & Zhu, 2004; Xie et al., 2006). The physical smashing method was performed by grinding the spores with an ultra smashing machine, the heat released during the process led to high temperature, which induced the decomposition of bioactive components. The soaking, ultrasonic and high pressure technology had disadvantages, such as low breaking yield, time-consumption and higher cost for special machines. The enzyme hydrolysis method was

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conducted by digesting the sporoderm with different types of enzymes released from mycelia, the process was slow and excessive digestion would decompose the bioactive components (Xie et al., 2006). In addition, it was reported that sporoderm-broken spores, generated by different breaking methods, exhibited different inhibitory effects on cancer cell growth (Xie et al., 2006), which indicated that the breaking method played an important role in the bioactivities of spores. Therefore, there is a need to develop an advanced and efficient method for sporoderm breaking of *G. lucidum*, which will provide high quality spore products. SC–CO_2 breaking of *G. lucidum* spores could be an alternative method because of the unique advantages of SC–CO_2.

SC–CO_2, an environment-friendly solvent, has a relatively high liquid-like density, low viscosity and high diffusivity (Lang & Wai, 2001). These properties provide a very special solvent that is effective both at dissolving materials and penetrating solid matrices (Taylor, 1996). In the process of SC–CO_2 treatment, the temperature is relatively low and the organic solvent is excluded, so decomposition of active components is avoided. Moreover, during the dynamic process, fresh SC–CO_2 flows through materials continuously; the mass transfer is intensive, which may result in a satisfactory performance. In view of these unique characteristics, SC–CO_2 processing of spores is proposed as a potential breaking method for the sporoderm of *G. lucidum*.

In the present study, sporoderm breaking of *G. lucidum* with SC–CO_2 is presented for the first time. The polysaccharides were extracted and determined to evaluate the performances of SC–CO_2 processing under different operating conditions. The effects of various process parameters, including operating pressure, operating temperature, time and CO_2 flow rate, were investigated for the optimization of the SC–CO_2 breaking conditions. The morphological changes between the intact and processed spores were observed by SEM.

2. Materials and methods

2.1. Plant material and reagents

G. lucidum spores were collected from the forestry centre of Northeast Forestry University and authenticated by Prof. Shao-Quan Nie from the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, Harbin, PR China. Voucher specimens were deposited in the herbarium of this Key Laboratory. The spores were dried at 50 °C for 24 h. Phenol (analytical reagent) and sulphuric acid (chemical reagent) were bought from Tianjin Kermel chemical reagents development centre (China). Carbon dioxide (purity 99.99%) was purchased from Liming gas corp. (China). Glucose was purchased from the Institute of Medicine and Bio-product of China. Stock solution of glucose was made by dissolving 1 mg standard in 10 ml of distilled water. Respective solutions for the calibration curve were prepared by serial dilutions of the stock solution with distilled water.

2.2. SC–CO_2 breaking of spores

A HA121-50-01 SFE device (Hua'an Supercritical Fluid Extraction corp., Nantong, China) was used to break *G. lucidum* spores. The operating methodology was as follows: liquid CO_2 was cooled by ethanol to −5 °C before being pressurized and passed into the device, the entire device was also pre-pressurized. Approximately 200 g of spores were loaded into a steel cylinder equipped with mesh filters (6.5 μm) on both ends, the filled cylinder was then introduced into the extraction vessel, and CO_2 was let in. During the breaking process, the operating pressure and CO_2 flow rate were controlled by adjusting the valves according to the pressure metre and flow metre; the temperature was set by the temperature controller. When the scheduled time was achieved, the extraction vessel was depressurized and the spores were taken out and collected.

2.3. Extraction of polysaccharides

The intact and processed *G. lucidum* spores (2 g) were respectively extracted with 100 ml of distilled water at 75 °C for 6 h under stirring. The extracts were finally concentrated under vacuum with a rotary evaporator device. The polysaccharides-enriched fractions were precipitated by adding ethanol, filtering, and solid polysaccharides were obtained on the filter paper. Distilled water was added to the solid polysaccharides to get a sample solution for determination.

2.4. Quantification of polysaccharides

The total polysaccharides concentration was determined by a modification of the phenol–sulphuric acid method (Taylor, 1995). A calibration curve was constructed, based on six different concentrations of glucose over the range 1.25–12.5 μg/ml. The curve was obtained by plotting the glucose absorbance at 488 nm versus glucose concentration in μg/ml, and a good linearity was found. The regression equation was $Y = 0.0519X - 0.0003$ ($r = 0.9954$, $n = 6$), where X represents glucose concentration (μg/ml) and Y represents the glucose absorbance.

One millilitre of the polysaccharides sample solution obtained above was prepared in triplicate; 5% phenol–sulphuric reagent (1 ml) and concentrated H_2SO_4 (5 ml) were subsequently added, and the mixture cooled to room temperature. The absorbance at 488 nm for each polysaccharides sample was determined with a Shimadzu UV-2550 UV–visible spectrophotometer (Shimadzu corp., Japan).

2.5. SEM observation

The intact and processed spores were examined by SEM to observe any morphological change that occurred in the sporoderm during the SC–CO_2 treatments. The spores were mounted on aluminium stubs, coated with gold-palladium in a sputter coater and viewed at 15 kV accelerating voltage in a Hitachi S-520 field emission scanning electron microscope (Hitachi Inst., Inc., San Jose, CA).

2.6. Experimental design

Orthogonal array design (OAD) was used to arrange the experiments and optimize the breaking parameters for *G. lucidum* spores. The effects of operating pressure, temperature and time on the breaking performance were investigated. A L_{16} (4^5) orthogonal matrix with three factors (two unarranged columns were used for error variance estimation); each factor, containing four levels, was selected to arrange the experiments. Pressures (P) were 27, 30, 33, 35 MPa, temperatures, (T) were 25, 30, 35, 40 °C, and time (t) were 1, 2, 3, 4 h.

3. Results and discussion

3.1. Effect of CO_2 flow rate

The effect of CO_2 flow rate on the breaking performance was investigated under the following conditions: 200 g *G. lucidum* spores were processed for 1, 2, 3, and 4 h, with the CO_2 flow rate being 4, 6, 8, 10, 12, 14 kg/h, respectively. The processed spores were extracted and the extraction yields of polysaccharide were determined, the results are shown in Fig. 1.

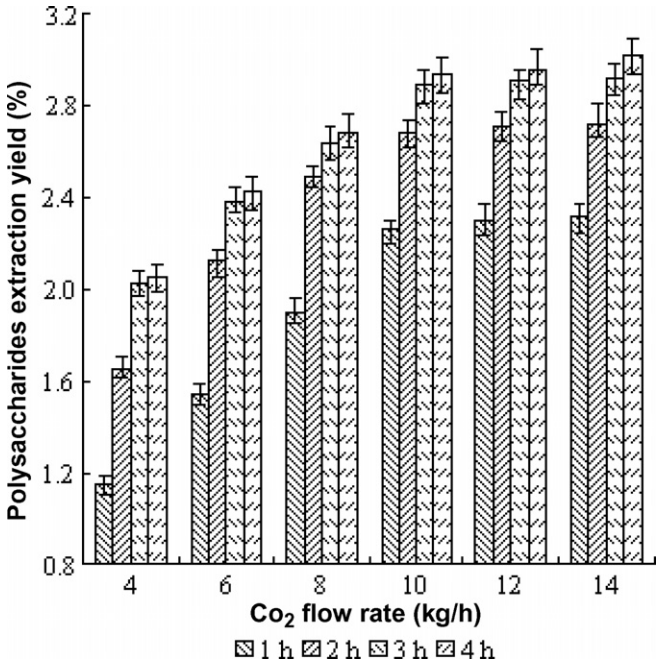

Fig. 1. Effect of CO_2 flow rate on the extraction yields of polysaccharides.

It can be seen from Fig. 1, for all four different times, that the effects of CO_2 flow rate on polysaccharide extraction yields were similar i.e., in the range 4–10 kg/h, the yields obviously increased with the CO_2 flow rate. When CO_2 flow rate exceeded 10 kg/h, the yields did not increase much further. It was known that, when the flow rate was higher, more SC–CO_2 passed through the spores at any time; thereby the effect on penetrating the sporoderm was stronger, resulting in a subsequent higher extraction yield. Similar results for supercritical fluid extraction (SFE) of jojoba seed oil and sunflower seed oil have been reported; the mass transfer parameter increased with the supercritical fluid flow rate, normally, until it reached an asymptotic value (Cocero & García, 2001; Salgın, 2007). In this study, treatment at the CO_2 flow rate of 10 kg/h gave close to the highest extraction yield of polysaccharides.

3.2. Effects of operating pressure, temperature and time

The effects of three factors, including operating pressure, temperature and time, on the breaking performance were investigated using a L_{16} (4^5) orthogonal matrix with the CO_2 flow rate at 10 kg/h. The results are summarized in Table 1. The results of analysis of variance (ANOVA) are shown in Table 2. The degree of significance of each factor is represented in Table 2 by its p-value; when a factor has a p-value smaller than 0.05, it influences the process in a significant way for a confidence level of 0.95.

Table 2 indicates that operating pressure has the most significant influence on the extraction yield of polysaccharides ($p = 0.000001$); time has significant influence ($p = 0.0004$), while the operating temperature has less influence ($p = 0.284$). Hence, it can be concluded that operating pressure and time are the main variables that influence the breaking performance.

The extraction yields of polysaccharides increased when the operating pressure was increased from 27 to 35 MPa. Higher pressure increased the density of the SC–CO_2 and the penetrating power, which enhanced the breaking process. In previous investigations on SFE of olive husk oil (Esquível, Bernardo-Gil, & King, 1999), celery seed oil (Papamichail, Louli, & Magoulas, 2000), coriander seed oil (Illés, Daood, Perneczki, Szokonya, & Then, 2000) and sunflower oil (Salgın, Döker, & Çalımlı, 2006), the effect of pressure has been reported. Our results are consistent with these data. Other studies have shown that temperature has a significant effect on the extraction yields of some compounds (Macías-Sánchez et al., 2007; Vági et al., 2007). The yields at different temperatures depended on a complex balance between the varying of the SC–CO_2 density and the solubility of compounds in it. In our study, the operating temperature did not show significant effects on the yield of polysaccharides. The possible reason was that CO_2 served to penetrate the hard sporoderm rather than dissolving the polysaccharides in the SC–CO_2 fluids. From Table 1, it can be observed that the highest extraction yield (2.98%) was obtained at the operating pressure of 35 MPa, operating temperature 25 °C, and time 4 h.

3.3. Empirical correlation

Empirical correlation was presented using the experimental data and Statistica 6.0 program. The correlation related the extraction yield of polysaccharides to the main variables-operating pressure and time.

Eq. (1) is expressed as follows:

$$Y = -11.3092 + 0.7271P + 0.3188t - 0.0096P^2 - 0.0031Pt - 0.02t^2, \quad (1)$$

where Y is the extraction yield of polysaccharides (% dry weight of spores), P is the operating pressure (MPa), and t is time (h). The

Table 1
Experimental matrix and value of observed responses ($n = 3$)

No.	Factors			Errors		Extraction yield (%)	RSD for extraction yield (%)
	P (MPa)	T (°C)	t (h)	$E1$	$E2$		
1	1 (27)	1 (25)	1 (1)	1	1	1.54	1.97
2	1 (27)	2 (30)	2 (2)	2	2	1.72	2.16
3	1 (27)	3 (35)	3 (3)	3	3	1.86	1.59
4	1 (27)	4 (40)	4 (4)	4	4	1.96	1.88
5	2 (30)	1 (25)	2 (2)	3	4	2.26	2.85
6	2 (30)	2 (30)	1 (1)	4	3	2.22	1.74
7	2 (30)	3 (35)	4 (4)	1	2	2.54	1.36
8	2 (30)	4 (40)	3 (3)	2	1	2.52	1.98
9	3 (33)	1 (25)	3 (3)	4	2	2.62	2.54
10	3 (33)	2 (30)	4 (4)	3	1	2.74	2.36
11	3 (33)	3 (35)	1 (1)	2	4	2.44	1.91
12	3 (33)	4 (40)	2 (2)	1	3	2.52	1.87
13	4 (35)	1 (25)	4 (4)	2	3	2.98	2.23
14	4 (35)	2 (30)	3 (3)	1	4	2.92	1.54
15	4 (35)	3 (35)	2 (2)	4	1	2.90	2.07
16	4 (35)	4 (40)	1 (1)	3	2	2.58	2.51

Table 2
ANOVA results for the extraction yields of polysaccharides

Source of variance	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
P	2.5098	3	0.8366	275.92	0.000001
T	0.0146	3	0.0049	1.61	0.284
t	0.2994	3	0.0998	32.92	0.0004
E1	0.0110	3	0.0030		
E2	0.0072	3			
Total	2.842	15			

correlation coefficient is 0.9686, and the observed value versus predicted value of this model is shown in Fig. 2.

Eq. (1) is represented graphically in Fig. 3 for the different operating conditions. Eq. (1) predicts that the highest extraction yield is obtained at the operating pressure of 37.2 MPa and the breaking time of 4.1 h, which are quite close to the experimental data, the operating pressure of 35 MPa and treatment time of 4 h, respectively. Therefore, Eq. (1) is appropriate for revealing the relationship between the extraction yield of polysaccharides and the operating pressure and processing time.

From the above results, the appropriate operating conditions to obtain a better breaking performance are, pressure 35 MPa, temperature 25 °C, time 4 h, and CO_2 flow rate 10 kg/h. Under the optimum conditions, the extraction yield of polysaccharides is 2.98%, which is significantly higher than the 0.94% for the intact ones.

3.4. SEM images

The intact and processed *G. lucidum* spores were examined by SEM to investigate the morphological changes in the surface of sporoderm during the breaking process. SEM images of untreated and treated spores are presented in Fig. 4. Under SEM, the intact spores were ovate-oblong or ovoid with truncated apex or blunt taper. There were some sinuous depressions or mini holes on the surface of the spores. The processed spores showed morphological changes. Some spores had crevices on the sporoderm, some had a gap and some were totally broken.

A comparison of the SEM images of intact spores and processed spores under 35 MPa for 2, 3, and 4 h (see Fig. 4a–c and f) showed that the mini holes on the processed spores were larger than those in the intact ones. The holes on the sporoderm of the processed spores were obviously larger, with longer breaking time. Fig. 4a, d, e and f show the SEM images of the intact spores and spores processed for 4 h under different pressures. It can be observed that processed spores had crevices and hollows and the effect was obvious on the high pressure processed ones. From Fig. 4, we conclude that high pressure and long breaking time are helpful for changing the shape of the spores and the status of the sporoderm.

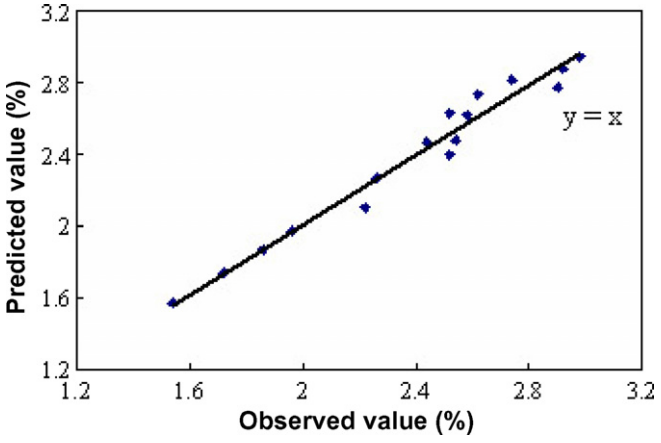

Fig. 2. Observed value versus predicted value for the extraction yields of polysaccharides.

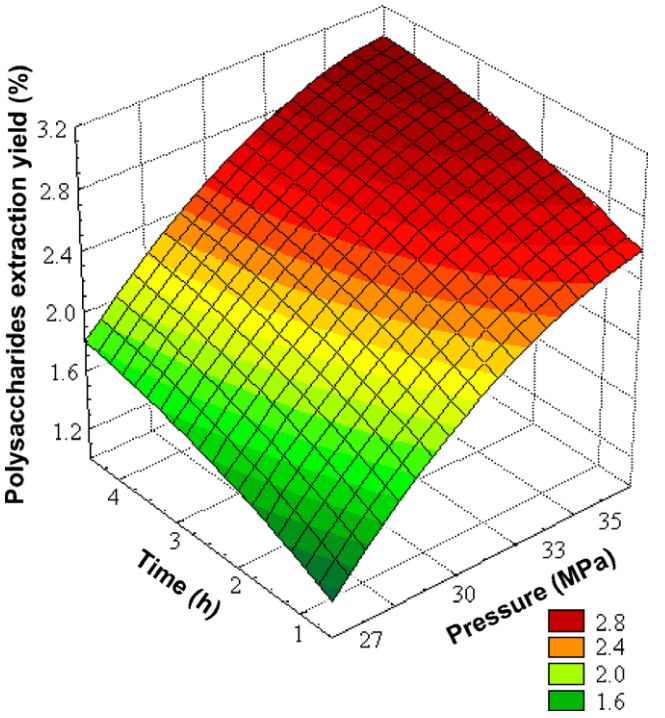

Fig. 3. Three-dimensional plot of the response surface for the extraction yields of polysaccharides. The scales of the *x*- and *y*-axes are coded value.

Although the total number of spores broken by SC–CO_2 was not high (approximately 10% at a high pressure of 35 MPa), the polysaccharides extraction yield of processed spores was much higher than that of the intact ones. The reason is that the SC–CO_2 treatment improved the permeability of the spores and enlarged the mini holes on the sporoderm. This facilitates the extraction of polysaccharides from the spores. Our results also show that the morphological changes on the sporoderm can improve the release of bioactive components from broken spores.

SC–CO_2 is helpful in sporoderm breaking of *G. lucidum* spores. With this processing, the highly active components in spores are freely available in traditional Chinese medicine (TCM) decoctions, extracts from TCM obtained by boiling it in water. CO_2 is safer than organic solvents, and much less decomposition of active components occurs under the low temperature breaking process.

4. Conclusions

In the present study, SC–CO_2 breaking of *G. lucidum* spores was investigated. Better breaking performance was obtained with the operating pressure 35 MPa, temperature 25 °C, time 4 h, and CO_2 flow rate 10 kg/h. Under these optimum breaking conditions, the

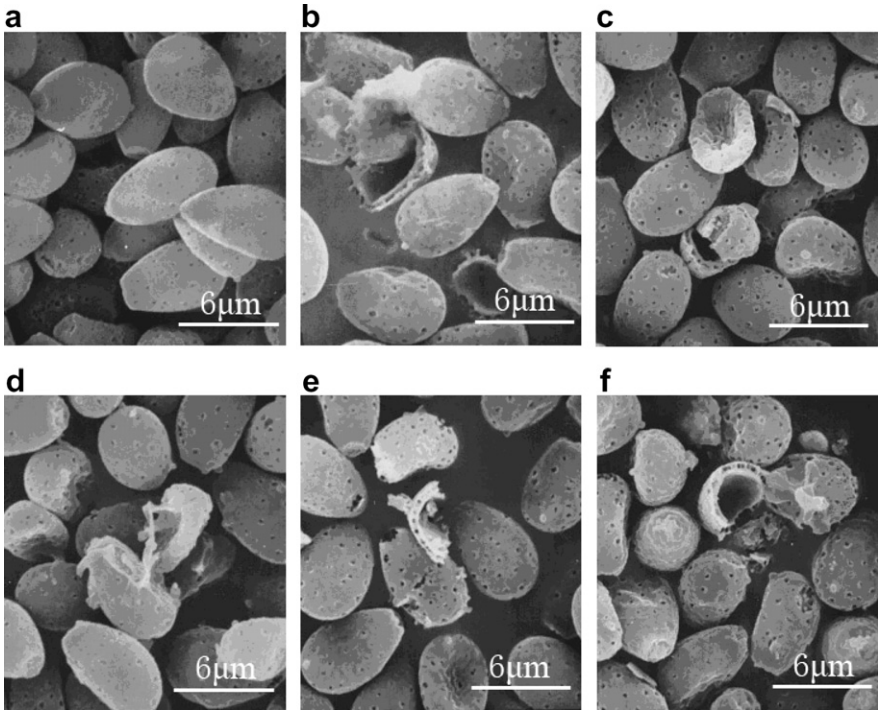

Fig. 4. SEM images of *Ganoderma lucidum* spores under different operating pressures and time, bar = 6 μm: (a) intact; (b) 35 MPa, 2 h; (c) 35 MPa, 3 h; (d) 30 MPa, 4 h; (e) 33 MPa, 4 h; and (f) 35 MPa, 4 h.

extraction yield of polysaccharides in treated spores reached 2.98%, which was three times greater than that in the intact ones (0.94%). Obvious morphological changes on the sporoderm of processed spores were observed compared to the intact spores. According to the above results, it can be concluded that SC–CO_2 is a fast and efficient method for breaking the sporoderm and improving the release of polysaccharides from *G. lucidum* spores. It also provides a new technique for the production of sporoderm-broken spores of *G. lucidum* for the market.

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