

Full Length Research Paper

Relationship between mycelium morphology and extracellular polysaccharide production of medicinal mushroom *Ganoderma lucidum* in submerged culture

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The medicinal mushroom *Ganoderma lucidum* is a source of polysaccharides used in disease treatment. The objective of this study was to investigate the relationships between *G. lucidum* mycelium morphology and the production of extracellular polysaccharides (EPS) in submerged fermentation. Mycelium pellets were classified according to diameter as either S, M or L pellets. M pellets were the main source of EPS. Changes in mycelium morphology and EPS production were obtained when cells were grown in different culture media; medium that contained a high concentration of glucose and low peptone enhanced the formation of M pellets. The proportion of M pellets in the culture was influenced greatly by KH_2PO_4 concentration and increase was accompanied by enhanced EPS production. Dry cell weight was affected mostly by the percentage of total M and L pellets present, while S pellet numbers had little effect. The production of EPS and the accumulation of mycelia were influenced mainly by *G. lucidum* pellet morphology. This study suggests that in large scale fermentation, high yields of EPS would be obtained by the control of mushroom morphology.

Key words: Medicinal mushroom, *Ganoderma lucidum*, morphology, extracellular polysaccharides, submerged fermentation.

INTRODUCTION

Ganoderma lucidum (Fr.) Krast (Polyporaceae) is a well known traditional Chinese mushroom used as a treatment for various diseases. It possesses medicinal value because of the presence of bioactive substrates such as polysaccharides and terpenoids. Polysaccharides have been shown to have antitumor, hypoglycaemic and antioxidative effects (Berovic et al., 2003; Chang et al., 2006; Hikino et al., 1989; Liu et al., 2011; Mohammed et al., 2007; Sone et al., 1985; Zhang et al., 2011) and are a recent area of research interest.

Traditional production of *G. lucidum* polysaccharide employs solid-state fermentation (SSF) using waste

(Mayszumi et al., 1993) such as sawdust, wheat bran, and wood as substrate. Submerged fermentation has several advantages compared with SSF, such as a short cultivation time and easy control of polysaccharide quality. In submerged fermentation, polysaccharide production and mycelium growth are greatly affected by culture conditions (Yang and Liau, 1998). Glucose and peptone are favourable carbon and nitrogen sources for biomass and extracellular polysaccharides (EPS) accumulation in various fungal strains (Lin and Chen, 2007). Moreover, the presence of K^+ and Mg^{2+} favours conditions for biomass and EPS production (Simonić et al., 2008; Kwon et al., 2009).

Fungal mycelia exhibit a range of phenotypes, including filamentous, clumps and pellets, under different submerged fermentation conditions. Change in morphology has a considerable effect on metabolic

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Table 1. Classification of different mycelium forms.

| Diameter (mm) | | | Roundness | |
|---------------|---------------|------------|--------------|---------------|
| S-Fraction | M-Fraction | L-Fraction | Rough pellet | Smooth pellet |
| D < 0.8 | 0.8 ≤ D < 2.5 | D ≥ 2.5 | R > 1.5 | R ≤ 1.5 |

product synthesis (Papagianni, 2004). The effect of mycelium morphology on the synthesis of metabolic products has been investigated previously (Park et al., 1999; Papagianni et al., 1999; Domingues et al., 2000). Developments of image analysis technology software, such as IPlab spectrum (Park et al., 1999) and Image-pro plus (Oncu et al., 2007), have provided a favourable platform to study the relationship between morphology and metabolic yield. However, little research has focused on the relationship between polysaccharide production and morphology in *G. lucidum* mycelia.

In this study, we investigated changes in morphology and EPS production under different medium compositions. A new aspect of *G. lucidum* mycelium morphology and EPS production was found based on form regularity, which suggested that high yields of polysaccharide could be realized by the control of mycelium morphology.

MATERIALS AND METHODS

Organisms and media

G. lucidum was maintained on potato dextrose agar (PDA) slants. The slants were inoculated and incubated at 30°C for 16 days, then stored at 4°C. Seed medium consisted of 20 g L⁻¹ glucose, 10 g L⁻¹ peptone, 4.5 g L⁻¹ KH₂PO₄ and 2 g L⁻¹ MgSO₄·7H₂O. The composition of the basic fermentation medium was same as that of the seed medium, except concentrations of glucose, peptone, KH₂PO₄ and MgSO₄·7H₂O were varied for different experiments. All media were sterilized at 121°C for 20 min; the glucose was sterilized separately to prevent the Maillard reaction.

Culture conditions

For the seed culture, 80 ml medium with an initial pH of 6.0 was prepared in a 250 ml flask, approximately 3 cm × 3 cm of mycelia from a PDA slant was inoculated into the culture; cultures were incubated for 16 days at 30°C on a rotary shaker (150 rpm). Next, 5 ml of the seed culture medium was inoculated into the fermentation culture and grown in a 500 ml shake flask that contained 150 ml liquid medium. This culture was incubated at 30°C on a rotary shaker at 150 rpm for 8 days.

Analytical methods

Determination of dry weight and exopolysaccharide

Dry cell weight (DCW) was obtained by centrifugation (4186 × g, 5 min); cells were washed three times with distilled water, and dried at 60°C to a constant weight. The residual sugar was assayed by the dinitrosalicylic acid method (Miller, 1959) and the total sugar content

was determined by phenol–sulfuric acid assay according to the method developed by Dubois et al. (1956).

Image analysis and morphology classification

In order to investigate the pellet morphology, 20 ml of culture broth was transferred to a Petri dish using a graduated cylinder. A CCD camera (IXUS115, Canon, Japan) was used to take images for every sample.

Pellet morphology was characterized using image analysis (Cox and Thomas, 1992), and Image-Pro Plus 6.0 software (Media Cybernetics Inc., Silver Spring, MD, USA). The pellets in the culture broth were classified into three morphological forms according to diameter (D). Pellets were classified as rough or smooth (Table 1) based on roundness (R).

Diameter was calculated as follows:

$$D = \sqrt{\frac{4 \times \text{Area}}{\pi}}$$

Roundness was calculated as follows:

$$R = \frac{P^2}{4\pi \times \text{Area}}$$

Where P is pellet perimeter.

RESULTS

Effect of time course of cultivation on morphology and EPS

G. lucidum mycelia were classified into three forms depending on diameter (Table 1). The S pellet form predominated initially in culture broth, while M pellet was the main form during fermentation (Figure 1). At day 7, the L pellet form was the predominant mycelium form in the culture broth and the percentage of M pellets declined sharply.

After day 7, the ratio of different size pellets was stable again, but the L pellet form predominated. EPS concentration reduced in the first 3 days and then increased to the maximum value at day 6 (Table 2). After a second reduction in concentration, EPS concentration was stable until the end of fermentation. Changes in EPS concentration were linked to morphological changes in *G.*

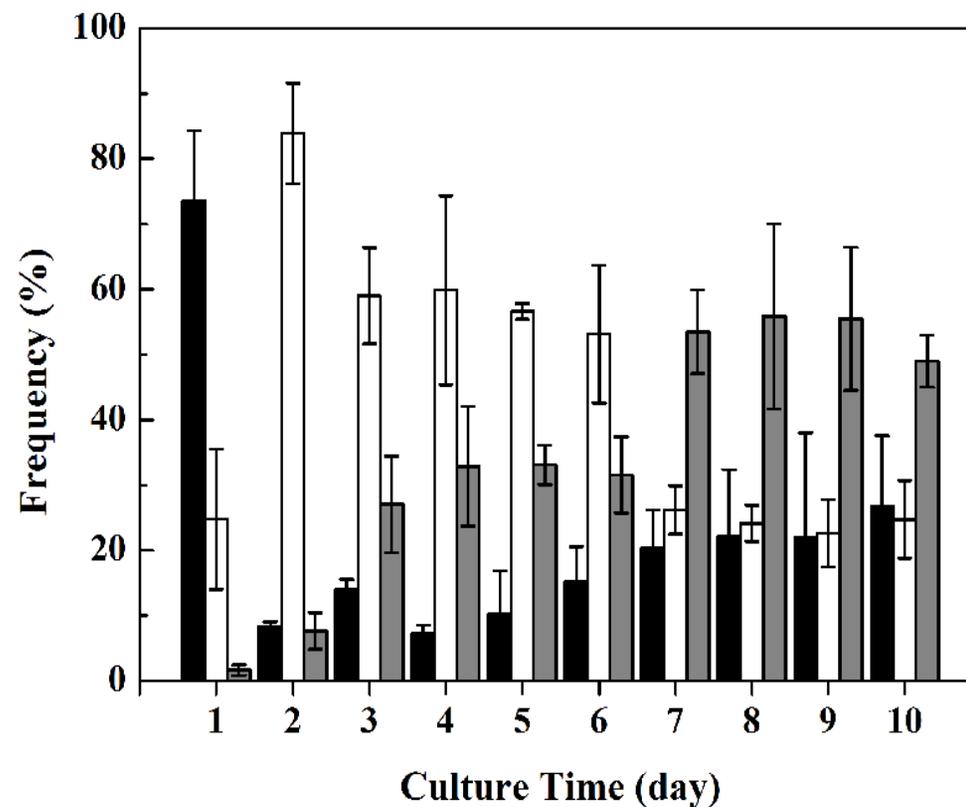


Figure 1. Change of mycelium morphology during submerged culture of *G. lucidum*. The error bars represent the standard deviation of three independent experiments. (■) S pellet; (□) M pellet; (■) L pellet.

Table 2. DCW and EPS concentration and percentage of rough pellets during fermentation*.

| Parameter | Day of fermentation | | | | | | | | | |
|-----------------------------|---------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| DCW (g L ⁻¹) | 0.11 ± 0.05 | 0.99 ± 0.15 | 1.74 ± 0.2 | 2.53 ± 0.2 | 3.11 ± 0.47 | 3.43 ± 0.36 | 4.78 ± 0.32 | 6.05 ± 0.42 | 7.32 ± 0.57 | 8.12 ± 0.51 |
| EPS (g L ⁻¹) | 0.60 ± 0.02 | 0.56 ± 0.05 | 0.43 ± 0.01 | 1.16 ± 0.01 | 2.15 ± 0.03 | 2.42 ± 0.01 | 1.61 ± 0.06 | 1.15 ± 0.07 | 0.87 ± 0.07 | 0.81 ± 0.04 |
| Rough pellet percentage (%) | 12.40 | 45.45 | 98.36 | 96.05 | 53.01 | 30.41 | 19.79 | 28.28 | 52.90 | 58.42 |

*Error bars represent the standard deviation of three independent experiments. DCW, dry cell weight; EPS, extracellular polysaccharides.

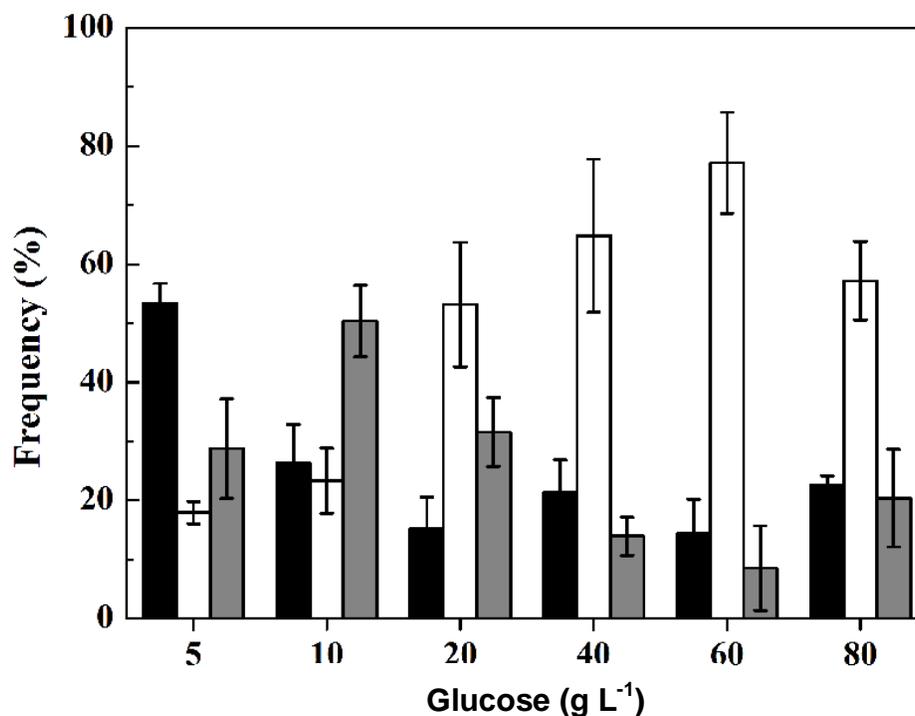


Figure 2. Effect of initial glucose concentration on the mycelium morphology during submerged culture of *G. lucidum*. The error bars represent the standard deviation of three independent experiments. (■) S pellet; (□) M pellet; (▒) L pellet.

lucidum mycelia. EPS was consumed when S pellets grew into M pellets, resulting in a reduction in EPS concentration at day 7.

Effect of glucose concentration on morphology and EPS

S and L pellets formed easily at low glucose concentrations, while high glucose triggered the formation of M pellets. The presence of M pellets improved the production of EPS greatly in comparison with S and L pellets (Figure 2 and Table 3). Low concentration glucose was beneficial to obtain high DCW; however, the concentration of this carbon source had little effect on pellet roundness (data not shown).

Effect of peptone concentration on morphology and EPS

Figure 3 shows that M pellets formed easily at low peptone concentration. High peptone concentration was beneficial for formation of L pellets and increase in the percentage of L pellets resulted in DCW accumulation. At day 1, most *G. lucidum* mycelia were in the S pellet form but DCW was only 0.11 ± 0.05 g.L⁻¹. However, the DCW increased markedly after days 6 and 10.

Effect of KH₂PO₄ and MgSO₄·7H₂O concentration on morphology and EPS

Addition of KH₂PO₄ and MgSO₄·7H₂O mineral salts enhanced EPS production, but had little effect on the accumulation of DCW (Table 3). KH₂PO₄ promoted greatly a change in the proportion of M pellets (Figure 4), while MgSO₄·7H₂O affected the morphology of *G. lucidum* only slightly (data not shown).

DISCUSSION

Wagner et al. (2004) reported that spherical hyphae give rise to protuberances during fermentation giving the pellet a 'rough' starburst appearance. Detached protuberances became S pellets. Roughness of pellets in culture reflected a change in morphology. From days 1 to 3, mycelia grew into the M pellet form and the percentage of rough pellets increased. Casas et al. (2005) reported that pellets that attained a size of several centimetres were internally hollow because of fungal biomass necrosis caused by lack of oxygen. Thus, M pellet may be the main mycelia producing EPS. The production of EPS by M pellet not only supplied the consumption of EPS but also accumulated the EPS in the culture broth.

The effects of glucose concentration on *Aspergillus niger* mycelium morphology have been reported

Table 3. Effect of culture medium nutrients on DCW and EPS production.

| Nutrients | Concentration (g L ⁻¹) | DCW (g L ⁻¹)* | EPS (g L ⁻¹)* |
|---------------------------------------|------------------------------------|---------------------------|---------------------------|
| Glucose | 5 | 2.80 ± 0.13 | 1.67 ± 0.03 |
| | 10 | 2.63 ± 0.85 | 2.34 ± 0.05 |
| | 20 | 3.43 ± 0.36 | 2.42 ± 0.01 |
| | 40 | 1.90 ± 0.04 | 2.63 ± 0.02 |
| | 60 | 1.47 ± 0.23 | 2.45 ± 0.03 |
| | 80 | 2.30 ± 0.88 | 2.39 ± 0.01 |
| Peptone | 2.5 | 2.16 ± 0.61 | 2.16 ± 0.06 |
| | 5 | 2.28 ± 0.32 | 2.41 ± 0.02 |
| | 10 | 3.57 ± 0.40 | 2.50 ± 0.02 |
| | 20 | 4.68 ± 0.15 | 1.80 ± 0.03 |
| | 40 | 5.41 ± 0.36 | 1.27 ± 0.04 |
| KH ₂ PO ₄ | 0 | 3.27 ± 0.25 | 1.51 ± 0.04 |
| | 1.5 | 4.03 ± 0.05 | 1.54 ± 0.06 |
| | 3 | 3.73 ± 0.60 | 1.62 ± 0.05 |
| | 4.5 | 3.52 ± 0.31 | 2.47 ± 0.04 |
| | 6 | 3.37 ± 0.35 | 2.05 ± 0.01 |
| MgSO ₄ · 7H ₂ O | 0 | 3.70 ± 0.61 | 0.54 ± 0.02 |
| | 1 | 3.90 ± 0.21 | 1.92 ± 0.08 |
| | 2 | 3.40 ± 0.36 | 2.44 ± 0.04 |
| | 3 | 3.60 ± 0.17 | 2.54 ± 0.01 |
| | 4 | 3.20 ± 0.53 | 2.45 ± 0.07 |

*Error bars represent the standard deviation of three independent experiments. DCW, dry cell weight; EPS, extracellular polysaccharides.

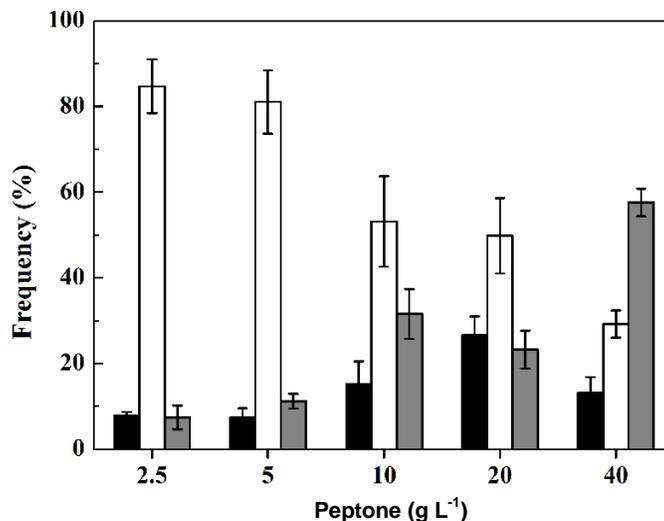


Figure 3. Effect of initial peptone concentration on the mycelium morphology during submerged culture of *G. lucidum*. The error bars represent the standard deviation of three independent experiments. (■) S pellet; (□) M pellet; (▒) L pellet.

previously by Papagianni and Matthey (2004). Our finding were similar to that reported by Fang and Zhong (2002), who suggested that medium with a high initial glucose concentration was beneficial to the formation of M pellets

and to EPS production. When initial glucose concentration exceeded a certain level, however, high initial osmotic pressure induced a reduction in cell growth and EPS production (Zhang et al., 1995). Peptone is

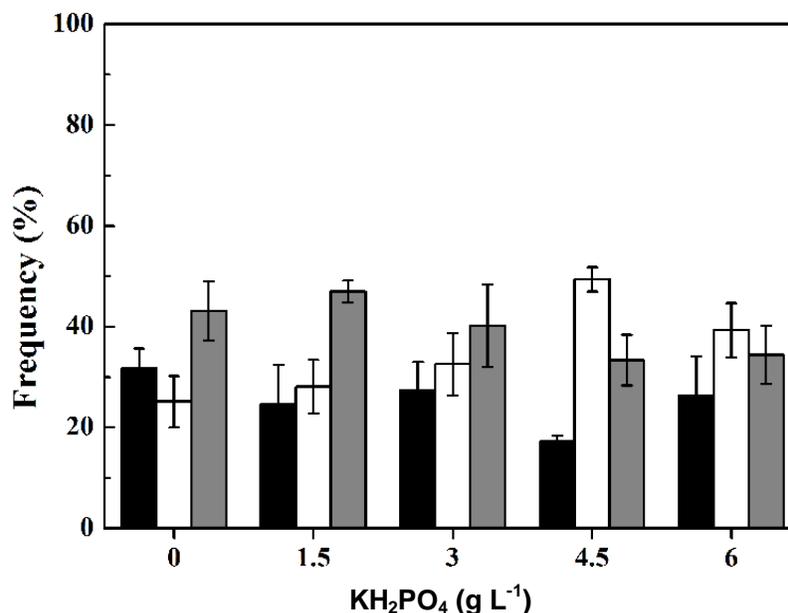


Figure 4. Effect of initial KH_2PO_4 concentration on the mycelium morphology during submerged culture of *G. lucidum*. The error bars represent the standard deviation of three independent experiments. (■) S pellet; (□) M pellet; (▒) L pellet.

known to have a positive effect on pellet formation (Liao et al., 2007) and plays a key role on *G. lucidum* pellet morphology and EPS production. A relatively lower concentration of peptone was beneficial to the formation of smooth L pellets and the synthesis of EPS. Previous work into *Rhizobium nigricans* fermentation reported that low concentrations of nitrogen sources resulted in small and light pellets (Znidarsic et al., 2000), and this option was also optimal for cell growth and EPS production in *G. lucidum* (Simonić et al., 2008). High peptone concentration was beneficial to the formation of L and rough pellets, resulting in increased DCW. In a previous study, the effect of peptone in promoting cell growth was attributed to the presence of amino acids (Tsvileva et al., 2004). However, the inhibitory effect surpassed the stimulatory effect of peptone at high concentrations (Fang and Zhong, 2002).

Hsieh et al. (2006) reported that DCW and EPS were enhanced by addition of KH_2PO_4 and MgSO_4 . However, the addition of KH_2PO_4 and MgSO_4 enhanced the production of EPS effectively, while DCW was not clearly influenced. Positive effects of Mg^{2+} and K^+ on EPS production may be attributed to their role as cofactors for the key enzyme α -phosphoglucomutase, which then leads to EPS accumulation (Tang and Zhong, 2002). A similar result was obtained by Tang et al. (2008).

KH_2PO_4 had a great effect on *G. lucidum* mycelium morphology and promoted an increase in the percentage of M pellets, but had little effect on the roundness of the pellets. This conclusion was in accordance with previous

research that reported that a rise in KH_2PO_4 concentration increased pellet perimeter (Papagianni et al., 1999).

In conclusion, this study demonstrated that EPS production and DCW accumulation was related to *G. lucidum* mycelium morphology. EPS was produced mainly by M pellets, while S pellets consumed the produced EPS during growth to M pellets. EPS accumulated to a maximum concentration when the percentage of all three pellet forms remained stable. During the course of fermentation, DCW was influenced mostly by the percentage of M and L pellet, while S pellets had little effect on weight. Culture conditions, including carbon source, nitrogen source, KH_2PO_4 and MgSO_4 , influenced the production of EPS and cell growth via changes in *G. lucidum* morphology.

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REFERENCES

Berovic M, Habijanac J, Zore I, Wraber B, Hodzar D, Boh B, Pohleven F

- (2003). Submerged cultivation of *Ganoderma lucidum* biomass and immunostimulatory effects of fungal polysaccharides. *J. Biotechnol.*, 103: 77-86.
- Casas L JL, Sanchez PJA, Fernandez SJM, Rodriguez PEM, Chisti Y (2005). Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus*. *J. Biotechnol.*, 116: 61-77.
- Chang MY, Tsai GJ, Houg JY (2006). Optimization of the medium composition for the submerged culture of *Ganoderma lucidum* by Taguchi array design and steepest ascent method. *Enzyme. Microb. Technol.*, 38: 407-414.
- Cox PW, Thomas CR (1992). Classification and measurement of fungal pellets by automated image analysis. *Biotechnol. Bioeng.*, 39: 945-952.
- Domingues FC, Queiroz JA, Cabral JMS, Fonseca LP (2000). The influence of culture conditions on mycelial structure and cellulose production by *Trichoderma reesei* Rut C-30. *Enzyme. Microb. Technol.*, 26: 394-401.
- Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, 28: 350-356.
- Fang QH, Zhong JJ (2002). Submerged fermentation of higher fungus *Ganoderma lucidum* for production of valuable bioactive metabolites—ganoderic acid and polysaccharide. *Biochem. Eng. J.*, 10: 61-65.
- Hikino H, Ishiyama M, Suzuki Y, Konno C (1989). Mechanisms of hypoglycemic activity of ganoderan B: a glycan of *Ganoderma lucidum* fruit bodies. *Planta Med.*, 55: 423-428.
- Hsieh C, Tseng MH, Liu CJ (2006). Production of polysaccharides from *Ganoderma lucidum* (CCRC 36041) under limitations of nutrients. *Enzyme. Microb. Technol.*, 38: 109-117.
- Kwon JS, Lee JS, Shin WC, Lee KE, Hong EK (2009). Optimization of culture conditions and medium components for the production of mycelia biomass and exo-polysaccharides with *Cordyceps militaris* in liquid culture. *Biotechnol. Bioproc. E.*, 14: 756-762.
- Liao W, Liu Y, Frear C, Chen SL (2007). A new approach of pellet formation of a filamentous fungus—*Rhizopus oryzae*. *Bioresour. Technol.*, 98: 3415-3423.
- Lin ES, Chen YH (2007). Factors affecting mycelial biomass and exopolysaccharide production in submerged cultivation of *Antrrodia cinnamomea* using complex media. *Bioresour. Technol.*, 98: 2511-2517.
- Liu GQ, Ren GP, Wang XL, Zhao Y (2011). Statistical optimization of the key medium components by response surface methodology to promote ganoderic acid formation by medicinal mushroom *Ganoderma sinense* in submerged culture. *J. Med. Plants Res.*, 5: 425-431.
- Mayszumi F, Okamoto H, Mizuno T (1993). Cultivation of reddish reishi (*Ganoderma lucidum*, Red). *Food. Rev. Int.*, 13: 365-382.
- Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31: 426-428.
- Mohammed A, Adelaiye AB, Abubakar MS, Abdurahman EM (2007). Effects of aqueous extract of *Ganoderma lucidum* on blood glucose levels of normoglycemic and alloxan-induced diabetic wistar rats. *J. Med. Plants Res.*, 1: 34-37.
- Oncu S, Tari C, Unluturk S (2007). Effect of various process parameters on morphology, rheology, and polygalacturonase production by *Aspergillus sojae* in a batch bioreactor. *Biotechnol. Prog.*, 23: 836-845.
- Papagianni M (2004). Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnol. Adv.*, 22: 189-259.
- Papagianni M, Matthey M (2004). Physiological aspects of free and immobilized *Aspergillus niger* cultures producing citric acid under various glucose concentrations. *Proc. Biochem.*, 39: 1963-1970.
- Papagianni M, Matthey M, Kristiansen B (1999). The influence of glucose concentration on citric acid production and morphology of *Aspergillus niger* in batch and culture. *Enzyme. Microb. Technol.*, 25: 710-717.
- Park EY, Koike Y, Higashiyama K, Fujikawa S, Okabe M (1999). Effect of nitrogen source on mycelia morphology and arachidonic acid production in cultures of *Mortierella alpina*. *J. Biosci. Bioeng.*, 88: 61-67.
- Simonić J, Stajić M, Glamočlija J, Vukojević J, Duletić-Laušević S, Brčeski I (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. (*Aphyllorhizomycetideae*). *Int. J. Med. Mushrooms*, 10: 351-360.
- Sone Y, Okuda R, Wada N, Kishida E, Misaki A (1985). Structures and antitumor activities of the polysaccharides isolated from fruiting body and the growing fermentation of mycelium of *Ganoderma lucidum*. *Agr. Biol. Chem.*, 49: 2641-2653.
- Tang YJ, Zhong JJ (2002). Exopolysaccharide biosynthesis and related enzyme activities of the medicinal fungus, *Ganoderma lucidum*, grown on lactose in a bioreactor. *Biotechnol. Lett.*, 24: 1023-1026.
- Tang YJ, Zhu LL, Liu RS, Li HM, Li DS, Mi ZY (2008). Quantitative response of cell growth and Tuber polysaccharides biosynthesis by medicinal mushroom Chinese true *Tuber sinense* to metal ion in culture medium. *Bioresour. Technol.*, 99: 7606-7615.
- Tsvileva OM, Pankratov AN, Nikitina VE, Garibova LV (2004). Relationship between the molecular structure of the nitrogen source and the activity of the extracellular lectins of *Lentinus edodes* (Berk.) Sing [*Lentinula edodes* (Berk.) Pegler] upon submerged cultivation. *Microbiol.*, 73: 410-413.
- Wagner R, Mitchell DA, Sasaki GL, Amazonas MALA (2004). Links between morphology and physiology of *Ganoderma lucidum* in submerged culture for the production of exopolysaccharide. *J. Biotechnol.*, 114: 153-164.
- Yang FC, Liao CB (1998). Effects of cultivation conditions on the mycelia growth of *Ganoderma lucidum* in submerged flask cultures. *Bioproc. Eng.*, 19: 233-236.
- Zhang LX, Fan C, Liu SC, Zang ZF, Jiao LL, Zhang LP (2011). Chemical composition and antitumor activity of polysaccharide from *Inonotus obliquus*. *J. Med. Plants Res.*, 5: 1251-1260.
- Zhang YH, Zhong JJ, Yu JT (1995). Effect of osmotic pressure on cell growth and production of ginseng saponin and polysaccharide in suspension cultures of *Panax notoginseng*. *Biotechnol. Lett.*, 17: 1347-1350.
- Znidarsic P, Komel R, Pavko A (2000). Influence of some environmental factors on *Rhizopus nigricans* submerged growth in the form of pellets. *World J. Microbiol. Biotechnol.*, 16: 589-593.