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Role of oxygen supply in submerged fermentation of *Ganoderma lucidum* for production of *Ganoderma* polysaccharide and ganoderic acid

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Abstract

The effects of oxygen supply on the submerged fermentation of *Ganoderma lucidum*, a famous traditional Chinese medicinal mushroom, for simultaneous production of bioactive compounds—*Ganoderma* polysaccharide and ganoderic acid (GA) were studied. An initial volumetric oxygen transfer coefficient ($K_L a$) value within the range of 16.4–96.0 h^{-1} had a significant effect on the cell growth, cellular morphology and metabolites biosynthesis. At an initial $K_L a$ of 78.2 h^{-1}, a maximal cell concentration of 15.62 g l^{-1} by dry weight was obtained, as well as a maximal intracellular polysaccharide (IPS) production of 2.19 g l^{-1} and its maximal productivity of 217 mg l^{-1} per day. An increase of initial $K_L a$ led to a bigger size of mycelia aggregates and a higher production and productivity of GA. The GA production and productivity at an initial $K_L a$ of 96.0 h^{-1} was 1.8-fold those at an initial $K_L a$ of 16.4 h^{-1}. Dissolved oxygen tension (DOT) also affected the fermentation process. The cell growth of *G. lucidum* was significantly inhibited when DOT was controlled ∼10% of air saturation, which was due to the oxygen limitation in mycelia aggregates. The production of extracellular polysaccharide (EPS) and contents of IPS and GA ∼10% of DOT were higher than those ∼25% of DOT. However, the total production and productivity of IPS and GA at a low DOT were lower than those at a high DOT. The fundamental information obtained in this study will be useful for submerged fermentation of *G. lucidum* on a large scale.

Keywords: *Ganoderma lucidum*; Fermentation technology; Ganoderic acid; *Ganoderma* polysaccharide; Medicinal mushroom; Dissolved oxygen; Oxygen transfer

1. Introduction

Among the sources of bioactive metabolites, less intensively investigated organisms like medicinal fungi seem to be promising for new structures with biological activities [1–7]. *Ganoderma lucidum* (Fr.) Krast (Polyporaceae) is a famous traditional Chinese medicinal mushroom. Polysaccharides and ganoderic acids (GAs) are two of its major bioactive components [1]. Interestingly, recent studies show that GAs have new biological activities including anti-tumor and anti-HIV-1 [2,3]. Because it usually takes several months to cultivate the fungus and the product yield is low in soil cultivation, submerged fermentation of *G. lucidum* is viewed as a promising alternative for efficient production of *Ganoderma* polysaccharide and GA [1,4,5].

Submerged fermentation of higher fungi is characterized by an increase in broth viscosity with time, either as a consequence of increased cell concentration, changes in microbial morphology, or because of the accumulation of extracellular products that alter the rheological characteristics of fermentation broth. After these changes, there will be a series of problems that should be considered and solved, especially oxygen supply. Oxygen affects cell growth, cellular morphology, nutrients uptake, and metabolite biosynthesis. Ishmetentskii et al. [8] reported that a high oxygen transfer rate (OTR) could reduce, enhance, or have no effect on the production of pullulan, depending on the strain ploid. In cultivation of filamentous fungus *Schizophyllum commune*, Rau et al. [9] reported that sufficient oxygen supply resulted in an increase in the specific growth rate and a decrease in the production rate of extracellular glucan. When oxygen partial pressure in the culture broth decreased to almost zero, the fungus responded to this oxygen limitation by reduced cell growth and increased glucan accumulation. In the submerged fermentation of *Monascus ruber*, Hajjaj et al. [10] reported that improving the oxygen supply increased the biomass yield, consumption of nitrogen source and production of secondary metabolites (red pigment and citrinin). In the submerged culture of *Lentinus edodes*, Yoshida et al. [11–13] reported that small pellets grew more quickly than larger pellets, and the aspects of oxygen utilization by mycelia changed in accordance with

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the variation of the growth type (in fibrous mycelia suspension or in mycelia pellets suspension).

Until now, there are no reports on effects of oxygen supply on simultaneous production of *Ganoderma* polysaccharide and GA. In this article, we investigated the impacts of initial volumetric oxygen transfer coefficient ($K_L a$) and dissolved oxygen tension (DOT) on fermentation of *G. lucidum* in order to obtain useful information for large-scale production of those bioactive compounds by the bioprocess.

2. Materials and methods

2.1. Maintenance and preculture of G. lucidum

The strain of *G. lucidum* CCGMC 5.616 was maintained on potato-agar-dextrose slants. The slant was inoculated with mycelia and incubated at 28 °C for 7 days, then stored at 4 °C for about 2 weeks. Preculture medium consisted of the following components (g l^{-1}): glucose 35, peptone 5, yeast extract 2.5, $KH_2PO_4 \cdot H_2O$ 1, $MgSO_4 \cdot 7H_2O$ 0.5, and Vitamin B_1 0.05. For the first preculture, 40-ml medium with initial pH of 5.5 was prepared in a 250-ml flask, and then 10-ml mycelium suspension from a slant culture was inoculated, and followed by 5-day incubation at 30 °C on a rotary shaker (120 rpm). For the second preculture, 45-ml medium was prepared in a 250-ml flask, and inoculated with 5-ml first preculture broth (with ca. 600–700 mg dry weight of cells per litre), then followed by 3-day incubation at 30 °C on a rotary shaker (120 rpm) [1,4,5].

2.2. Submerged fermentation in bioreactor

The bioreactor used was a 3.5-l (working volume) agitated bioreactor with two six-bladed turbine impellers (6.5 cm i.d.). The vertical distance between two impellers was 7.0 cm and the lower impeller was 5.0 cm above the reactor bottom. Aeration was through a ring sparger with a pore size of 0.8 mm, which was located 2.5 cm above the reactor bottom. Fermentation was conducted at 30 °C in the dark. Fermentation medium consisted of the following components (g l^{-1}): lactose 35, peptone 5, yeast extract 2.5, $KH_2PO_4 \cdot H_2O$ 1, $MgSO_4 \cdot 7H_2O$ 0.5, and Vitamin B_1 0.05 [1,4,5].

2.3. Effect of initial $K_L a$

The initial $K_L a$ was determined using the dynamic gassing-in and gassing-out method [14]. The cultures were all agitated at the same speed (200 rpm), and the aeration rate was set at 220, 1050, 1750 and 3500 ml min^{-1} to obtain the desired initial $K_L a$ values of 16.4, 60.0, 78.2 and 96.0 h^{-1}, respectively.

2.4. Effect of DOT

The DOT in the culture broth was monitored by using a polarographic DO probe and regulated by changing aeration rate (0.03–1.0 vvm) and agitation speed (100–250 rpm) during fermentation.

2.5. Sampling, determination of dry weight, medium sugar, and pellet size

For sampling, about 20–30 ml of broth was taken once from each reactor. Dry cell weight (DW) and residual sugar concentration were measured by gravimetric method and phenol–sulfuric acid method, respectively [1,4,5]. The mycelia aggregates from samples were sieved into different sizes by using stainless-steel sieves, and their size distribution frequency was calculated based on the ratio of DW as measured.

2.6. Measurements of Ganoderma polysaccharide and GA

For the determination of extracellular polysaccharide (EPS), after removal of mycelia by centrifugation, the crude EPS was precipitated with addition of 95% (v/v) ethanol by four times of volume, then separated by centrifugation at 13,000 × *g*. The insoluble components were suspended in 1 M NaOH at 60 °C for 1 h, and the supernatant was measured by phenol–sulfuric acid method [1,4,5].

The contents of intracellular polysaccharide (IPS) and GA in mycelia were analyzed. Details of these measurements have been reported elsewhere [1,4,5]. Although the GAs present in the mycelia of *G. lucidum* were a complicated mixture, they usually have α,β-unsaturated carbonyl group, whose UV absorbance is maximal ∼230–260 nm. The mixture of GA was detected at 245 nm by using analytical pure thymol (Shanghai Chemical Reagents Co., China) as standard.

3. Results and discussion

Effect of initial $K_L a$ on *G. lucidum* cultures was studied by setting various initial $K_L a$ values. All cell cultures were agitated at 200 rpm and aeration rate was adjusted over a range of 220–3500 ml min^{-1} to produce the desired initial $K_L a$ values from 16.4 to 96.0 h^{-1}. Fig. 1A shows the cell growth kinetics at initial $K_L a$ values of 16.4, 60.0, 78.2 and 96.0 h^{-1}. The cell growth at an initial $K_L a$ of 96.0 h^{-1} appeared to be slow as the culture period continued for up to 13 days, whereas it was 8 or 10 days for the other cultures. Initial $K_L a$ affected the biomass level, and its peak value of 15.62 g DW cells l^{-1} was obtained at an initial $K_L a$ of 78.2 h^{-1}. The results indicated that initial $K_L a$ had a significant effect on the cell growth during fermentation and an initial $K_L a$ of 78.2 h^{-1} seemed to be best for cell growth of *G. lucidum*.

In parallel with the changes in cell growth were changes in cellular morphology during the cultivation at various initial $K_L a$ values. It appeared that cultures at a higher initial $K_L a$ value were more aggregated than those at lower initial

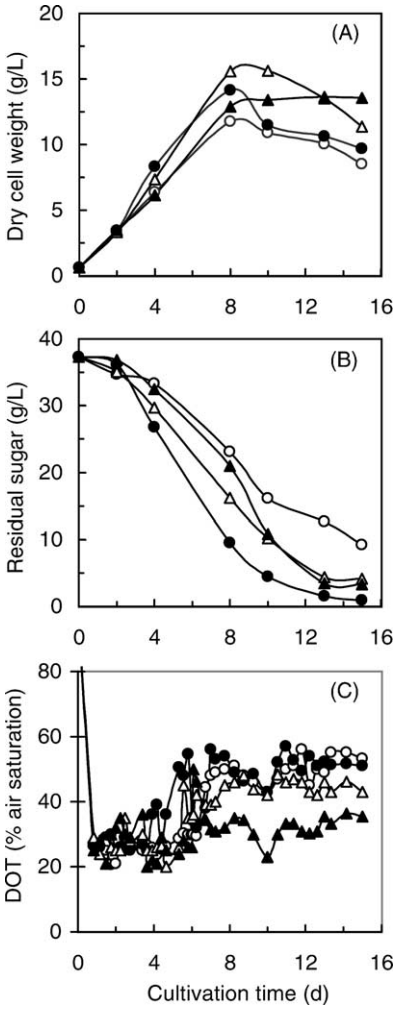

Fig. 1. Time profiles of DW (A), residual sugar concentration (B), and DOT (C) during submerged fermentation of *G. lucidum* at various initial $K_L a$ levels in a turbine-agitated bioreactor. Symbols for initial $K_L a$ values (h^{-1}): 16.4 (○), 60.0 (●), 78.2 (△) and 96.0 (▲).

$K_L a$ values. As shown in Fig. 2, an increase of initial $K_L a$ increased the percentage of mycelia aggregates over the size of 0.50 mm and between the size of 0.50–0.25 mm in diameter, while the percentage of mycelia aggregates below the size of 0.25 mm was decreased. Also, there was a big increase in the percentage of mycelia aggregates over 0.50 mm in diameter at an initial $K_L a$ of 96.0 h^{-1}. It was clear that initial $K_L a$ greatly affected the cellular morphology. In plant cell cultures of *Catharanthus roseus*, Leckie et al. [15] also reported that different size distribution of cell aggregates was observed at different initial $K_L a$ levels.

Limitation of oxygen within mycelia pellets was expected if mycelia pellets were larger than a certain critical size. The critical size can be calculated according to the following equation [16]:

$$d_{crit} = \sqrt{\frac{24 \times C_{O_2} \times D_{eff}}{R_{O_2}}} \quad (1)$$

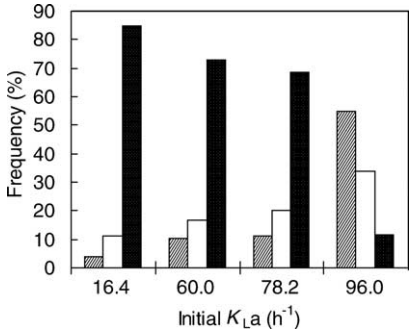

Fig. 2. Effect of initial $K_L a$ values on the distribution (expressed as frequency based on DW) of different mycelia aggregate sizes in the fermentation (Day 10). Symbols for mycelia aggregate sizes: diameter larger than 0.50 mm (hatched bar), diameter between 0.50–0.25 mm (blank bar), and diameter smaller than 0.25 mm (dark bar).

where d_{crit} is the critical diameter at which the internal oxygen limitation will occur, C_{O_2} the dissolved oxygen tension in medium, D_{eff} the effective diffusion coefficient of oxygen in mycelia pellets, and R_{O_2} is the oxygen consumption rate per pellet volume. D_{eff} is equal to the product of the molecular diffusion coefficient and the pellet porosity. R_{O_2} is equal to the product of specific oxygen uptake rate (SOUR) and the density of the mycelia pellets. On Day 10 of our *G. lucidum* fermentation at an initial $K_L a$ of 96.0 h^{-1}, DO was determined to be about 0.0732 mol m^{-3}, SOUR was measured as 0.785 mmol O_2 g^{-1} DW h^{-1}), the pellet density was 75 kg DW m^{-3}, and D_{eff} was assumed to be 1.9×10^{-9} m^2 s^{-1} [17]. According to Eq. (1), the critical pellet diameter was estimated to be 0.45 mm. At an initial $K_L a$ of 96.0 h^{-1}, about 55% (by dry weight) of mycelia pellets on Day 10 had a diameter larger than 0.50 mm (Fig. 2). The cells in the center of those pellets may be under oxygen limitation. Thus, the apparent cell growth was limited at an initial $K_L a$ of 96.0 h^{-1}.

Time courses of residual sugar concentration are compared in Fig. 1B. At an initial $K_L a$ of 60.0 h^{-1}, the substrate consumption rate was higher than in other cases, while it was the lowest at an initial $K_L a$ of 16.4 h^{-1}.

The dynamic changes of DOT are shown in Fig. 1C. The DOT at the later stage of culture at an initial $K_L a$ value of 96.0 h^{-1} was relatively lower. As we know, the DOT in the fermentation broth was directly related with OTR and oxygen uptake rate (OUR). During fermentation, the $K_L a$ value may change with the change of cellular morphology, viscosity of fermentation broth, and so on. These factors will lead to the phenomenon as observed in the work.

Fig. 3A shows the kinetics of EPS accumulation at various initial $K_L a$ levels. From Days 0 to 13, a rapid increase of EPS concentration was observed, and from Day 13 to the end of culture (Day 15), its accumulation level showed a slight decrease. The EPS production was 0.97, 0.69, 0.92 and 0.92 g l^{-1} in the cultures at initial $K_L a$ values of 16.4, 60.0, 78.2 and 96.0 h^{-1}, respectively, and its corresponding productivity was 73.1, 51.5, 69.2 and 69.2 mg l^{-1} per day.

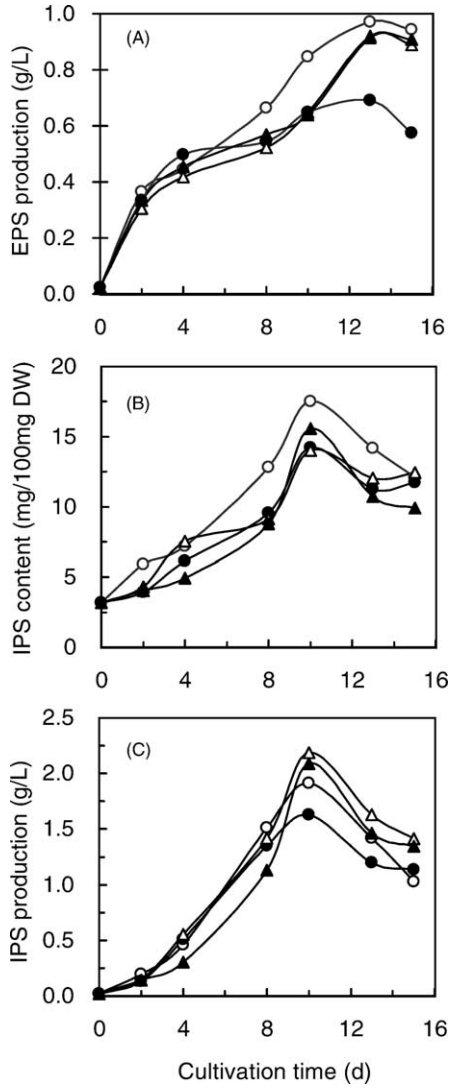

Fig. 3. Effect of initial $K_L a$ values on the EPS production (A), IPS content (B) and IPS production (C). Symbols for initial $K_L a$ values (h^{-1}): 16.4 (○), 60.0 (●), 78.2 (△) and 96.0 (▲).

Effect of initial $K_L a$ on the specific production (i.e. content) of IPS is shown in Fig. 3B. The maximum IPS content of 17.51, 14.20, 14.01 and 15.59 mg per 100 mg DW was reached on Day 10 in the cultures at an initial $K_L a$ value of 16.4, 60.0, 78.2 and 96.0 h^{-1}, respectively. Fig. 3C shows the dynamic profiles of total accumulation of IPS. The maximum IPS production of 1.91, 1.63, 2.19 and 2.09 g l^{-1} was reached on Day 10 in the culture grown at an initial $K_L a$ value of 16.4, 60.0, 78.2 and 96.0 h^{-1}, respectively, and its corresponding productivity was 189.0, 161.0, 217.0 and 207.0 mg l^{-1} per day. The results indicated that a relatively higher initial $K_L a$ value was favorable for IPS production and productivity.

Time profiles of GA content at various initial $K_L a$ values are shown in Fig. 4A. At an initial $K_L a$ of 96.0 h^{-1}, in parallel with a large increase in the percentage of mycelia aggregates over the size of 0.50 mm (Fig. 2) was an increase

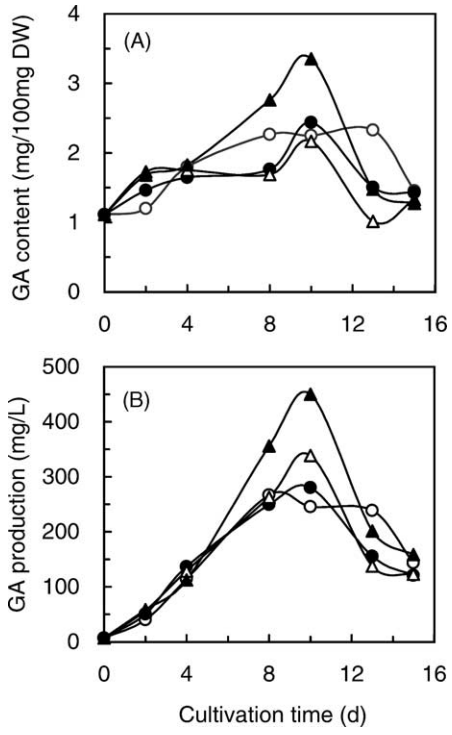

Fig. 4. Effect of initial $K_L a$ values on the specific production (i.e. content) (A) and total production (B) of GA. Symbols for initial $K_L a$ values (h^{-1}): 16.4 (○), 60.0 (●), 78.2 (△) and 96.0 (▲).

in the GA content on Day 10. The maximum GA content in the culture at initial $K_L a$ values of 16.4, 60.0, 78.2 and 96.0 h^{-1} was 2.33, 2.44, 2.17 and 3.36 mg per 100 mg DW as obtained on Days 13, 10, 10 and 10, respectively. As mentioned above, for the cultures at an initial $K_L a$ of 96.0 h^{-1} on Day 10, the cells in the center of about 55% mycelia pellets may be under oxygen limitation, which was considered to be the key factor affecting GA synthesis [18]. In a previous study, it was also suggested that a larger size of mycelia aggregates was beneficial for the GA biosynthesis, which may be due to the oxygen limitation in the pellet center [18]. The kinetics of total GA production is shown in Fig. 4B. Although the highest biomass was obtained at an initial $K_L a$ of 78.2 h^{-1}, the maximum GA production was obtained at an initial $K_L a$ of 96.0 h^{-1} because of the high GA content obtained in the latter case (Fig. 4A). The total GA production of 245.5, 280.2, 338.5 and 450.1 mg^{-1} was attained on Day 10 in the culture grown at initial $K_L a$ values of 16.4, 60.0, 78.2 and 96.0 h^{-1}, respectively, and their corresponding productivity was 23.9, 27.3, 33.2 and 44.3 mg l^{-1} per day. An increase in initial $K_L a$ led to an increased production and productivity of GA. The GA production and productivity at an initial $K_L a$ value of 96.0 h^{-1} was 1.8-fold those at an initial $K_L a$ value of 16.4 h^{-1}. It is clear that an initial $K_L a$ value of 96.0 h^{-1} was optimal for both GA production and productivity.

The DOT at the later stage of culture at an initial $K_L a$ value of 96.0 h^{-1} was lower than other three cases (Fig. 1C),

and the GA content in this case (on Days 8 and 10) was higher than that in the other cases (Fig. 4A). There seems to exist certain relationship between specific GA production (i.e. content) and DOT level. Further experiments were conducted to explore this potential relationship.

Fig. 5A shows two different DOT profiles in the submerged fermentation. Compared with a low DOT (∼10% of air saturation), the cells of *G. lucidum* grew more quickly when DOT was kept at a higher level (i.e. ∼25% of air saturation) (Fig. 5B). The maximum cell density was 4.08±0.23 and 14.70 ± 0.20 g DW cells l^{-1} ∼10 and 25% of DOT, respectively. The results indicated that the cell growth of *G. lucidum* was significantly inhibited when DOT was controlled ∼10% of air saturation. In the culture of *Pseudomonas putida* and *Amycolatopsis orientalis*, Choi et al. [19] and McIntyre et al. [20] also reported that DOT should be kept above 20% of air saturation for achieving optimum rates of cell growth and accumulation of *cis*,*cis*-muconate [19] and vancomycin [20].

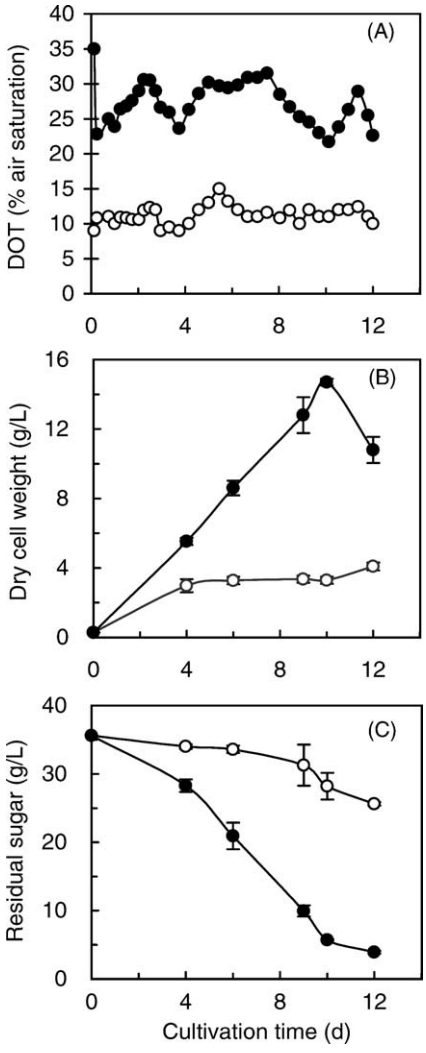

Fig. 5. Time courses of DOT (A), DW (B) and residual sugar concentration (C) during the submerged fermentation of *G. lucidum* in a turbine-agitated bioreactor. Symbols for DOT: ∼10% of air saturation (○) and ∼25% of air saturation (●). The error bars in the figure indicate maximum errors from two independent samples of two reactors.

The DOT gradient in the mycelia pellet can be calculated according to the following equation [16,17]:

$$D\left(\frac{d^2C}{dr^2}+\frac{2}{r}\frac{dC}{dr}\right)=\rho(Q_{O_2})=\frac{\rho(Q_{O_2})_m C}{K_O+C} \quad (2)$$

where C is the DO concentration over the pellet cross sections at a distance r from the pellet center, ρ the density of cells in the pellet, D the coefficient of oxygen diffusion along the liquid channels inside the mycelia pellet, Q_{O_2} the oxygen consumption rate per pellet volume, $(Q_{O_2})_m$ the maximal oxygen consumption rate per pellet volume, and K_O is oxygen constant. When DOT was controlled ∼10% of air saturation, DO concentration was determined to be 2.44×10^{-2} mol m^{-3}, the radius of mycelia pellet was about 1.9×10^{-4} m on an average, the maximal SOUR was measured as 1.85 mmol O_2 g^{-1} DW h^{-1}, the pellet density was 75 kg DW m^{-3}, D was assumed to be 1.9×10^{-9} m^2 s^{-1}, and K_O was 4.47×10^{-5} mol m^{-3} [17]. From Eq. (2), the DOT in the center of mycelia pellet was estimated to be zero, which indicted that the cells in the mycelia pellet were under oxygen limitation.

Time courses of residual sugar are compared in Fig. 5C. The substrate consumption corresponded well to the cell growth. Compared with low DOT (∼10%), the cells consumed lactose more quickly when DOT was controlled ∼25%. Around 10% of DOT, at the end of fermentation (Day 12) there still remained a high level of residual sugar, i.e. 25.63±0.23 g l^{-1}. In contrast, ∼25% of DOT, almost all the sugar was utilized at the end of fermentation. The cell yield on sugar was 0.38 ± 0.01 and 0.48 ± 0.00 g DW g^{-1} lactose ∼10 and 25% of DOT, respectively. An increase of DOT led to a higher lactose consumption rate and a higher cell yield against sugar. Similar phenomena were also observed in the culture of *Azotobacter vinelandii* [21].

Kinetics of EPS accumulation is indicated in Fig. 6A. After inoculation, a rapid increase of EPS concentration was observed. DOT level affected the final production of EPS. EPS production and productivity obtained ∼10% of DOT (0.70±0.02 g l^{-1} and 56.3±0.3 mg l^{-1} per day) were higher than those ∼25% of DOT (0.60 ± 0.04 g l^{-1} and 48.0 ± 2.0 mg l^{-1} per day). The IPS content ∼10% of DOT was also higher than that ∼25% of DOT (Fig. 6B). The results suggest that oxygen limitation was beneficial for the metabolic flux towards the polysaccharide biosynthesis. Fig. 6C shows the dynamic profiles of total IPS accumulation. The total IPS production reached 0.60 ± 0.06 and 1.56 ± 0.04 g l^{-1} ∼10 and 25% of DOT, respectively, and their corresponding productivity was 49.2 ± 4.2 and 155.0 ± 3.0 mg l^{-1} per day.

Time profiles of GA content are shown in Fig. 7A. The GA content ∼10% of DOT was higher than that ∼25% of DOT. Around 10% of DOT, after inoculation GA content increased until Day 9, when a maximum GA content of

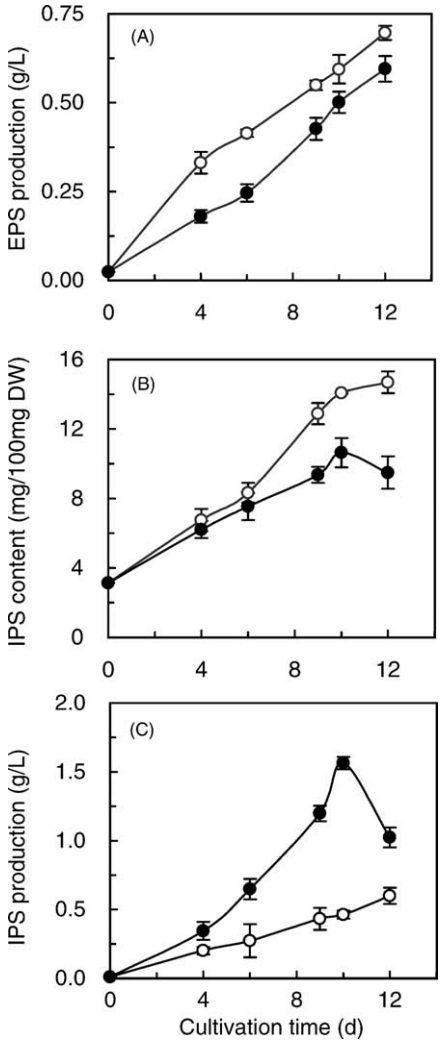

Fig. 6. Effect of DOT on the EPS production (A), IPS content (B) and total IPS production (C). Symbols for DOT: ∼10% of air saturation (○) and ∼25% of air saturation (●). The error bars in the figure indicate maximum errors from two independent samples of two reactors.

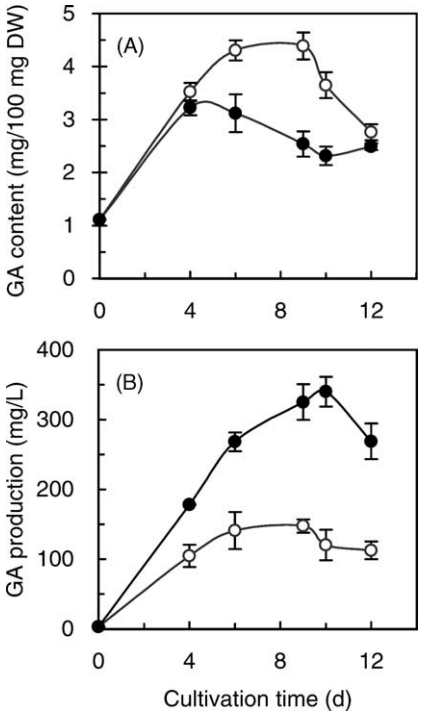

Fig. 7. Effect of DOT on the GA content (A) and its total production (B). Symbols for DOT: ∼10% of air saturation (○) and ∼25% of air saturation (●). The error bars in the figure indicate maximum errors from two independent samples of two reactors.

4.39 ± 0.25 mg per 100 mg DW was obtained. Later, it decreased sharply. In the case ∼25% of DOT, after inoculation GA content increased until Day 4, when a maximum GA content of 3.22 ± 0.14 mg per 100 mg DW was reached. After that, GA content decreased slowly until Day 10, and it had a little increase at the end of fermentation (Day 12). The data indicated that a relatively lower DOT, where the cells in the center of mycelia pellet could be under oxygen limitation, was beneficial for the GA biosynthesis. The results imply that oxygen limitation was beneficial for the metabolic flux towards the GA biosynthesis. The kinetics of total GA production is shown in Fig. 7B. The total GA accumulation reached 147.5±9.7 and 340.1±21.2 mg l^{-1} ∼10 and 25% of DOT, respectively, and their corresponding productivity was 16.0±0.7 and 33.7±1.8 mg l^{-1} per day. It is clear that a relatively high DOT led to a higher GA production and productivity.

When DOT was controlled ∼10% of air saturation, the cells of mycelia pellet had relatively bigger sizes than those at 25% of DOT (data not shown), which were under oxygen limitation as discussed above. They did not grow after Day 4 (Fig. 5B) while maintaining the cellular activities, and the metabolic flux was more shifted towards GA biosynthesis, which led to a higher GA content in this case compared to that at 25% of DOT (Fig. 7A). Although a higher content of IPS and GA (Fig. 7A) was obtained ∼10% of DOT, a much higher cell density was obtained ∼25% of DOT (Fig. 5B), therefore, a higher total production of IPS and GA (Fig. 7B) was reached in the latter case. The highest IPS and GA production was reached on the same day (Day 10) as the biomass. The production and productivity of GA and IPS ∼25% of DOT were about two-fold those ∼10% of DOT. The results indicated that a high cell density was desirable for total production of intracellular products in order to increase the metabolite productivity.

The present work demonstrated that oxygen supply significantly affected the cell growth, cellular morphology and metabolite biosynthesis during submerged fermentation of *G. lucidum* in a bioreactor. The experiments on both initial $K_L a$ values and DOT were also repeated, and the same conclusions were obtained. The results obtained are considered useful for the simultaneous, highly efficient production of the cell mass, *Ganoderma* polysaccharide, and GA on a large scale.

Acknowledgments

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