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# Performance analyses of a pH-shift and DOT-shift integrated fed-batch fermentation process for the production of ganoderic acid and *Ganoderma* polysaccharides by medicinal mushroom *Ganoderma lucidum*

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ABSTRACT

Investigations on *Ganoderma lucidum* fermentation suggested that the responses of the cell growth and metabolites biosynthesis to pH and dissolved oxygen tension (DOT) were different. The ganoderic acid (GA) production of 321.6 mg/L was obtained in the pH-shift culture by combining a 4-day culture at pH 3.0 with the following 6-day culture at pH 4.5, which was higher by 45% and 300% compared with the culture at pH 3.0 and 4.5, respectively. The GA production of 487.1 mg/L was achieved in the DOT-shift culture by combining a 6-day culture at 25% of DOT with a following 6-day culture at 10% of DOT, which was higher by 43% and 230% compared with the culture at 25% and 10% of DOT, respectively. A fed-batch fermentation process by combining the above-mentioned pH-shift and DOT-shift strategies resulted in a significant synergistic enhancement of GA accumulation up to 754.6 mg/L, which is the highest reported in the submerged fermentation of *G. lucidum* in stirred-tank bioreactor.



## 1. Introduction

Medicinal mushrooms are abundant sources of a wide range of useful native products and new compounds with interesting biological activities (Wasser, 2002; Lindequist et al., 2005). *Ganoderma lucidum* (Fr.) Krast (Polyporaceae) is a famous traditional Chinese medicinal mushroom. Ganoderic acid (GA) and *Ganoderma* polysaccharides are two of its major bioactive components (Paterson, 2006). Interestingly, recent studies show that GA has new biological activities including suppressing the growth of human solid tumor and the proliferation of a highly metastatic lung cancer cell line (95-D) (Tang et al., 2006), and anti-HIV-1 (El-Mekkawy et al., 1998). Currently, commercial products from medicinal mushrooms are mostly obtained through the field-cultivation of the fruiting body. However, in this case it is difficult to control the quality of the final product. So, mushroom submerged fermentation is viewed as a promising alternative for the efficient production of their valuable products (Zhong and Tang, 2004; Tang et al., 2007).

In *G. lucidum* fermentation, Zhong together with his colleagues demonstrated the response of the cell growth and metabolites biosynthesis to process parameters (i.e., pH, dissolved oxygen tension, substrate feeding) was different. In the shake flask fermentation of *G. lucidum*, Fang and Zhong (2002a) found an initial pH of 6.5 was the best for mycelial growth and GA biosynthesis, while an initial pH of 3.5 was beneficial for the accumulation of *Ganoderma* polysaccharides. In the stirred-tank bioreactor cultivation of *G. lucidum*, Tang and Zhong (2003a) observed that 25% of dissolved oxygen tension (DOT) was beneficial for *G. lucidum* growth, while 10% of DOT was favorable for the specific GA biosynthesis (i.e., GA content). Based on the favorable effect of oxygen limitation on the specific GA biosynthesis, Fang and Zhong (2002b) developed a two-stage fermentation process by combining conventional shake flask fermentation (i.e., the first-stage culture) with static culture (i.e., the second-stage culture), and GA production was greatly enhanced in the two-stage culture process. Then, the first-stage culture was scaled-up in the conventional stirred-tank bioreactor (Tang and Zhong, 2003a), and the second-stage culture was successfully scaled-up in the novel multi-layer static bioreactor (Tang and Zhong, 2003b). Tang and Zhong (2002) demonstrated the inhibition of GA biosynthesis by a relatively higher initial lactose concentration (i.e., >35 g/L) was avoided and GA production markedly improved by lactose supplementation when its residual level was around 10–5 g/L.

Medicinal mushroom cells have the ability to respond to environment alterations. Provided that these alterations proceed with

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the acceptable ranges, in which apoptosis or necrosis is not induced, the physiological response of *G. lucidum* cells towards changes in pH, DOT, and nutrient supply is mostly related to alterations in the cell growth and metabolites biosynthesis. Different environment alterations have different physiological impacts on the metabolism of *G. lucidum* cells. Therefore, well-directed process parameters shift can provoke physiological changes that positively affect process performance, thus representing a valuable control strategy for the cell growth and metabolites biosynthesis. Among the process control strategy developed for enhancing metabolite biosynthesis, pH-shift and DOT-shift have been shown to be the most efficient strategies. pH-shift strategy has been developed for efficient production of bioactive metabolites in submerged fermentation of *G. lucidum* (Lee et al., 1999; Kim et al., 2006), *Streptoverticillium mobaraense* (Zheng et al., 2002), *Xanthophyllomyces dendrorhous* (Hu et al., 2006), *Aspergillus terreus* (Lai et al., 2005), *Clostridium sporogenes* (Montville et al., 1985), *Escherichia coli* K235-WXJ (Zhan et al., 2002) and *Bacillus licheniformis* (Çalık et al., 2002). DOT-shift strategy was demonstrated to be an important process control strategy for cordycepin accumulation in the submerged fermentation of *Cordyceps militaris* (Mao and Zhong, 2004).

However, until now there is no report on the integrated strategy of combining process control parameters shift with sugar feeding for the efficient production of metabolites by medicinal mushroom submerged fermentation. Therefore, it would be interesting to investigate whether the integrated strategy with simultaneous using of pH-shift strategy, DO-shift strategy and carbon source feeding strategy will lead to a quantum improvement in GA and *Ganoderma* polysaccharides production by the submerged fermentation of *G. lucidum*. Based on our previous results, a multi-stage integrated fed-batch fermentation process of *G. lucidum* by combining pH-shift, DOT-shift with lactose feeding was developed in this work, and the fermentation efficiency was greatly improved in the conventional stirred-tank bioreactors. This work is very useful to the large-scale fermentation of *G. lucidum* for the simultaneous production of GA and *Ganoderma* polysaccharides. Such work will be very helpful to other mushroom fermentations for useful metabolite production.

## 2. Methods

### 2.1. Maintenance and preculture of *G. lucidum*

The strain of *G. lucidum* CGMCC 5.616 was purchased from China General Microbiological Fermentation Collection Center (Beijing, China). The details of the preculture medium and preculture conditions have been previously described (Tang and Zhong, 2002).

### 2.2. Fermentation in the stirred-tank bioreactor

The stirred-tank bioreactor used was a 5.5-L (working volume) BioFlo 110 New Brunswick Scientific (NJ, USA) agitated bioreactor with two six-bladed Rushton impellers (5.9 cm i.d.). The lower impeller was 2.5 cm above the reactor bottom, and the vertical distance between two impellers was 8.5 cm. The reactor was aerated through a ring sparger with a pore size of 1.0 mm, which was located 2.2 cm above the reactor bottom. The bioreactor was equipped with the probes of pH (Mettler-Toledo GmbH, Switzerland), DO (Mettler-Toledo GmbH, Switzerland), temperature and foam.

Four cultures were carried out simultaneously in the stirred-tank bioreactors with homogeneous cell source under well-controlled process conditions but at different investigating culture conditions. The identical cell source and process conditions other than the investigating condition made it possible to endure accurate head-to-head comparisons. The results presented were confirmed to be reproducible in another experiment (data not shown).

#### 2.2.1. Effect of culture pH

The significance of culture pH on *G. lucidum* growth and metabolites accumulation was studied by setting culture pH at 2.0, 2.5, 3.0, 4.5, and 5.5 during the whole fermentation process in the stirred-tank bioreactors. For comparison, the control experiment was initially run at a pH of 5.5, and since then pH was not controlled (i.e., at an initial pH of 5.5). Initially the aeration and agitation rate was 0.1 vvm and 50 rpm, respectively. In order to strictly control DOT, the gas blending technique was applied to control inlet gas composition, where air was proportionally mixed with $N_2$ or $O_2$ in the inlet. DOT was set between 25 ± 5% of air saturation and controlled by adjusting agitation speed, aeration rate and inlet gas composition during fermentation, while time profiles of agitation rate were the same in order to diminish the impact of shear stress and mixing. At the end of fermentation, the aeration and agitation rate was around 1.0 vvm and 400 rpm, respectively.

#### 2.2.2. pH-shift culture

Based on the impact of culture pH, a pH-shift culture was proposed by combining a 4-day culture at a pH of 3.0 (i.e., the first-stage) with the following culture at pH 3.0, 4.5, and 5.5 (i.e., the second-stage). The control experiment was conducted without the pH control in the second-stage culture for comparison. The other culture conditions were the same as in the above experiments.

#### 2.2.3. DOT-shift culture

According to our previous work (Tang and Zhong, 2003a), a DOT-shift culture was developed by combining the cell growth phase (i.e., the first-stage) cultured at 25% of DOT with GA accumulation phase (i.e., the second-stage) cultured at 10% of DOT. The impact of shift time between the cell growth phase and GA accumulation phase was investigated by setting the shift time on day 4, day 6, day 8 and day 10. Culture pH was controlled at 3.00 ± 0.01 throughout the fermentation process. The other culture conditions were the same as in the above experiments.

#### 2.2.4. Lactose feeding strategy

When residual sugar concentration decreased to around 10–5 g/L on day 9, which was predicted from previous fermentations and confirmed by off-line assay, a fed-batch process with a pulse feeding of highly concentrated lactose solution into a reactor was conducted to increase the residual lactose concentration by 15 g/L (Tang and Zhong, 2002). The other culture conditions were the same as in the above experiments.

#### 2.2.5. Integration of pH-shift, DOT-shift and lactose feeding

From the viewpoint of total GA accumulation, the above results indicated that the optimal pH-shift culture was combining the 4-day culture at pH 3.0 with the following culture at pH 4.5; and the optimal DOT-shift culture was coupling the 6-day culture at 25% of DOT and the following culture at 10% of DOT. Here, in order to further enhance total GA production, integration of pH-shift, DOT-shift and lactose feeding were conducted by combining their specific optimal strategies. There are four kinds of integrated strategy, i.e., interacting pH-shift and DOT-shift, combining pH-shift with lactose feeding, coupling DOT-shift and lactose feeding, simultaneously using pH-shift, DOT-shift and lactose feeding.

### 2.3. Metabolites measurements

The extracellular polysaccharides (EPS) production, the content of intracellular polysaccharides (IPS) and ganoderic acid (GA) were

measured as the methods previously reported (Tang and Zhong, 2002).

## 3. Results and discussion

### 3.1. Effect of culture pH

Fang and Zhong (2002a) investigated the impact of initial pH on the performance of submerged fermentation of *G. lucidum* in shake flask. As we know, culture pH in shake flask could not be controlled, and furthermore Fang and Zhong (2002a) reported that culture pH showed a sharp decrease to 3.2 during the first 4 days of cultivation in all cases. It is essential to study the effect of pH on the performance of *G. lucidum* submerged fermentation in stirred-tank bioreactor, in which culture pH could be controlled well.

Fig. 1A demonstrates that EPS production was obviously inhibited at the pH of 2.0 and 2.5, and the highest EPS production (i.e., 1.30 g/L) and productivity (i.e., 63.9 mg/L per day) were attained at the pH of 3.0. A different phenomenon was observed in the cultivation of *G. lucidum* ASI 7004, Lee et al. reported the highest EPS production of 13.58 g/L was obtained at the pH of 6 (Lee et al., 1999). As we confirmed, their measurement of EPS concentration in the culture broth by gravimetric method was not accurate due to the impurity residues in the samples, and it could overestimate the polysaccharide amount by almost 9–10-folds of that by using a conventional phenol-sulfuric acid method as we used.

Fig. 1B shows culture pH significantly affected specific IPS biosynthesis (i.e., IPS content), and a pH of 3.0 was beneficial for the specific IPS biosynthesis. As shown in Fig. 1C, the maximal IPS production of 2.70 g/L was obtained on day 10 at the pH of 3.0 because of the highest biomass (data not shown) and IPS content (Fig. 1B) on day 10.

Time courses of GA content under various culture pH are compared in Fig. 2A. After inoculation, GA content quickly increased up to the peak values on day 2. After that, a gradual decrease of GA content was observed in all cases, except that GA content at the pH of 3.0 showed a sharply increase after day 10. GA content at the pH of 3.0 was higher than the other three cases after the culture of day 12, which was the main contribution to the higher total GA production at pH 3.0 (Fig. 2B). Fig. 2B clearly demonstrates culture pH significantly affected total GA accumulation. The highest GA production was obtained at the pH of 3.0 because of a relatively higher cell density (data not shown) and higher GA content at the later-stage fermentation of *G. lucidum* (Fig. 2A).

To conclude, the highest biomass (i.e., 16.01 g DW/L), the production of EPS (i.e., 1.30 g/L), IPS (i.e., 2.70 g/L) and GA (i.e., 221.4 mg/L) were obtained at the culture pH of 3.0, which was higher by 49%, 155%, 120% and 100% compared to the culture at an initial pH of 5.5, respectively.

### 3.2. pH-shift culture

By compared the kinetics of *G. lucidum* cell growth (data not shown) and specific GA biosynthesis (Fig. 2A) at the pH of 3.0, we observed that GA content quickly decreased from day 2 to day 10, while *G. lucidum* cell grew quickly; a quickly increase of GA content was observed after day 10, while *G. lucidum* stop to grow and biomass decreased. This indicated that the optimal culture pH for *G. lucidum* cell growth phase may be different from that for GA biosynthesis phase, which suggested that culture pH at different phase should be adjusted according to the demand of cell growth or GA biosynthesis in order to enhance total GA accumulation. A gradual decrease of GA content was observed after day 2 (Fig. 2A), so it was supposed that pH should be shifted not later than day 2. While, preliminary experiment results indicated that

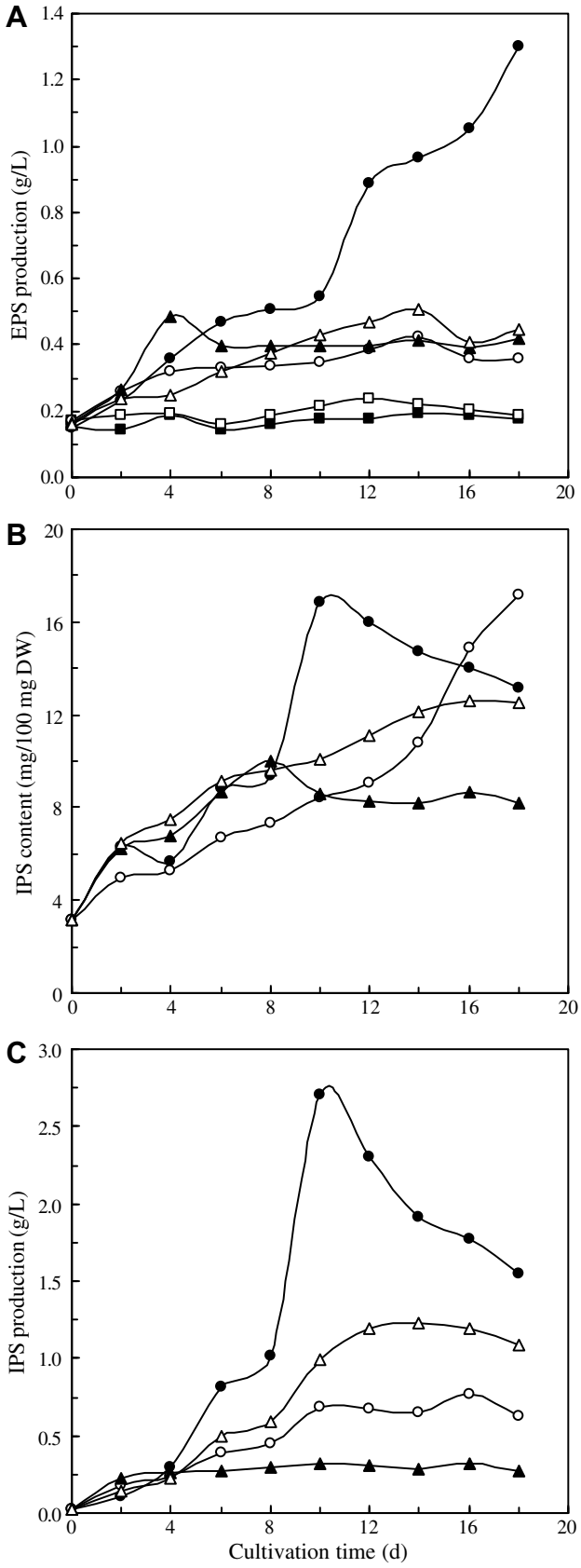


**Fig. 1.** Time courses of EPS production (A), IPS content (B) and IPS production (C) under various culture pH values during the submerged fermentation of medicinal mushroom *G. lucidum* in stirred-tank bioreactor. Symbols for culture pH: 2.0 (■), 2.5 (□), 3.0 (●), 4.5 (○), 5.5 (▲), and the control experiment (△) which was initially run at the pH of 5.5, then without pH control (i.e., at an initial pH of 5.5).

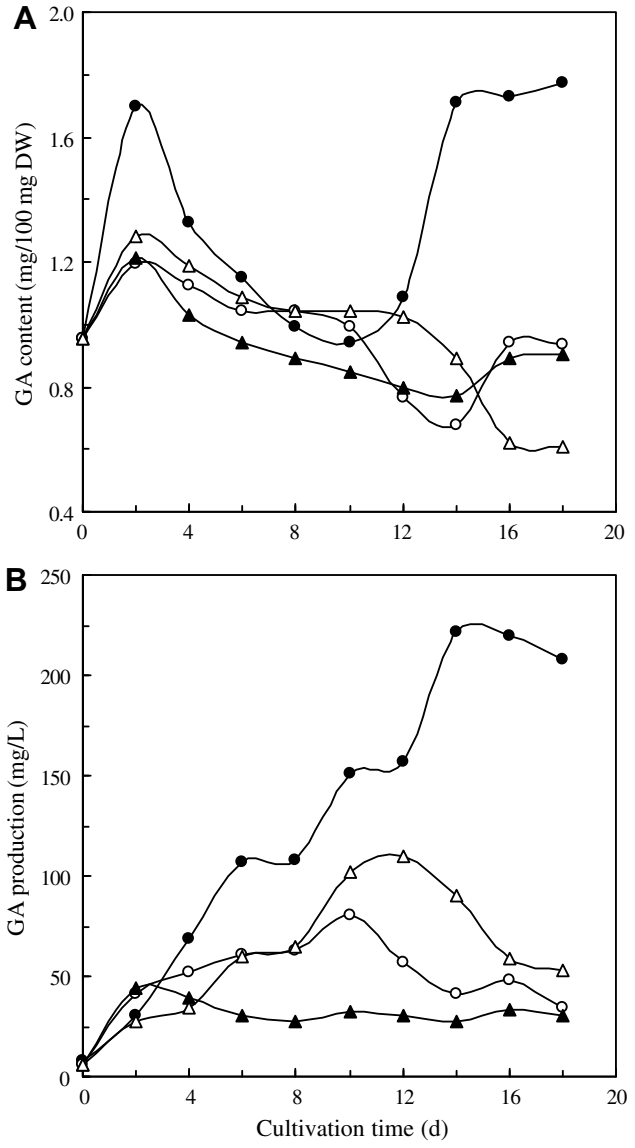


**Fig. 2.** Dynamic profiles of specific GA biosynthesis (i.e., GA content) (A) and total GA accumulation (B) under various culture pH values. The symbols for culture pH are the same as those in Fig. 1.

culture pH shifted on day 2 was not favorable for the cell growth and metabolites accumulation (data not shown) perhaps because the biomass was too low on day 2. Based on the above analysis, the first 4 days culture at the pH of 3.0 (i.e., the first-stage) for *G. lucidum* growth was shifted to the second-stage culture in which pH was controlled at 3.0, 4.5, and 5.5 for GA biosynthesis.

As shown in Fig. 3A, EPS production was remarkably affected by the second-stage culture pH. Not only EPS production (i.e., 2.35 g/L) and EPS yield on lactose (i.e., 68.4 mg/g lactose), but also EPS productivity (i.e., 158.1 mg/L per day) and specific productivity (i.e., 15.7 mg/g DW per day) was the highest when pH was not controlled in the second-stage culture. In the cultivation of *G. lucidum* ASI 7004, Lee et al. reported the pH-shift from 3.0 to 6.0 on day 2 was best for EPS biosynthesis (Lee et al., 1999).

By compared Fig. 3B and C, it was concluded that the pH-shift from 3.0 to 4.5 on day 4 was beneficial for the specific and total IPS production.

Fig. 4A clearly shows that GA content was significantly affected by the second-stage culture pH. The GA content continued to increase after day 2 when pH was shifted from 3.0 to 4.5 or 5.5 on

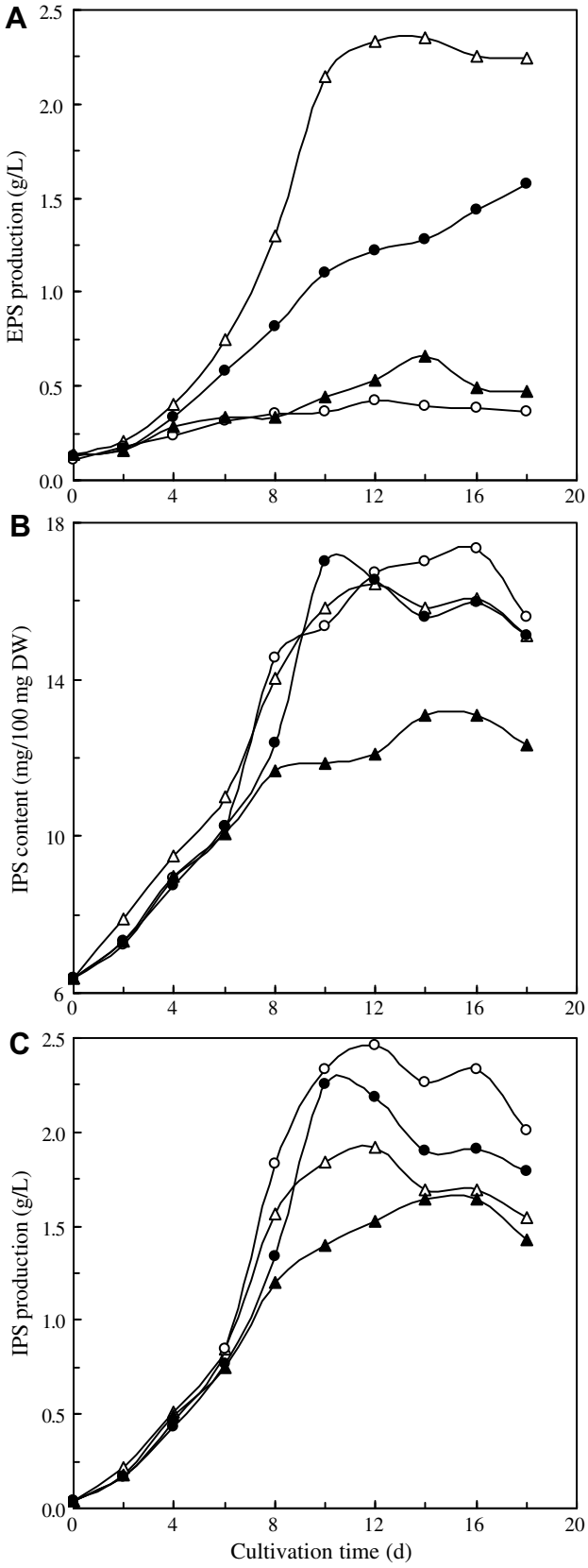


**Fig. 3.** Kinetics of EPS production (A), IPS content (B) and IPS production (C) in the pH-shift culture by combining the 4-day culture at pH 3.0 (i.e., the first-stage) with the second-stage culture at the pH of 3.0 (●), 4.5 (○), 5.5 (▲), and the control experiment (△) which was run without pH control in the second-stage.

day 4, while there was a sharp decrease in GA content when pH was controlled at 3.0 or without control in the second-stage culture. This suggested that GA biosynthesis metabolism was turned around when pH was shifted from 3.0 to 4.5 or 5.5 on day 4. As shown in Fig. 4B, total GA accumulation was significantly affected by second-stage culture pH. Not only the maximal GA production (i.e., 321.6 mg/L) and productivity (i.e., 31.5 mg/L per day), but also GA yield on lactose (i.e., 8.5 mg/g lactose) was obtained at the shift of pH from 3.0 to 4.5 on day 4. In the submerged fermentation of *X. dendrorhous*, Hu et al. reported that pH-shift from 6.0 to 4.0 on the 80th h improved astaxanthin production to 27.05 mg/L, which was 24.1% higher than constant pH fermentation (Hu et al., 2006).

A pH-shift culture of *G. lucidum* was successfully developed for enhancing GA production. The highest GA production of 321.6 mg/L was obtained when pH was shifted from 3.0 to 4.5 on day 4, which was increased by 45% and 300% compared with the cultures at the pH of 3.0 and 4.5, respectively. This demonstrated that a pH-shift culture by combining the first 4 days culture at the pH of 3.0 with the following culture at the pH of 4.5 was useful for enhancing total GA accumulation.

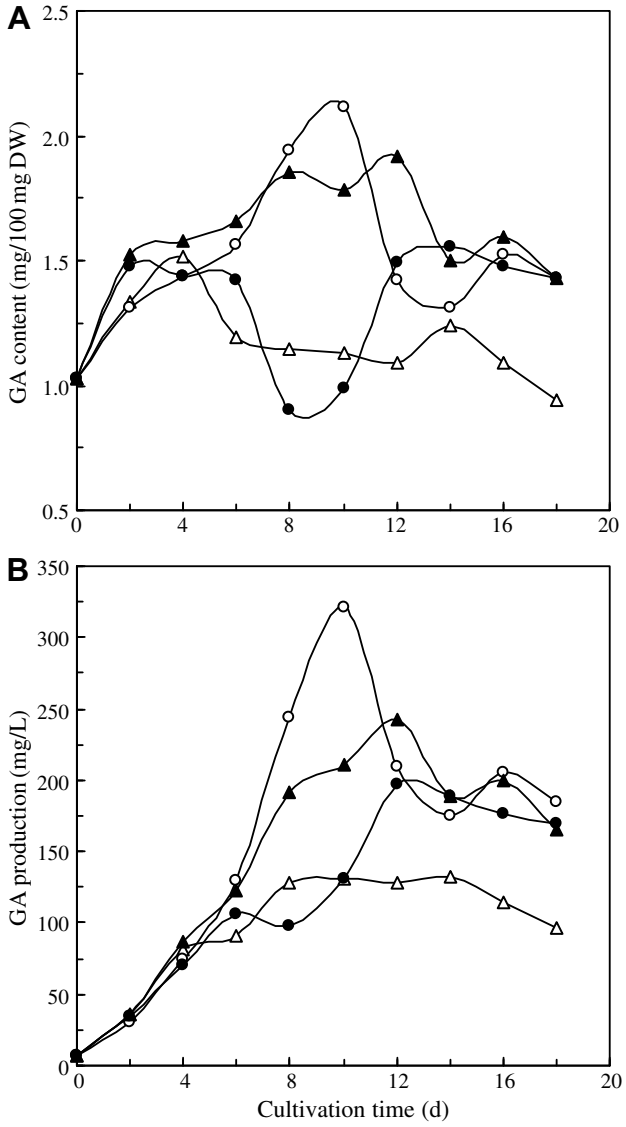


**Fig. 4.** Dynamic profiles of the specific GA biosynthesis (i.e., GA content) (A) and total GA accumulation (B) in the pH-shift culture by combining the 4-day culture at pH 3.0 (i.e., the first-stage) with the second-stage culture at the pH of 3.0 (●), 4.5 (○), 5.5 (▲), and the control experiment (△) which was run without pH control in the second-stage.

### 3.3. DOT-shift culture

Tang and Zhong (2003a) demonstrated that 25% of DOT was beneficial for the cell growth of *G. lucidum*, while 10% of DOT was favorable for specific GA biosynthesis (i.e., GA content). Based on the results, a DOT-shift culture by combining the first-stage culture at 25% of DOT with the following second-stage culture at 10% of DOT was proposed in order to increase total GA accumulation. Here, effect of shift time (i.e., day 4, 6, 8 and 10) between the first-stage and the second-stage was tested.

The kinetics of EPS production under different shift time is compared in Fig. 5A. In all cases, a gradual increase of EPS production was observed within the cultivation time. Not only the maximal EPS production and yield, but also EPS productivity and specific productivity were attained at the shift time of day 4. This indicated that the first-stage culture at 25% of DOT should be shifted to the second-stage culture at 10% of DOT on day 4 from the viewpoint of EPS production.

As shown in Fig. 5B, the maximal IPS content was 8.5, 12.4, 16.8 and 15.4 mg/100 mg DW at the shift time of day 4, 6, 8 and 10, respectively. The dynamic profiles of total IPS production under different shift time are compared in Fig. 5C. The IPS production reached its peak value (i.e., 0.77, 1.84, 2.88 and 2.05 g/L) at the shift time of day 4, 6, 8 and 10, respectively. Not only the maximal IPS content and production, but also its productivity and specific productivity were obtained at the shift time of day 8. These results indicated that IPS biosynthesis was stimulated by the DOT-shift from 25% to 10% on day 8.

Fig. 6A clearly shows that the specific GA biosynthesis (i.e., GA content) was significantly affected by shift time. In all cases, after inoculation, GA content quickly increased up to about 2.5 mg/100 mg DW on day 2. After that, a gradual decrease trend of GA content was observed at the shift time of day 8 or day 10, while a quickly increase of GA content occurred at the shift time of day 4 or day 6. So, GA content at the shift time of day 4 or day 6 was much higher than the other two cases in the later-stage culture, which was the main contribution to the higher total GA production at the shift time of day 6 (Fig. 6B). The results indicated that the DOT-shift culture on day 6 was favorable for the specific GA biosynthesis (i.e., GA content).

Fig. 6B clearly demonstrates that shift time obviously affected total GA accumulation. The highest GA production (i.e., 487.1 mg/L) was obtained at the shift time of day 6 because of a relatively higher cell density (data not shown) and higher GA content (Fig. 6A) at the later-stage fermentation of *G. lucidum*. In the cultivation of mushroom *C. militaris* to produce cordycepin, Mao and Zhong reported that DOT-shift from 60% to 30% when specific cordycepin formation rate decreased improved cordycepin production and productivity to 201.1 and 15.5 mg/L per day, which was 15% and 30% higher than conventional DOT control experiment, respectively (Mao and Zhong, 2004).

To conclude, a DOT-shift culture of *G. lucidum* was successfully developed. The highest GA production of 487.1 mg/L was obtained when DOT was shifted from 25% to 10% on day 6, which was increased by 43% and 230% compared with the cultures at the 25% and 10% of DOT, respectively (Tang and Zhong, 2003a).

### 3.4. Integrated strategy of pH-shift, DOT-shift and lactose feeding

An integrated strategy of combining of well-directed process parameters shift with carbon source feeding can provoke physiological changes that positively affect process performance, thus representing a valuable integrated strategy for the cell growth

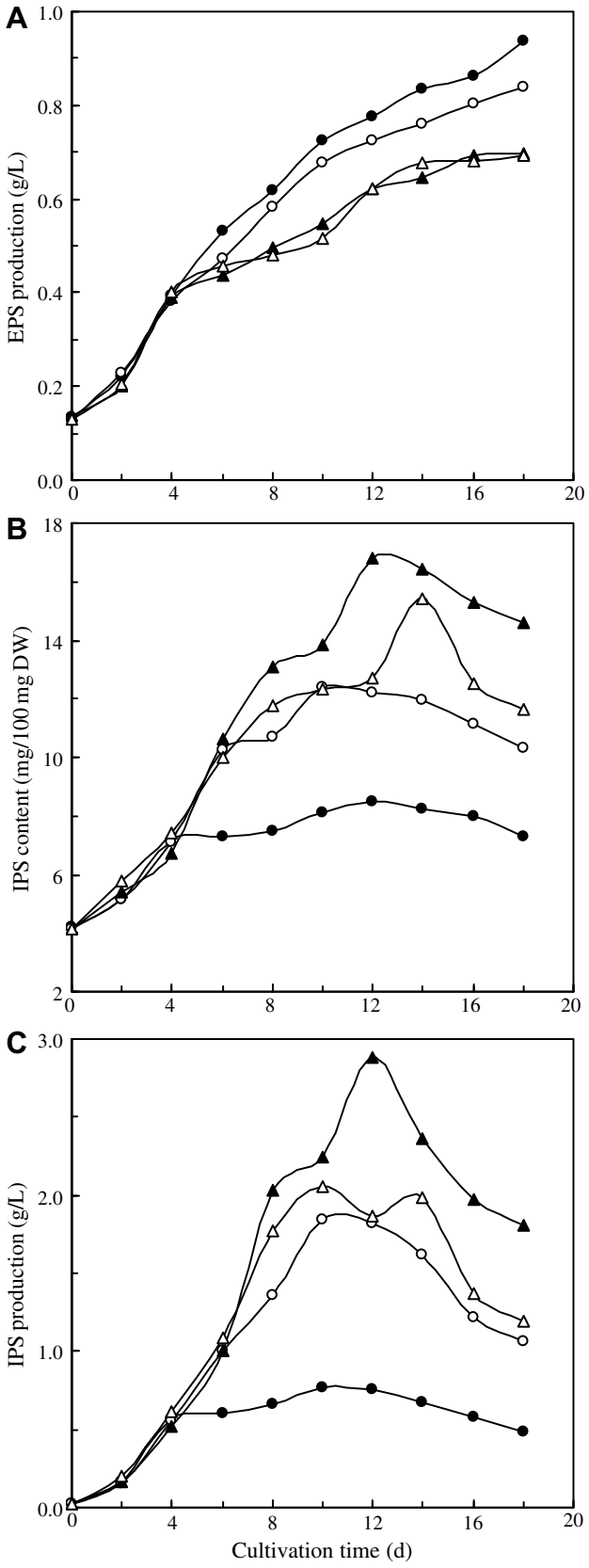


**Fig. 5.** Dynamic profiles of EPS production (A), IPS content (B) and IPS production (C) under various shift time during the DO-shift culture of *G. lucidum*. Symbols for the shift time: day 4 (●), day 6 (○), day 8 (▲), and day10 (△).

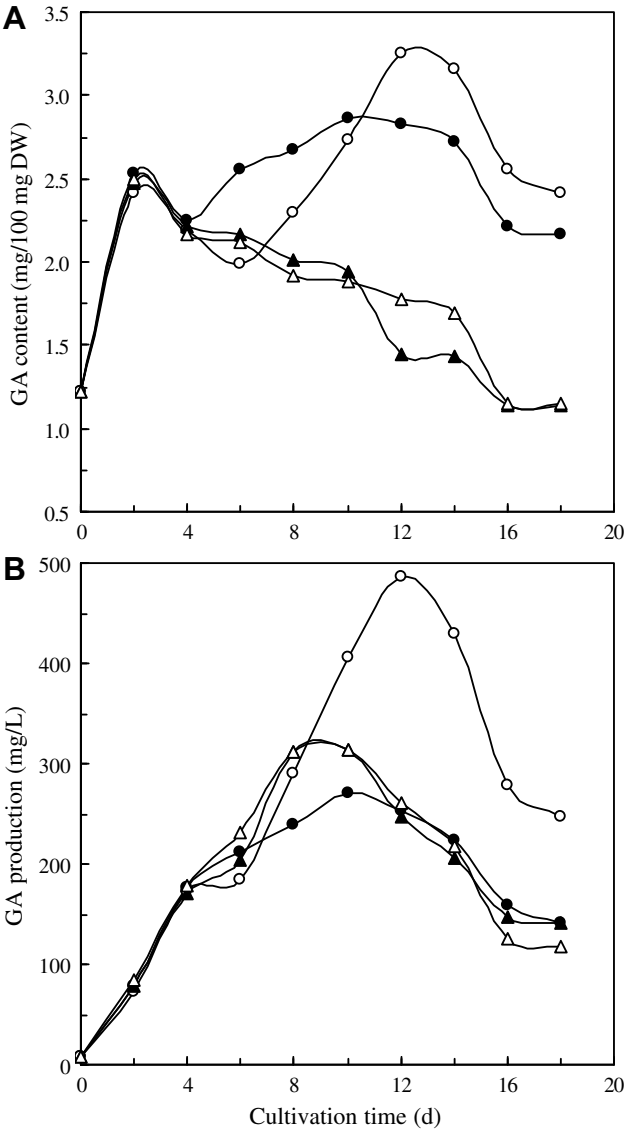


**Fig. 6.** Time profiles of GA content (A) and total GA accumulation (B) under various shift time during the DO-shift culture of *G. lucidum*. The symbols for the shift time are the same as those in Fig. 5.

and metabolites biosynthesis. In CHO cells cultivation for expressing Epo-Fc, Trummer et al. developed such a process with coupling pH-shift strategy and temperature-shift strategy for enhancing Epo-Fc accumulation, and the maximum Epo-Fc production of 5344 pmol/mL was attained, which was 2.2-fold higher than that of control (Trummer et al., 2006). Mohamad et al. reported another integrated process control strategy with simultaneous use of carbon source feeding strategy and pH-shift strategy for the production of kojic acid in submerged fermentation of *Aspergillus flavus*, and the maximum kojic acid production of 31.00 g/L was obtained, which was about 8-fold higher than that for batch fermentation (Mohamad et al., 2002). Liang et al. demonstrated an integrated strategy of combining cysteine feeding with DO-shift for efficient production of glutathione (Liang et al., 2008).

In the pH-shift culture, the maximal GA production of 321.6 mg/L was obtained through a 4-day culture at the pH of 3.0 followed by 6-day culture at the pH of 4.5. In the DOT-shift culture, the maximal GA production of 487.1 mg/L was achieved by

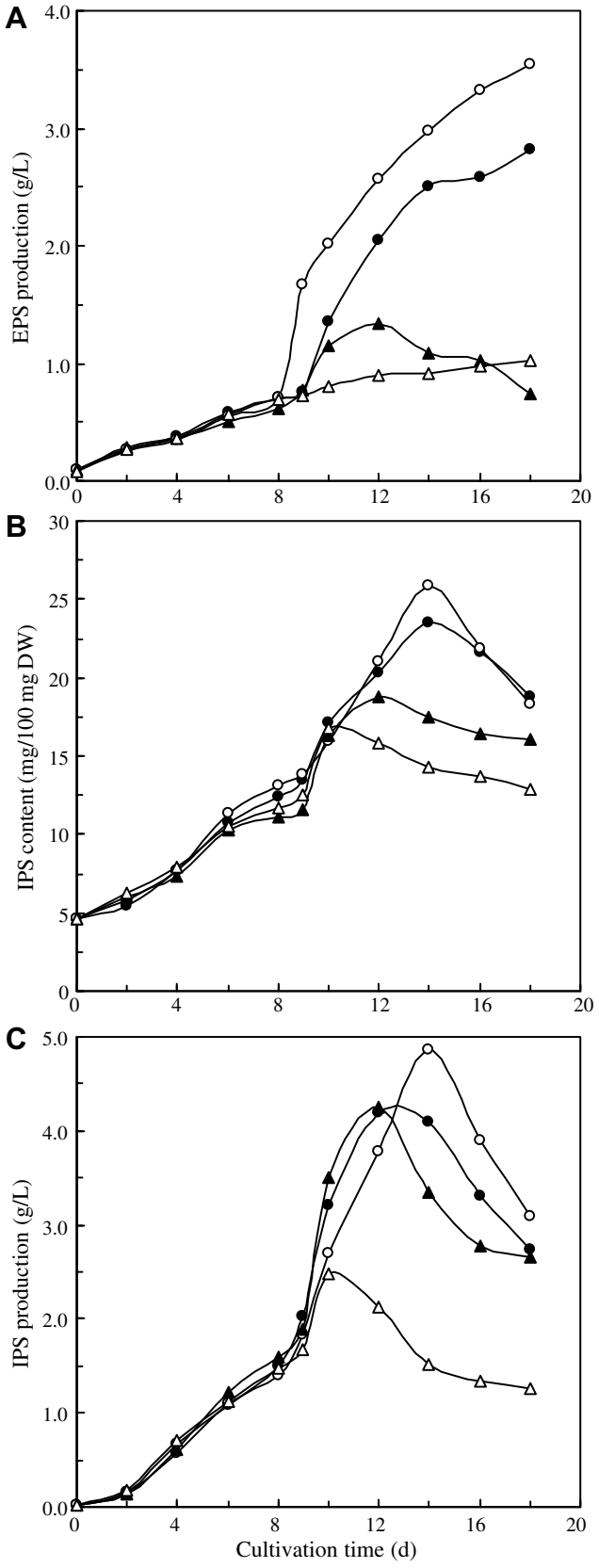


**Fig. 7.** Time profiles of EPS production (A), IPS content (B) and IPS production (C) under various integrated strategy of combining pH-shift, DOT-shift and lactose feeding. Symbols for integrated strategy: interacting pH-shift and DOT-shift (△), combining pH-shift with lactose feeding (▲), coupling DOT-shift and lactose feeding (○), and simultaneously using pH-shift, DOT-shift and lactose feeding (●).

combining a 6-day culture at 25% of DOT with a following 6-day culture at 10% of DOT. Lactose feeding has been already identified to be an efficient strategy for enhancing GA production by the submerged fermentation of *G. lucidum* (Tang and Zhong, 2002). Here, in order to further enhance total GA accumulation, we studied the synergic effects of the above-mentioned optimal strategy of pH-shift, DOT-shift and lactose feeding by coupling pH-shift with DOT-shift, combining pH-shift with lactose feeding, interacting DOT-shift with lactose feeding and simultaneously using pH-shift, DOT-shift and lactose feeding, respectively.

The kinetics of EPS production under different integrated strategy is compared in Fig. 7A. The maximal EPS production was 1.02, 1.34, 3.54 and 2.82 g/L at the integrated strategy of coupling pH-shift with DOT-shift, combining pH-shift with lactose feeding, interacting DOT-shift with lactose feeding, and simultaneously using pH-shift, DOT-shift and lactose feeding, respectively, and the corresponding EPS productivity was 51.7, 103.8, 191.3 and 151.6 mg/L per day. This indicated that DOT-shift and lactose feeding should be combined from the viewpoint of EPS biosynthesis.

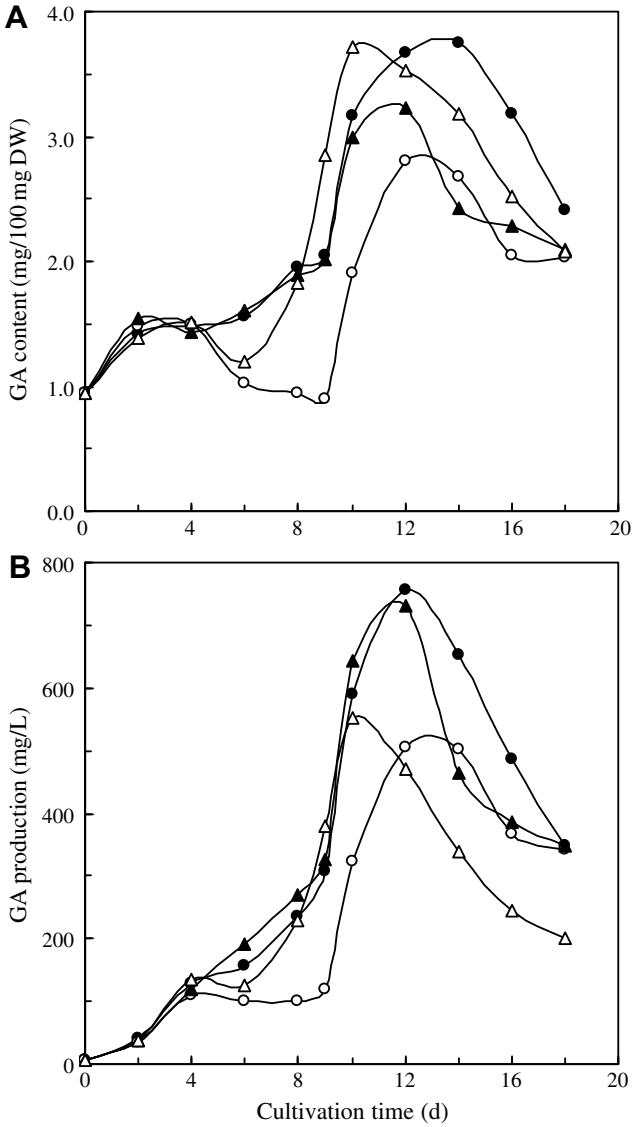


**Fig. 8.** Dynamic profiles of GA content (A) and total GA accumulation (B) under various integrated strategy of combining pH-shift, DOT-shift and lactose feeding. The symbols for the integrated strategy are the same as those in Fig. 7.

By compared Fig. 7B and C, it was concluded that not only the maximal IPS content, but also the maximal IPS production was obtained at the integrated strategy of interacting DOT-shift with lactose feeding. It was suggested that DOT-shift and lactose feeding had a synergistically favorable effect on the IPS biosynthesis.

Fig. 8A shows integrated strategy significantly affected the specific GA biosynthesis (i.e., GA content). The maximal GA content was attained at interacting pH-shift and DO-shift or simultaneously using pH-shift, DOT-shift and lactose feeding. This suggested the specific GA biosynthesis was synergistically improved by interacting pH-shift with DOT-shift. As shown in Fig. 8B, total GA accumulation was significantly affected by the integrated strategy. The maximal total GA production was 550.9, 730.4, 504.7 and 754.6 mg/L at the integrated strategy of coupling pH-shift and DOT-shift, combining pH-shift with lactose feeding, interacting DOT-shift with lactose feeding, and simultaneously using pH-shift, DOT-shift and lactose feeding, respectively. Not only the maximal GA production, but also the GA productivity was obtained at the integration of pH-shift, DOT-shift and lactose feeding.

By the integrated strategy of pH-shift, DOT-shift and lactose feeding in the submerged fermentation of *G. lucidum,* the maximal GA production of 754.6 mg/L was achieved, which was 2.3-fold, 1.5-fold and 2.1-fold compared to those obtained in the culture of pH-shift, DOT-shift and fed-batch fermentation, respectively.

## 4. Conclusion

In this work, a novel integrated strategy for the efficient production of ganoderic acid (GA) by the submerged fermentation of medicinal mushroom *G. lucidum* in the conventional stirred-tank bioreactor was demonstrated by simultaneously adopting the pH-shift, DOT-shift and lactose feeding strategy. Compared with the liquid static culture of *G. lucidum* in the multi-layer static bioreactor (Tang and Zhong, 2003b), the novel multi-stage stirred-tank bioreactor cultivation process of *G. lucidum* developed in this work is more easy to scale-up in the pilot and industrial scale bioreactors, and its scale-up is under the way in our laboratory.

Furthermore, the synergic effect of pH-shift, DOT-shift, and lactose feeding strategy demonstrated that the multi-stage control strategy could provoke physiological changes that positively affect GA biosynthesis, although further studies are needed to elucidate the synergistic mechanisms. This work also suggests an efficient avenue for the development of similar integrated strategies to advance the other culture process for the commercial bioactive components production.

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