J Ind Microbiol Biotechnol (2014) 41:1305–1310
DOI 10.1007/s10295-014-1460-1

SHORT COMMUNICATION

SIMB
Society for Industrial Microbiology and Biotechnology

Integrated strategy of pH-shift and glucose feeding for enhanced production of bioactive Antrodin C in submerged fermentation of *Antrodia camphorata*

Huan Zhang · Yong-Dan Hu · Rui-Qiu Lu · Yong-Jun Xia · Bo-Bo Zhang · Gan-Rong Xu

Received: 10 March 2014 / Accepted: 8 May 2014 / Published online: 29 May 2014

Abstract Antrodin C is one of the most potent bioactive components produced by the medicinal mushroom *Antrodia camphorata*. However, almost all studies in this field have focused on the biological activity of Antrodin C and relatively rare information has been reported regarding the biosynthetic process of Antrodin C. In this study, the strategies of pH-shift and glucose feeding for enhanced production of Antrodin C in submerged fermentation of *A. camphorata* were successfully applied in stirred bioreactors. The critical parameters for pH-shift and glucose feeding were systematically investigated. On one hand, the optimal culture pH for cell growth was distinct with Antrodin C biosynthesis and the maximum Antrodin C production was obtained by maintaining the first-stage culture at initial pH 4.5 and adjusted to 6.0 at day 8. On the other hand, it was beneficial for the Antrodin C accumulation with the initial glucose concentration of 40 g/L and feeding glucose to keep the residual sugar above 10 g/L. The maximum Antrodin C production (1,549.06 mg/L) was about 2.1-fold higher than that of control in 15-L stirred bioreactors by taking advantage of the integrated strategy of pH-shift and glucose feeding. These results would be helpful for the design of a highly efficient Antrodin C biosynthesis process.

H. Zhang · Y.-D. Hu · R.-Q. Lu · B.-B. Zhang (✉) · G.-R. Xu (✉)
Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, People's Republic of China
e-mail: superzbobo@gmail.com

G.-R. Xu
e-mail: grxu527@126.com

Y.-J. Xia
School of Medical Instrument and Food Engineering, University of Shanghai for Science and Technology, Shanghai 200093, People's Republic of China

Keywords *Antrodia camphorata* · Antrodin C · Submerged fermentation · pH-shift · Glucose feeding

Introduction

Antrodia camphorata, a valuable mushroom unique in Taiwan, has been well known as a traditional medicine for treatment of diverse diseases [1, 2]. Many types of bioactive compounds have been revealed from *A. camphorata* including polysaccharides, terpenoids, succinic and maleic derivatives, etc. [3–5]. Among these bioactive metabolites, Antrodin C has been recognized as one of the most potential bioactive components, which possesses notable antiproliferative activity against Lewis lung carcinoma tumor cell line and potent inhibitory activity on hepatitis C virus [5, 6]. In spite of these potential pharmaceutical applications, relatively rare information has been reported hitherto regarding the biosynthetic process of Antrodin C. This might be partially due to the fastidious nature of *A. camphorata* that its fruiting body only grows slowly on the inner heartwood wall of the *Cinnamomum kanehirai Hayata* [7]. Furthermore, although some success has been achieved by solid-state culture of *A. camphorata*, the growth period is still too long and it is also difficult to control the product quality. Thus, submerged fermentation might be a simple, fast and efficient alternative approach for the production of various valuable metabolites of *A. camphorata* including Antrodin C. To our best knowledge, there is only one study so far related to the bioprocess of Antrodin C production reported by our group, in which a biphasic fermentation system was used for alleviating the product inhibition [8].

However, in our previous study, the cultivation was conducted in shaking-flasks and the yield of Antrodin C was

relatively low. Therefore, a further investigation on the process parameters mostly related to the metabolites biosynthesis should be carried out in the bioreactors. Among the process that control strategies, pH-shift and sugar feeding have been shown to be the most efficient strategies [9–16]. On one hand, the pH-shift strategy is usually applied in submerged fermentation due to the fact that the optimum pH for the cell growth and metabolites biosynthesis are often different [16]. On the other hand, the sugar feeding strategy will lead to an improvement of the metabolite yield by avoiding the inhibitory effect of a relatively higher level of initial sugar [15].

In this study, the pH-shift and sugar feeding strategy were applied for the submerged fermentation of *A. camphorata* in stirred bioreactors. This approach will be of great significance for the efficient production of bioactive metabolites in the large-scale fermentation of *A. camphorata*.

Materials and methods

Microorganism

Antrodia camphorata was from the collection of Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University (China).

Fermentation conditions

Fermentation in the shaking-flasks

The fermentation condition in the shaking-flasks was the same as that of our previous work [8].

Fermentation in the stirred-tank bioreactors

The stirred-tank bioreactors used were 5-L and 15-L agitated bioreactor with two six-bladed Rushton impellers. The airflow rate and rotation speed was 4.0 vvm and 130 rpm, respectively. The other conditions were the same as that of shaking-flask culture.

Effect of initial pH

For studying the effect of different initial pH values, the medium pH values were adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 by adding 1.0 mol/L HCl or 1.0 mol/L NaOH.

pH-shift strategy

A pH-shift experiment was proposed in a 5-L stirred-tank bioreactor by combining a first-stage culture at initial pH 4.5 for 7 days with a second-stage culture at pH 5.0, 6.0 and 7.0, respectively. It is worth mentioning that the second-stage culture pH values were adjusted at the beginning of day 8 and since then the pH was not further controlled.

Effect of initial glucose concentration

For the investigation on the effects of different initial glucose concentrations, 25, 40, 55 and 70 g/L of glucose were tested in 5-L stirred-tank bioreactor, respectively.

Glucose feeding strategy

When the residual sugar concentration decreased to 5, 10, 15 and 20 g/L, a fed-batch process with a pulse feeding of a highly concentrated glucose solution into a reactor was conducted to increase the residual glucose concentration by 10 g/L.

Integration of pH-shift and glucose feeding

Based on the results of above experiments, an integration of pH-shift and glucose feeding was conducted in a 15-L stirred-tank bioreactor for further enhancement of Antrodin C production.

Substance measurements

Determination of biomass and residual sugar

For determination of the biomass concentration, the mycelia were filtered through a pre-weighted filter paper under suction and washed with distilled water, then collected and dried at 50 °C to a constant weight. Residual sugar in the filtrate was determined by dinitrosalicylic acid (DNS) method.

Table 1 Effect of different initial pH values on the biomass and Antrodin C production

Groups	pH		Biomass (g/L)	Antrodin C (mg/L)
	Initial pH	Final pH		
Control	4.49 ± 0.01	3.61 ± 0.03	12.17 ± 0.10^{a}	231.75 ± 2.01^{A}
1	2.00 ± 0.01	1.74 ± 0.03	10.76 ± 0.09^{b}	114.63 ± 1.87^{B}
2	3.00 ± 0.01	2.66 ± 0.04	11.01 ± 0.08^{b}	153.58 ± 1.45^{C}
3	4.00 ± 0.01	3.19 ± 0.03	12.05 ± 0.11^{a}	217.47 ± 1.99^{D}
4	5.00 ± 0.01	3.67 ± 0.04	11.93 ± 0.07^{a}	248.10 ± 2.35^{E}
5	6.00 ± 0.01	4.30 ± 0.03	11.18 ± 0.09^{b}	223.91 ± 2.47^{F}
6	7.00 ± 0.01	4.71 ± 0.05	9.56 ± 0.07^{c}	189.26 ± 2.23^{G}
7	8.00 ± 0.01	5.89 ± 0.04	6.01 ± 0.10^{d}	76.04 ± 1.98^{H}

Conditions: The initial pH of the medium in control was about 4.5 without adjustment. Each culture was carried out at 28 °C for 10 days at 130 rpm. Value (mean ± standard deviation, $n = 3$) within each column having different lowercase/capital letters have significant difference (ANOVA Tukey's test; $p < 0.05$)

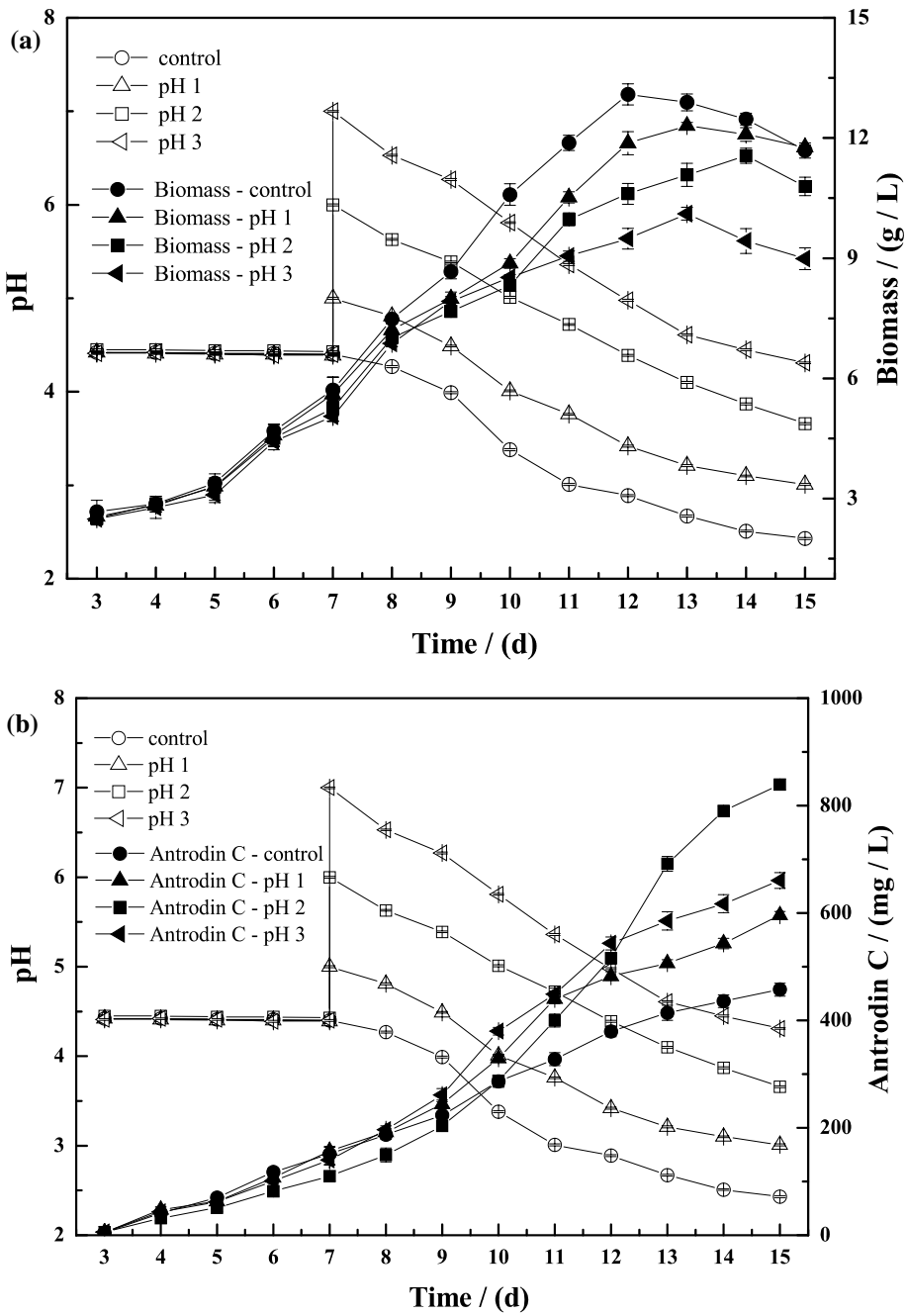

Fig. 1 Time profiles of biomass (**a**) and Antrodin C (**b**) in submerged fermentation of *A. camphorata* under different pH-shift in 5-L stirred bioreactors. *Control* initial pH 4.5 without control, *pH 1* controlled pH shifting from 4.5 to 5.0 at the beginning of day 8, *pH 2* controlled pH shifting from 4.5 to 6.0 at the beginning of day 8, *pH 3* controlled pH shifting from 4.5 to 7.0 at the beginning of day 8

Extraction and analysis of Antrodin C

The extraction and analysis of Antrodin C were based on our previously described method [8].

Results and discussion

Effects of initial pH

The effects of different initial pH values on the biomass and production of Antrodin C were investigated in the shaking-flask and the results were shown in Table 1. The biomass was obviously inhibited when the initial pH was adjusted below 3.0 or above 6.0. An optimal initial pH for cell growth occurred at 4.5 (an original pH of culture medium without adjustment) with the biomass of 12.17 g/L, while the highest Antrodin C production (248.10 mg/L) was obtained at the initial pH of 5.0. The results indicated that cell growth might be better at a lower pH range while the Antrodin C formation might be beneficial at a relatively higher pH range, suggested that a pH-shift strategy was necessary for the enhancement of Antrodin C production. It was in agreement with

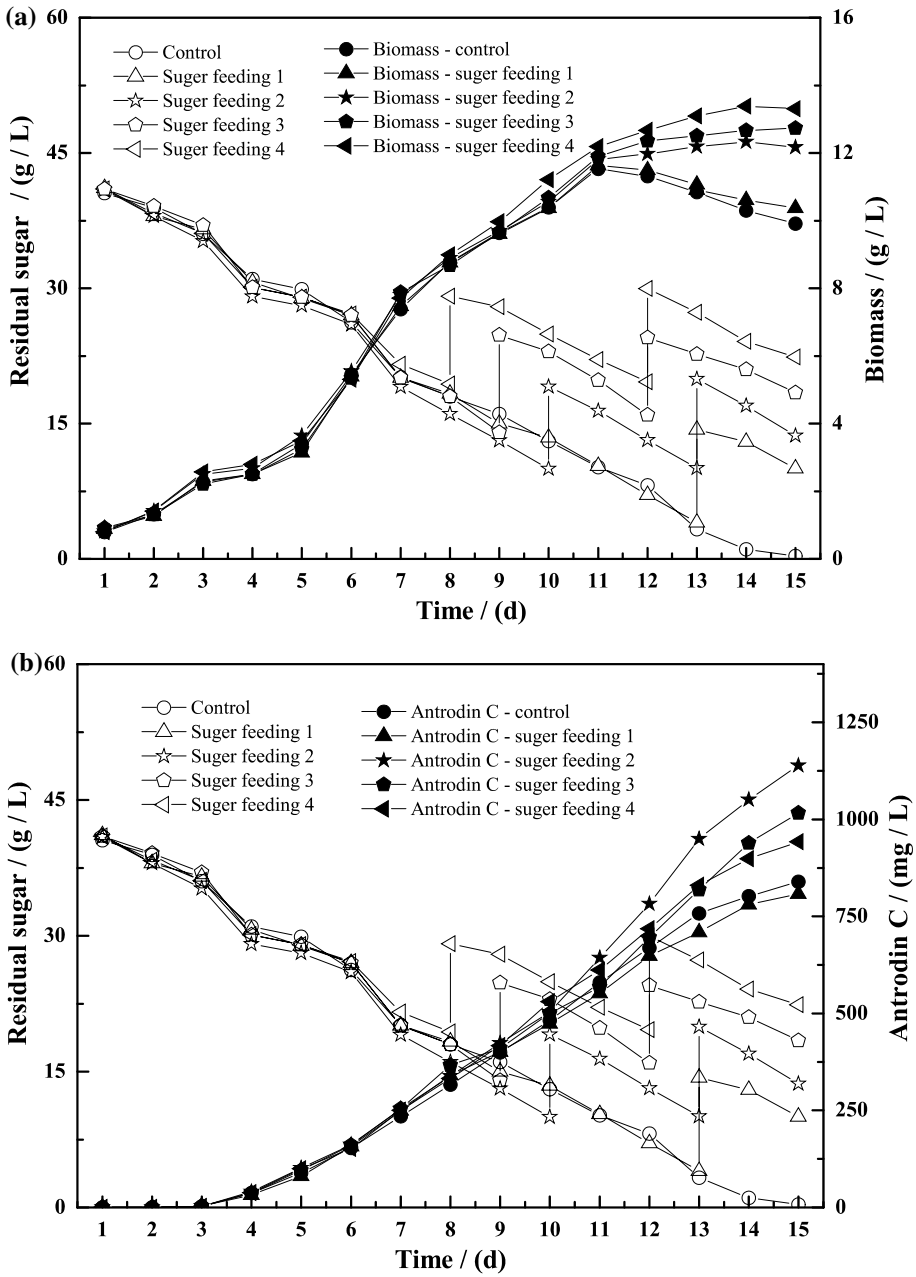

Fig. 2 Time course of the biomass (**a**) and Antrodin C production (**b**) at different feeding time during submerged fermentation of *A. camphorata* in 5-L stirred bioreactors. *Symbols for glucose feeding time* sugar feeding 1, 2, 3 and 4 corresponded to the feeding of glucose at the residual sugar of 5, 10, 15 and 20 g/L, respectively

the results reported by Shu and Lung [16], in which the preferable pH values for mycelial growth and exopolysaccharide production of *A. camphorata* were 4.0 and 5.0, respectively.

pH-shift strategy in 5-L bioreactors

Based on the preliminary results of the time course study, the pH of fermentation broth remained relatively constant at 4.5 during the first 7 days, after which it showed an obvious decrease and reached 2.43 at the end of fermentation (data not shown). Therefore, different degrees of pH-shift were applied in 5-L stirred bioreactors and the results were shown in Fig. 1. It was found that both the biomass and Antrodin C production were remarkably influenced by different second-stage culture pHs. The biomass decreased obviously when the pH shifted from 4.5 to 5.0, 6.0 and 7.0 on day 8, suggesting that the mycelial growth required a relatively acidic environment. In contrast, the application of pH-shift led to a significant increase in the Antrodin C yield. Thus, it was concluded that a pH-shift culture was successfully developed for enhancing Antrodin C accumulation. The highest Antrodin C production (839.31 mg/L) was obtained when the pH was shifted from 4.5 to 6.0 on day 8, which was increased by 83.47 % compared with the control. The present results were similar with the report of

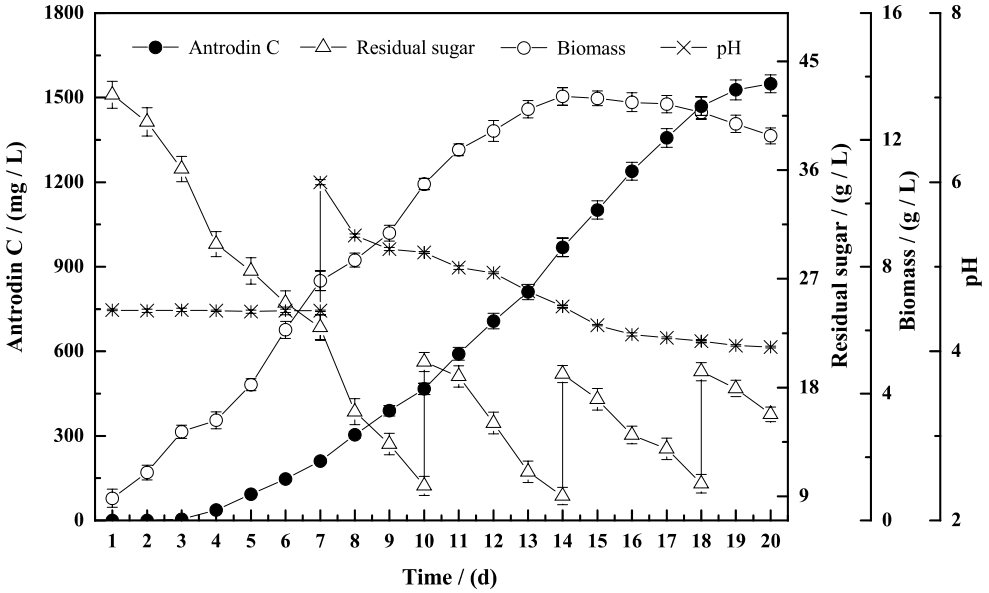

Fig. 3 Time profiles of biomass, pH, residual sugar and Antrodin C production of *A. camphorata* in 15-L stirred bioreactors with the integrated strategy of combining pH-shift and glucose feeding

Kim et al. [14], in which the maximum exopolysaccharides production of *Ganoderma lucidum* was achieved by shifting the controlled pH from 3.0 to 6.0 after day 4.

Glucose feeding strategy in 5-L bioreactors

The effects of initial glucose concentration were investigated for the further application of glucose feeding strategy. On one hand, the higher biomass could be obtained at a relatively higher initial glucose concentration, which may be attributed to rapid growth of mycelia in nutrition-rich environment (data not shown). On the other hand, the highest Antrodin C production was achieved at the initial glucose concentration of 40 g/L (data not shown). However, excessive supply of glucose was detrimental for the production of Antrodin C, possibly caused by the inhibitory effect of glucose on the related enzymes. As demonstrated in a fed-batch culture of *Ganoderma resinaceum* DG-6556 for production of exopolysaccharides, the application of sugar feeding was effective for preventing the product inhibition by the substrate [17].

Thus, the glucose feeding strategy would be useful for the enhanced production of Antrodin C. As shown in Fig. 2, it was found that the supply of glucose was beneficial for the mycelial growth and an earlier feeding of glucose led to a higher biomass, indicating that the mycelial growth was more susceptible to the glucose supplement at the logarithmic growth phase than at the stationary phase. Comparatively, the earliest feeding of glucose did not result in the highest Antrodin C yield, indicating that the supplement of glucose at too earlier fermentation time may be partly converted into biomass rather than metabolites biosynthesis. Based on the results, the maximum Antrodin C production (1,138.92 mg/L) was obtained when the glucose concentration maintained no less than 10 g/L.

Integrated strategy of pH-shift and glucose feeding in 15-L bioreactors

In order to further enhance Antrodin C biosynthesis, the integrated strategy of pH-shift and glucose feeding was applied in a 15-L bioreactor. As shown in Fig. 3, the biomass increased rapidly from day 1 to 14, after that the biomass kept relatively stable. Although the accumulation of Antrodin C was slow before day 4, the continued increase in its biosynthesis could be achieved during the subsequent fermentation period, by taking the advantages of the integrated strategy. The yield of Antrodin C reached 1,549.06 mg/L at the end of the fermentation, corresponded to an increase of 210 % when compared with that of the control (data not shown). These results indicated that the integrated strategy of pH-shift and glucose feeding had a synergistically favorable effect on the Antrodin C biosynthesis. Tang et al. [13] developed a similar process of a fed-batch fermentation process by combining pH-shift strategy and DOT-shift strategy that resulted in a significant synergistic enhancement of ganoderic acid accumulation in the submerged fermentation of *G. lucidum* in stirred-tank bioreactor. Thus, an integrated strategy of combining of well-directed process parameters shift with carbon source feeding can provoke physiological changes that positively affects process performance, representing a valuable

integrated strategy for the cell growth and metabolites biosynthesis [13, 18].

Conclusion

In this work, for the first time, the strategies of pH-shift and glucose feeding for the efficient production of Antrodin C were successfully demonstrated during the submerged fermentation of *A. camphorata* in bioreactors. It was found that the responses of the mycelial growth and Antrodin C biosynthesis to pH-shift and glucose feeding were different. A significant synergistic enhancement of Antrodin C production was achieved by combining the above-mentioned pH-shift and glucose feeding strategies in 15-L stirred bioreactors. The results obtained in this study offer a promising approach for high Antrodin C production and will be helpful for the design of a highly efficient Antrodin C biosynthesis process on a large scale.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Grant No. 21306065), the Natural Science Foundation of Jiangsu Province (Grant No. BK20130134), a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, the 111 Project (No. 111-2-06), and the Jiangsu province "Collaborative Innovation Center for Advanced Industrial Fermentation" industry development program.

References

1. Geethangili M, Tzeng YM (2011) Review of pharmacological effects of *Antrodia camphorata* and its bioactive compounds. J Evid-Based Complement Altern Med 2011:1–17
2. Huang CH, Chang YY, Liu CW, Kang WY, Lin YL, Chang HC, Chen YC (2010) Fruiting body of *Niuchangchih* (*Antrodia camphorata*) protects livers against chronic alcohol consumption damage. J Agric Food Chem 58:3859–3866
3. Chen YJ, Cheng PC, Lin CN, Liao HF, Chen YY, Chen CC, Lee KM (2008) Polysaccharides from *Antrodia camphorata* mycelia extracts possess immunomodulatory activity and inhibits infection of Schistosoma mansoni. Int Immunopharmacol 8:458–467
4. Hsieh YC, Rao YK, Peng JW, Huang CY, Shyue SK, Hsu SL, Tzeng YM (2011) Antcin B and its ester derivative from *Antrodia camphorata* induce apoptosis in Hepatocellular Carcinoma cells involves enhancing oxidative stress coincident with activation of intrinsic and extrinsic apoptotic pathway. J Agric Food Chem 59:10943–10954
5. Nakamura N, Hirakawa A, Gao JJ, Kakuda H, Shiro M, Komatsu Y, Sheu CC, Hattori M (2004) Five new maleic and succinic acid derivatives from the mycelium of *Antrodia camphorata* and their cytotoxic effects on LLC tumor cell line. J Nat Prod 67:46–48
6. Phuong DT, Ma CM, Hattori M, Jin JS (2009) Inhibitory effects of antrodins A-E from *Antrodia cinnamomea* and their metabolites on hepatitis C virus protease. Phytother Res 23:582–584
7. Chang TT, Chou WN (2004) *Antrodia cinnamomea* reconsidered and *A. salmonea* sp. nov. on *Cunninghamia konishii* in Taiwan. Bot Bull Acad Sin 45:347–352
8. Zhang H, Xia YJ, Wang YL, Zhang BB, Xu GR (2013) Coupling use of surfactant and in situ extractant for enhanced production of Antrodin C by submerged fermentation of *Antrodia camphorata*. Biochem Eng J 79:194–199
9. Fang QH, Zhong JJ (2002) Effect of initial pH on production of ganoderic acid and polysaccharide by submerged fermentation of *Ganoderma lucidum*. Process Biochem 37:769–774
10. Tang YJ, Zhong JJ (2002) Fed-batch fermentation of *Ganoderma lucidum* for hyperproduction of polysaccharide and ganoderic acid. Enzyme Microb Technol 31:20–28
11. Liu YP, Zheng P, Sun ZH, Ni Y, Dong JJ, Wei P (2008) Strategies of pH control and glucose-fed batch fermentation for production of succinic acid by *Actinobacillus succinogenes* CGMCC1593. J Chem Technol Biotechnol 83:722–729
12. Chen X, Liu L, Li JH, Liu J, Du GC, Chen J (2012) Optimization of glucose feeding approaches for enhanced glucosamine and *N*-acetylglucosamine production by an engineered *Escherichia coli*. J Ind Microbiol Biotechnol 39:359–365
13. Tang YJ, Zhan W, Zhong JJ (2009) 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*. Bioresour Technol 100:1852–1859
14. Kim HM, Park MK, Yun JW (2006) Culture pH affects exopolysaccharide production in submerged mycelial culture of *Ganoderma lucidum*. Appl Biochem Biotechnol 134:249–261
15. Papagianni M, Mattey 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
16. Shu CH, Lung MY (2004) Effect of pH on the production and molecular weight distribution of exopolysaccharide by *Antrodia camphorata* in batch cultures. Process Biochem 39:931–937
17. Kim HM, Paik SY, Ra KS, Koo KB, Yun JW, Choi JW (2006) Enhanced production of exopolysaccharides by fed-batch culture of *Ganoderma resinaceum* DG-6556. J Microbiol 44:233–242
18. Liang GB, Du GC, Chen J (2008) A novel strategy of enhanced glutathione production in high cell density cultivation of *Candida utilis*-cysteine addition combined with dissolved oxygen controlling. Enzyme Microb Technol 42:284–289