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The State Of The Art in the Production of Bioactive Metabolites from *In Vitro* Plant and Mushroom Cultures

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Abstract

In vitro cultivation of plant cells and mushrooms is an alternative to whole plant/mushroom extraction for obtaining valuable secondary metabolites. In recent years, strategies and methodologies for enhancing the production and productivity of useful metabolites in liquid cultures have been demonstrated. Use of new powerful elicitors could effectively induce ginsenoside and taxoid biosynthesis in cell cultures of *Taxus chinensis* and *Panax notoginseng* and alter the product heterogeneity of *P. notoginseng* cells. Two-stage cultivation was another interesting method for hyper-production of ganoderic acid (GA) by liquid fermentation of *Ganoderma lucidum*, a traditional Chinese medicinal mushroom. Such studies are important to the commercial utilization of plant cells and mushrooms for production of high-value-added bioactive metabolites.

Keywords: Plant cell culture, Medicinal mushroom, Ganoderma lucidum , Taxus chinensis, Panax notoginseng, Secondary metabolite

1.0 Introduction

Nowadays, people in developed countries spend more than $10 billion annually on phytomedicines and plant-based remedies in the form of teas, extracts, oils and capsules. Around the world, there has appeared a booming industry for herbal preparations, nutriceuticals, so-called functional foods, and other natural health products. Cultivation of plant cells and medicinal mushrooms has advantages as a method of producing valuable bioactive compounds, and bioengineering studies are very important to reach a highly efficient production process.

Strategies and methodologies for enhancing the production and productivity of useful metabolites in liquid cultures have been demonstrated in recent years. These include combination of elicitation (by methyl jasmonate) and substrate (sucrose) addition (1,2), repeated elicitation and its combination with sucrose feeding (3), signal transduction of newly synthesized elicitors and their application (4-6), and quantitative manipulation of external calcium level (7). Here, the cell cultures of *Taxus chinensis* for taxoid production and of *Panax notoginseng* for production of ginseng saponin and polysaccharide were taken as model systems of plant cell cultures.

In the case of mushroom cultures, we used *Ganoderma lucidum*, which is a famous traditional Chinese medicinal herb and produces bioactive compound ganoderic acid (GA).

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For the efficient production of GA, a two-stage cultivation process combining liquid fermentation and static culture was developed, which was a powerful strategy for GA hyper-production (8-10). Recently we have obtained new results regarding the GA accumulation by static cultures of *G. lucidum* cultured under different oxygen levels in gaseous phase. The above work is considered beneficial to the commercial utilization of plant cells and mushrooms for production of high-value-added bioactive metabolites. Two typical examples are shown as follows.

2.0 Materials and Methods

2.1 Example I: Use of a new powerful elicitor to induce ginsenoside biosynthesis and alter the product heterogeneity in cell cultures of P. notoginseng

Molecular diversity is a widely existing phenomenon in plant secondary metabolites, and intentional manipulation of the heterogeneity of secondary metabolites in cell cultures is a very important issue (11). Exogenously applied methyl jasmonate (MJA) could enhance production of secondary metabolites by a variety of plant species (1,12). Recently, Qian et al. (4,5) reported that the hydroxylation of MJA at its C-1 position resulted in a higher stimulatory activity on taxane biosynthesis in cell cultures of *T. chinensis.* However, it is interesting to understand whether or not the newly synthesized jasmonates (4,5) could effectively enhance the ginsenoside production and change the metabolite heterogeneity.

Recently we used chemically synthesized 2-hydroxyethyl jasmonate (HEJA) to induce the ginsenoside biosynthesis and to manipulate the product heterogeneity in cell cultures of *P. notoginseng* (13). HEJA was synthesized and purified as described earlier (4), and both MJA and HEJA contain the same ratio of stereoisomers (4).

The dose response and timing of HEJA elicitation were investigated in cell suspension cultures of *P. notoginseng*. The optimal concentration and timing of HEJA addition for both cell growth and ginsenoside accumulation was identified to be 200 μM added on day 4, which was the same as those of MJA.

A comparison of the effects of MJA and HEJA on cell growth, ginsenoside biosynthesis and UDPG-ginsenoside Rd glucosyltransferase (UGRdGT) activity was done. Both MJA and HEJA were added on day 4 to the cell cultures at 200 μM. The maximum production of individual ginsenoside on day 13 is shown in Table 1. With HEJA elicitation, a maximal production titer of ginsenoside Rg_1, Re, Rb_1 and Rd was 47.4±4.8, 52.3±4.4, 190±18 and 12.1±2.5 mg/L, about 1.3, 1.3, 1.7 and 2.1-fold that with MJA elicitation, respectively. It was clear that HEJA had stronger elicitation of ginsenoside biosynthesis than MJA.

Table 1. Effects of MJA and HEJA on the production of individual ginsenoside on day 13

Cultivation conditions	Ginsenoside production (mg/L)			
	Rg_1	Re	Rb_1	Rd
Control	22.1±1.3[a]	23.0±1.2[a]	28.1±3.1[a]	0[a]
MJA	35.2±4.0[b]	39.2±3.5[b]	113±10[b]	5.87±0.88[b]
HEJA	47.4±4.8[c]	52.3±4.4[c]	190±18[c]	12.1±2.5[c]

[a, b, c] Means with the same letter all noted in a single column are not significantly different according to Tukey's Honestly Significant Difference multiple-comparison test with a family

error rate of 0.05

A higher Rb/Rg ratio with HEJA elicitation was observed than that with MJA elicitation. The results indicated that compared to MJA, HEJA could lead to higher amounts of Rb group ginsenoside. This means that it can alter the distribution of heterogeneous ginsenosides more efficiently than MJA.

Studies on the signal events, including JA biosynthesis and oxidative burst, confirmed that the cells had similar defense response to HEJA elicitation as to MJA elicitation. The activity of Rb_1 biosynthetic enzyme, UGRdGT, was also higher in the former case. The information is useful for hyper-production of bioactive heterogeneous products in plant cell cultures.

2.2 Example II: Two-stage cultivation approach for hyper-production of GA by liquid fermentation of medicinal mushroom G. lucidum

Medicinal mushrooms are a promising source of new bioactive compounds. It usually takes several months to cultivate mushrooms and the product yield is low in soil cultivation. Cell culture of mushrooms is viewed as a promising alternative for efficient production of their valuable products.

G. lucidum (Leyss.:Fr.) Karst, a mushroom-like fungus, is one of the most famous traditional Chinese medicinal herbs. Because of its high medicinal value, *G. lucidum* has been popular as a health food and medicine in the Far East for more than 2000 years. GA produced by this higher fungus has a number of important biological functions including cytotoxicity to hepatoma cells, inhibition of cholesterol synthesis, and anti-HIV-1 and anti-HIV-protease activities. In contrast, the information on GA production by fermentation technology was very limited (10). There is a great need to enhance the valuable product titer for potential commercial application of the higher fungus fermentation.

Investigation on the impact of pellet size on the cellular oxygen uptake and GA accumulation suggested the favorable effect of oxygen limitation on GA formation by the higher fungus *G. lucidum* (Table 2). A two-stage fermentation process was thus proposed for enhancing the metabolite production by combining conventional shake-flask fermentation (first stage) with static culture (second stage). A high cell density of 20.9 g DW/L was achieved through a 4-day shake-flask fermentation followed by a 12-day static culture. A change in the cell morphology and a decrease in the sugar consumption rate were observed during the static culture. The GA production in the new two-stage process was considerably enhanced with its content increased from 1.36 (control) to 3.19 mg/100 mg DW, which was much higher than previously observed. In addition, from Table 3, it was clear that the cell yield on glucose (calculated between day 4 and the time of maximum biomass) was doubled in static fermentation. It suggested that the sugar utilization was more efficiently shifted to biomass formation in the static cultures. This work indicated that the new process was very promising for highly efficient production of the valuable bioactive product by *G. lucidum* cells.

Table 2 Effect of pellet size on the specific oxygen uptake rate (Sp. OUR) and cellular content of GA in shake flask fermentation of the higher fungus *G. lucidum*[a]

	Diameter of pellets (mm)		
	larger than 1.6	between 1.2~1.6	smaller than 1.2
Sp. OUR (mmol O_2/(g DW · h))	0.65±0.02	0.86±0.03	1.22±0.02
GA content (mg/100 mg DW)	1.62±0.21	1.27±0.14	0.98±0.12

[a] Standard deviation was calculated from 3 samples.

Table 3. Effects of static culture on the cell growth and yield as well as GA production[a]

	Control	Static culture after the 4th day of shake flask fermentation
Maximum DW (g/L)	15.7±0.42 (d 8)[b]	20.9±0.45 (d 16)[b]
Yx/s (g DW per g of glucose)	0.391±0.059	0.681±0.005
GA content (mg/100mg DW)	1.36±0.09 (d 8)[b]	3.19±0.24 (d 12)[b]

[a]Standard deviation was calculated from 3 independent samples. [b]The time (days) when the maximum value was obtained for each case.

Based on the above work, next it is essential and reasonable to study the scale-up of this novel process from a flask to a bioreactor for potential commercial application of the mushroom culture. The first-stage culture of *G. lucidum*, which is similar to a conventional bacterial fermentation process, was recently successfully conducted in a 3-L laboratory reactor (14). In order to scale-up the second-stage culture (liquid static culture), a systematic approach was taken by performing a series of experiments to identify the responsible factors.

Initial volumetric oxygen transfer coefficient (K_La) and area of liquid surface per liquid volume (A_s) were identified as key factors affecting cell growth and GA accumulation in liquid static cultures of *G. lucidum* (9). A multi-layer static bioreactor, which has multi-parallel growth surfaces and multi-fold culture area, was designed for hyper-production of GA based on those experimental findings.

At a low initial K_La level of 2.1 h^{-1}, a thick layer of white mycelia was formed on the liquid surface, and an optimal production of total GA (*i.e.* GA production in the liquid and on the liquid surface) was obtained. Both the formation of white mycelia and production of GA on the liquid surface were enhanced with an increase of A_s within the range as investigated (0.24-1.53 cm^2/mL). At an A_s value of 0.90 cm^2/mL, the total GA production reached maximum.

The cell growth, lactose consumption and GA accumulation in various-scale static bioreactors were all very similar (Table 4), and as a typical example the dynamic profiles obtained in a three-layer static bioreactor (with a working volume of 654 mL) are shown in Fig. 1. After inoculation, the cell density increased until day 20, when a maximal value was reached. The sugar consumption coincided with the cell growth pattern. The GA production titer also increased from the beginning until day 20. It reached 940-980 mg/L after 20 days of cultivation in various vessels, and their corresponding productivity was about 50 mg/(L·d) (Table 4).

A further study on the performance of static culture process of *G. lucidum* in a 7.5-L (working volume) three-layer static bioreactor was attempted by maintaining the initial $K_L a$ value at a low level of 2.1 h^{-1}. As summarized in Table 4, the specific GA production (*i.e.* content) in a 7.5-L static bioreactor was similar to that in small static bioreactors. The maximum cell dry weight of 20.3 g/L and GA production of 937 mg/L was attained after 20 days of cultivation in the bioreactor.

Table 4 Cell growth and GA accumulation by *G. lucidum* cells in various culture vessels

Cultivation system	50 mL T-flask	One-layer static reactor	Two-layer static reactor	Three-layer static reactor	Large three-layer static reactor
Total working volume (mL)	20	218	436	654	7500
Total cell density* (g/L)	20.1	20.8	20.4	19.7	20.3
Maximum GA content (mg/100 mg)	4.88	4.64	4.81	4.96	4.67
Total GA production (mg/L)	944	963	976	970	937
Total GA productivity (mg/L per day)	50.9	52.1	52.9	52.5	50.4

*The maximum dry cell weight was reached on day 20 in all cases.

From the above, it is clear that the static culture process was successfully scaled up from a 20-mL (working volume) T-flask to a 7.5-L (working volume) multi-layer static bioreactor based on initial $K_L a$. The maximum biomass (20.8±0.1 g DW/L), GA content (4.96±0.13 mg/100mg DW) and total GA production (976±35 mg/L) were attained in static bioreactors. In this work, both the content and production titer of GA obtained were much higher than those in previous reports (14-18). The work is considered useful for large-scale efficient production of GA towards industrial application. The information obtained may be also beneficial to other culture processes of medicinal and edible mushrooms, which have wide utilization and application in food and health care sectors.

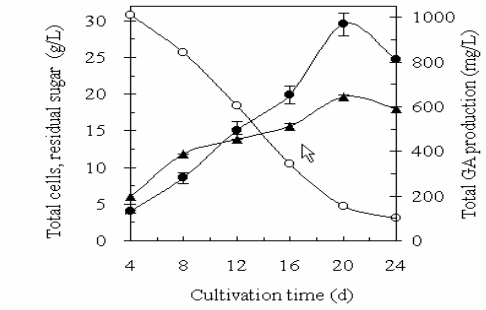

Fig 1 Time profiles of cell growth (dark triangle), residual sugar (open circle) and GA production (dark circle) in a three-layer static bioreactor. Error bars in the figure indicate standard deviations from 3 independent samples.

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