J. Plant Biotechnology(2001) *Vol. 3(2). pp. 107~112*

Journal of Plant Biotechnology

Production of Rosmarinic Acid, Lithospermic Acid B, and Tanshinones by Suspension Cultures of Ti-Transformed *Salvia miltiorrhiza* Cells in Bioreactors

Jian-Jiang Zhong[1*], Hui Chen[2], Feng Chen[2]

[1]*State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China;* [2]*Department of Botany, the University of Hong Kong, Pokfulam Road, Hong Kong, China.*

Key words: bioreactor engineering, plant cell suspension culture, phenolic compounds, tanshinone, *Salvia miltiorrhiza*, Ti-transformed cell line

Abstract

The kinetics of Ti-transformed *Salvia miltiorrhiza* cell cultures was studied in 250-mL shake flasks by using B5 medium with addition of 30 g/L of sucrose. In the cell cultures, the maximum cell mass obtained was 11.5 g DW/L on day 15. The highest amount of phenolic compounds - rosmarinic acid (RA) and lithospermic acid B (LAB) reached 871.3 mg/L (day 15) and 121.3 mg/L (day 13), respectively. The total tanshinone production, i.e., intracellular plus extracellular cryptotanshinone, tanshinone I, and tanshinone IIA, was 5.3 mg/L on day 13. For the cultivations in 2.4-L stirred bioreactors, the residual sugar level and medium conductivity were a little higher in a small turbine impeller reactor (T_S) than those in a large turbine impeller reactor (T_L), while a higher cell density was obtained in the T_L. For the production of tanshinones and phenolics, better results were obtained in the T_L than in the T_S. In the T_L, similar or even a little higher production titers of tanshinones and phenolic compounds were achieved compared to those in the flasks. The results suggest that the shake flask results could be successfully scaled up to the T_L reactor. Such a large impeller reactor like T_L may be better than a small impeller one for the large-scale production of the valuable metabolites by the suspension cultures of Ti transformed *S. miltiorrhiza* cells. This is considered due to the beneficial culture environment in the T_L, such as low shear rates as estimated theoretically.

Introduction

Plants produce more than 30,000 kinds of chemicals including pharmaceuticals, food ingredients and other fine chemicals. Many of these compounds are difficult to synthesize chemically, or difficult to produce or to increase their production even through genetic engineering. In recent years, the market of natural plant products expands rapidly. For example, the current world market of raw materials of ginseng has reached about one billion US$ per year (Wu and Zhong, 1999). This increasing trend will continue in the 21st century because more and more people prefer to natural products.

At present, many plant products are obtained from field cultivation. However, the field cultivation is a time-consuming and labor-intensive process and is subjected to several conditions such as soil, climate, pathogens and pests. Plant cell culture has been looked at as a potential alternative for efficient production of natural bioactive compounds (Choi et al., 2000; Yu et al., 2000), because it is unlimited by environmental, ecological or climatic conditions and the cells can grow at a higher rate than whole plants in cultivation.

Salvia miltiorrhiza Bunge (Labiatae) is an important Chinese medicinal plant, which grows wild in China. The dried root, Dan-shen, is prescribed in combination with

* Corresponding author, E-mail: jjzhong@ecust.edu.cn
Received Mar. 30, 2001; accepted May. 20, 2001.

other herbal drugs for chest congestion and coronary diseases. Its pharmaceutical product is one of the very few traditional Chinese medicines which were recently admitted to the US market by the FDA. This has received great attention both in China and abroad. The effective components of the roots can be classified as lipid-soluble and water-soluble ones. The lipid-soluble components are mainly tanshinones, the quinoid diterpenes, which impart the reddish-brown color to the roots. It is reported that tanshinone I and cryptotanshinone prevent the complications of myocardial ischemia, and tanshinones IIA has been successfully used for treatment of angina pectoris in China (Bruneton, 1995). In another aspect, the water-soluble components are caffeic acid and related phenolic compounds such as rosmarinic acid and lithospermic acid B. Recent studies indicate that rosmarinic acid and its salts have anti-HIV activities (Mazumder et al., 1997), and lithospermic acid B exhibits endothelium-dependent vasodilator and hypotensive effects (Kamata et al., 1993; Kamata et al., 1994) while magnesium lithospermic acid B shows uremia and hepatitis preventive effects in animal tests (Tanaka et al., 1989; Hase et al., 1997).

In our previous work (Chen et al., 1997), Ti-transformed cell cultures of *S. miltiorrhiza* were established, which showed fast growth rate and did not require exogenous phytohormones. The cell line was demonstrated to produce water-soluble effective compounds such as rosmarinic acid (RA) and lithospermic acid B (LAB) (Chen et al., 1999). Furthermore, the lipid-soluble valuable bioactive components such as tanshinones (cryptotanshinone, tanshinone I, tanshinone IIA) could be also accumulated in the cell cultures (Chen et al., 1997). In this paper, we first investigated the kinetics of the Ti-transformed suspension cultures of *S. miltiorrhiza* cells in shake flasks by using 30 g/L of sucrose, then we conducted the production of RA, LAB, and tanshinones by the cell cultures in a stirred bioreactor with a large or a small turbine impeller. The work is considered to be important to the simultaneous production of the above useful metabolites by the cell cultures on a large scale.

Materials and Methods

Cell cultures

Transformed cell cultures of *S. miltiorrhiza* were obtained after infecting the sterile plantlets of *S. miltiorrhiza* with *Agrobacterium tumefaciens* strain C58 as described previously (Chen et al., 1997). The transformed cell line A with a characteristic fast growth rate was used in this work. The cell line was cultured in hormone free B5 medium containing 30 g/L of sucrose. All cultivations were conducted in darkness at 25°C in 250-mL conical flasks containing 100-mL medium.

Bioreactor systems

The cell suspensions (aggregated cells) were cultured in a 2.4-L flat-bladed turbine reactor. It was a commercial available laboratory fermentor (Type L1523, Bioengineering AG, Switzerland) with a disc diameter of 3.9 cm (small one, T_S) or 4.8 cm (large one, T_L). The details of the reactor geometry are summarized in Table 1. A sintered glass sparger with pore sizes of about 30-80 μm was fitted at the reactor bottom. Aeration was conducted by compressed air sterilized through membrane air-filters.

Sampling and measurements of cell weight, medium sugar, and conductivity

For shake flask cultures, one or two flasks were taken each time for sampling. During bioreactor cultivations, because the cells form large aggregates (from 0.5 up to 2.0 cm), only the liquid medium was sampled once every 2-3 days, and the total cell mass was analyzed when harvested at the end of cultivation.

After sampling, the cell aggregates were filtered and washed several times with a sufficient amount of distilled water. The cell dry weight was measured by gravimetric method. The culture supernatants were used for analyses of residual sugar by a phenol-concentrated sulfuric acid method and medium conductivity (4020 Conductivity Meter, Jenway). The analytical procedures for medium sugar were the same as described previously (Zhang and Zhong, 1997).

HPLC analyses of phenolic compounds and tanshinones

For analyses of RA and LAB, about 50 mg of a dried sample was mashed and extracted with MeOH (10 mL) for 16 h at room temperature. Each extract, after filtration

Table 1. Geometric details of a stirred bioreactor used for cultivation of *S. miltiorrhiza* cells.

Reactor system	Dimension
Inner diameter of reactor	10.0 cm
Height of reactor	30.0 cm
Total volume	2.4 liter
Working volume	1.5 liter

through a 0.45 mm filter, was subjected to HPLC analysis on a Beckman System Gold liquid chromatograph equipped with a 125 Solvent Module pump and a 166 UV-VIS detector. The extract solution was separated and analyzed by using a Merck Superspher RP-18 endcapped column (2504.0 mm, 5 μm) at 30°C. The mobile phase consisted of solvent A (methanol/water/formic acid, 14.0:85.2:0.8, by volume) and solvent B (methanol/water, 65:35, by volume). The following gradient procedure was used: 0-2 min, 25% B; 2-27 min, linear change to 65% B. The flow rate was set at 1.0 mL/min. Peaks were detected at a wavelength of 280 nm. The retention times were 25.8 min for RA and 28.3 min for LAB.

Quantitative analysis of tanshinones was carried out as follows. Dried cells (ca. 50 mg) were mashed and extracted with 5 ml of MeOH-CH_2Cl_2 (3:1, v/v) twice at room temperature. HPLC was conducted on a Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array detector. The MeOH-CH_2Cl_2 extract solution was filtered through a 0.45 mm filter and separated by using a Resolve™ C_{18} column (Waters, 300 mm × 3.9 mm, 5 mm) at 25°C. The mobile phase consisted of acetonitrile (65%) and water (35%), and the flow rate was set at 1.0 mL/min. The filtered extracts were injected with a Rheodyne 7725 valve with a 20 μL loop. Peaks were detected at a wavelength of 270 nm to facilitate the simultaneous detection of various tanshinones (Cryptotanshinone, Tanshinone I, Tanshinone IIA). Chromatographic peaks were identified by comparing retention times and spectra against known standards.

Results and Discussion

Kinetics of Ti-transformed *Salvia miltiorrhiza* cell cultures in flasks

Previously, production of rosmarinic acid (RA) and lithospermic acid B (LAB) was studied in a shake flask by using B5 medium with 20 g/L of initial sucrose (Chen et al., 1999). Our preliminary experiments indicated that a moderate increase of initial sucrose concentration was beneficial to the cell cultures (data not shown). Here, we used 30 g/L of sucrose as the medium carbon source and investigated the kinetics of the Ti-transformed *S. miltiorrhiza* suspension cultures for production of both phenolic compounds (RA and LAB) and tanshinones (cryptotanshinone, tanshinone I, and tanshinone IIA).

As shown in Figure 1A, the cells almost did not grow for the first 3 days of cultivation, and they grew quickly during day 7-12. The cell mass reached its maximum on day 15, i.e., 11.5 g DW/L, which was higher than a previous report (Chen et al., 1999). For the medium sugar, it decreased from the beginning and there still remained a little at the end of cultivation (Figure 1A). The medium conductivity, which reflects the strength of total ions in the medium, showed a rapid decrease during day 7-12, which corresponded well to the cells' growth pattern (Figure 1A). From the data of medium conductivity, it can be seen that

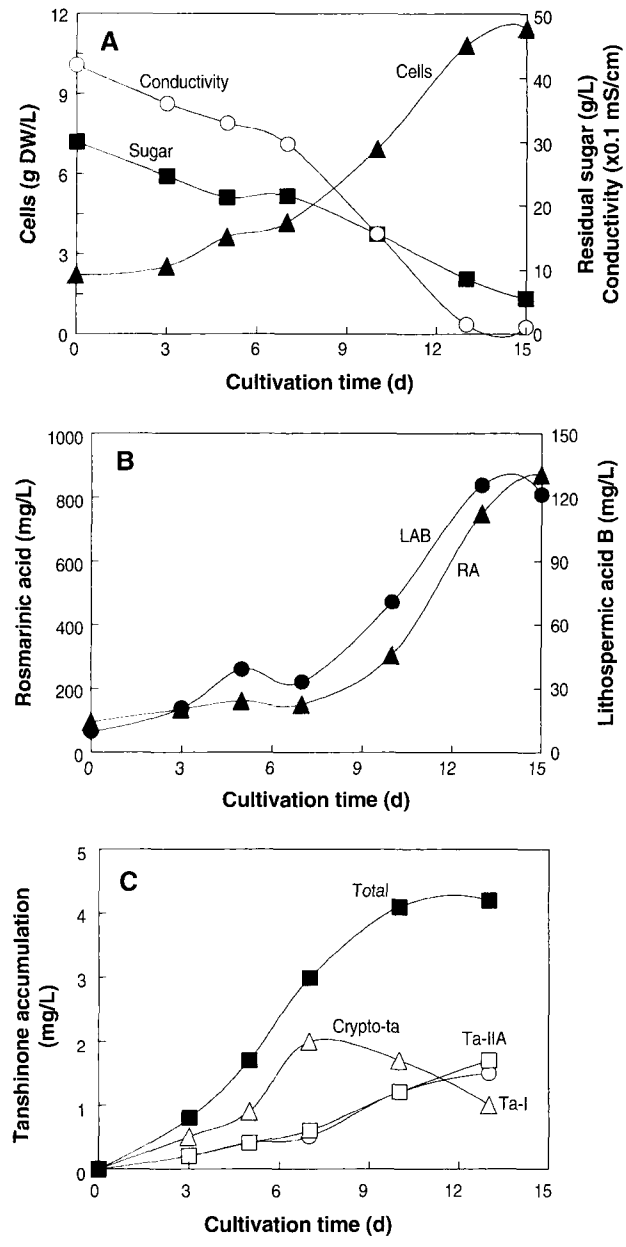

Figure 1. Kinetics of dry cell weight, residual sugar, medium conductivity (A), accumulation of rosmarinic acid (RA) and lithospermic acid B (LAB) (B), and production of cryptotanshinone (Crypto-ta), tanshinone I (Ta-I), tanshinone IIA (Ta-IIA), and total tanshinones (Total) (C) by suspension cultures of Ti-transformed *Salvia miltiorrhiza* cells in 250-mL shake flasks.

the medium ions were almost depleted near the end of cultivation.

The kinetic profiles of the intracellular formation of phenolic compounds, i.e., rosmarinic acid (RA) and lithospermic acid B (LAB), are shown in Figure 1B. Both RA and LAB were accumulated slowly during the first week of cultivation. After day 7, they increased rapidly and reached their maximum values around day 13-15. The highest amount of RA and LAB was 871.3 mg/L (day 15) and 121.3 mg/L (day 13), respectively, with their respective productivity of 51.7 mg/(L.d) and 8.9 mg/(L.d). In a previous report, only 481.5 mg/L of RA (day 20) and 85.0 mg/L of LAB (day 20) were obtained in the same B5 medium with addition of 20 g sucrose/L (Chen et al., 1999). The beneficial effects of a moderate enhancement in the initial sucrose concentration on secondary metabolite production were also reported in other cases of plant cell suspension cultures [such as Zhang and Zhong, 1997]. In addition, in the medium neither RA nor LAB was detected during the cell cultivations.

Figure 1C indicates the time profiles of the intracellular accumulation of cryptotanshinone, tanshinone I, tanshinone IIA, and total tanshinones by suspension cultures of Ti-transformed *S. miltiorrhiza* cells in the flasks. The cryptotanshinone formation reached its peak of 2.0 mg/L on day 7, and later it decreased gradually. In contrast, the formation of intracellular tanshinone I and IIA showed a different kinetic pattern from the cryptotanshinone. Both tanshinones I and IIA had a gradual increase in accumulation from the start to the end of cultivation, and they reached their highest values of 1.5 and 1.7 mg/L on day 13, respectively. For the total tanshinones (cryptotanshinone plus tanshinone I plus tanshinone IIA), the level increased rapidly from day 0 to day 10, and reached the highest (i.e., 4.2 mg/L) on day 13.

Different from phenolic compounds, a certain amount of intracellular tanshinones was detected to be released in the medium near the end of cultivation (Table 2). The total extracellular tanshinones reached 1.09 mg/L on day 13. Thus, the total intracellular plus extracellular tanshinones obtained here was 5.3 mg/L (day 13). For the same cell line (fast growing cell line) and same medium (B5 medium without addition of elicitors like yeast extract), in a previous work only 0.89 mg/L of total tanshinones was obtained after 16 days of cultivation (Chen et al., 1997).

Bioreactor Cultivations

Based on the above successful shake flask results, bioreactor cultivations were further carried out by using the same B5 medium with 30 g/L of sucrose as in the flasks. Figure 2 shows the time courses of medium sugar

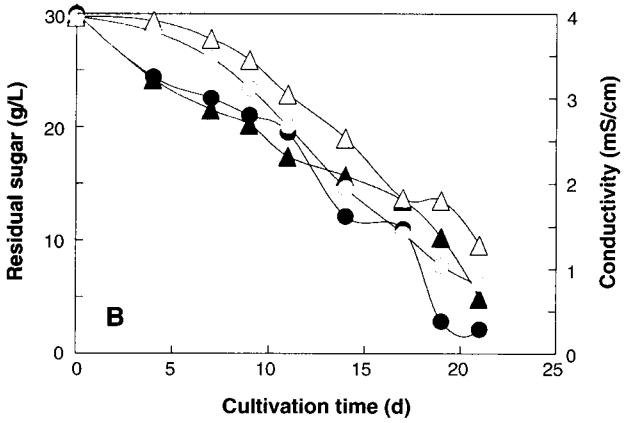

Figure 2. Time profiles of residual sugar and medium conductivity in suspension cultures of Ti-transformed *S. miltiorrhiza* cells in a 1.5-L stirred bioreactor with a large turbine impeller (T_L, circle) or a small turbine impeller (T_S, triangle). Symbols: dark for sugar; open for medium conductivity.

Table 2. Intracellular and extracellular accumulation of rosmarinic acid (RA), lithospermic acid B (LAB) and tanshinones (Cryptotanshinone, Tanshinone I, Tanshinone IIA) in 250-mL shake flask cultures of Ti-transformed *Salvia miltiorrhiza cells* (unit: mg/L).

Culture time	RA	LAB	Cryptotanshinone	Tanshinone I	Tanshinone IIA	Total tanshinones
In cells						
day 10	305.1	70.7	1.7	1.2	1.2	4.1
day 13	749.4	125.6	1.0	1.5	1.7	4.2
In medium						
day 10	ND	ND	0.34	0	0	0.34
day 13	ND	ND	0.30	0.28	0.51	1.09
Total (Intracellular + extracellular)						
day 10	305.1	70.7	2.0	1.2	1.2	4.4
day 13	749.4	125.6	1.3	1.8	2.2	5.3

[a]ND: not detected. Tanshinones were not detected in medium before day 10 in flask cultures. The accumulation of both phenolic compounds and tanshinones reached maximum on day 10 or day 13.

consumption and decrease of medium conductivity during the cell cultivations in a 2.4-L bioreactor with a small turbine impeller (T_S) or a large turbine impeller (T_L). In the T_S, the residual sugar level and medium conductivity were a little bit higher than those in T_L. Correspondingly, the final cell density obtained in T_S was also a little lower than that in T_L (Table 4). The higher cell growth in the large impeller reactor may be due to its low shear stress effected on the suspension cells (Kieran et al., 1997), which could be theoretically analyzed as follows.

During the cultivations, for the T_L, the agitation speed was ranged to be 60-100 rpm (from the beginning to the end of cultivation). But, a higher agitation speed for the T_S had to be set (i.e., from 150 rpm at the beginning to 200 rpm at the end of cultivation) in order to obtain a similar homogeneous liquid flow in both reactors. Therefore, the average shear rate (= $10 \cdot N$) (Zhong et al., 1994) in the T_S was much higher than that in the T_L, i.e., 10-16.7 vs. 25-33.3 per second. For the maximum shear rate, which can be evaluated by impeller tip speed (= $3.14 \cdot N \cdot D$) (Zhong et al., 1994), it was 15.1-25.1 cm/s for T_L while much higher for T_S (30.6-40.8 cm/s).

For the metabolites' accumulation, the tanshinones were detected in medium after 17 days of cultivation, while no phenolics were found in medium during the entire cultivations. Although tanshinone IIA was not detected in all the cases, both cryptotanshinone and tanshinone I were secreted into medium on day 19 and day 21 (Table 3). In T_S, the highest amount of total tanshinones in medium was 0.443 mg/L on day 19, while it was 0.365 mg/L on day 21 for T_L (Table 3). This may be also due to the relatively higher shear environment in T_S, which could affect the viability and outer structures of the cultured cells and made them easily release intracellular metabolites into the liquid medium. The secretion of secondary metabolite under a high shear environment was also reported in other cases of plant cell cultures (such as summarized by Kieran et al., 1997).

For the intracellular accumulation of the metabolites, almost all of them (except LAB) were of higher levels in T_L compared to those in T_S (Table 4). This was also true for the total production of these metabolites in the two bioreactors (Table 5). By comparing Table 2 and Table 5, it can be seen that the RA and LAB production titers were similar in both T_S and a shake flask, but the accumulation of tanshinones was greatly reduced in the bioreactor (T_S). In contrast, for the T_L, similar or even a little better results were obtained compared with the flasks. In addition, the

Table 3. Accumulation of tanshinones in medium in a stirred bioreactor with a small turbine impeller (T_S) or a large one (T_L)[a].

Reactor type	Culture time (d)	Cryptotanshinone (mg/L)	Tanshinone I (mg/L)	Tanshinone IIA (mg/L)	Total tanshinones (mg/L)
T_S	17	0	0	0	0
	19	0.193	0.250	0	0.443
	21	0.145	0.269	0	0.414
T_L	17	0	0	0	0
	19	0.096	0.115	0	0.211
	21	0.106	0.259	0	0.365

[a]Tanshinones were not detected in medium before day 17 of cultivation. Phenolic compounds were not detected in medium.

Table 4. Final cell density and intracellular contents of RA, LAB and tanshinones (Cryptotanshinone, Tanshinone I, Tanshinone IIA) in a stirred bioreactor with a small turbine impeller (T_S) or a large one (T_L) (on day 21).

Reactor type	Cell density (g DW/L)	RA (mg/g)	LAB (mg/g)	Cryptotanshinone (mg/g)	Tanshinone I (mg/g)	Tanshinone IIA (mg/g)
T_S	8.1	76.5	15.5	0.15	0.09	0.08
T_L	10.5	107.3	14.2	0.26	0.13	0.10

Table 5. Total accumulation (extracellular plus intracellular) of RA, LAB and tanshinones (Cryptotanshinone, Tanshinone I, Tanshinone IIA) in bioreactors (on day 21).

Reactor type	RA (mg/L)	LAB (mg/L)	Cryptotanshinone (mg/L)	Tanshinone I (mg/L)	Tanshinone IIA (mg/L)	Total tanshinones (mg/L)
T_S	619.7	125.6	1.36	1.0	0.65	3.01
T_L	1126.7	149.1	2.84	1.63	1.05	5.52

better performance of T_L than T_S was confirmed in a repeated experiment (data not shown). The results are considered due to the beneficial culture environment in the T_L such as low shear stress, which was theoretically estimated in a previous paragraph. The results indicate that a large-impeller reactor (like T_L) may be better than a small-impeller one for the efficient production of the valuable tanshinones and phenolic compounds by the suspension cultures of Ti transformed *S. miltiorrhiza* cells on a large scale.

Acknowledgements

The financial aid from the University of Hong Kong Committee on Research and Conference Grants (CRCG) is gratefully acknowledged. We would also like to thank the support from the Invitation and Exchange Program under the Ministry of Education of China (MOE). J.J.Z. also thanks the Cheung Kong Scholars Program (CKSP) of MOE.

References

Bruneton J (1995) Pharmacognosy, Phytochemistry, Medicinal Plants. Intercept Ltd., UK.

Chen H, Yuan JP, Chen F, Zhang YL, Song JY (1997) Tanshinone production in Ti-transformed *Salvia miltiorrhiza* cell suspension cultures. J Biotechnol 58: 147-156.

Chen H, Chen F, Zhang YL, Song JY (1999) Production of rosmarinic acid and lithospermic acid B in Ti transformed *Salvia miltiorrhiza* cell suspension cultures. Process Biochem 34: 777-784.

Choi SM, Son SH, Yun SR, Kwon OW, Seon JH, Paek KY (2000) Pilot-scale culture of adventitious roots of ginseng in a bioreactor system. Plant Cell Tissue Org Cult 62: 187-193.

Hase K, Kasimu R, Basnet P, Kadota S, Namba T (1997) Preventive effect of lithospermate B from *Salvia miltiorrhiza* on experimental hepatitis induced by carbon tetrachloride or D-galactosamine/lipopolysaccharide. Planta Med 63: 22-26.

Kamata K, Iizuka T, Nagai M, Kasuya Y (1993) Endothelium-dependent vasodilator effects of the extract from *Salviae miltiorrhizae Radix*. A study on the identification of lithospermic acid B in the extracts. Gen Pharmacol 24: 977-981.

Kamata K, Noguchi M, Nagai M (1994) Hypotensive effects of lithospermic acid B isolated from the extract of *Salviae miltiorrhiza Radix* in the rat. Gen Pharmacol 25: 69-73.

Kieran PM, MacLoughlin PF, Malone DM (1997) Plant cell suspension cultures: some engineering considerations. J Biotechnol 59: 39-52.

Mazumder A, Neamati N, Sunder S, Schulz J, Pertz H, Eich E, Pommier Y (1997) Curcumin analogs with altered potencies against HIV-1 integrase as probes for biochemical mechanisms of drug action. J Med Chem 40: 3057-3063.

Tanaka T, Morimoto S, Nonaka G, Nishioka I, Yokozawa T, Chung HY, Oura H (1989) Magnesium and ammonium-potassium lithospermates B, the active principles having a uremia-preventive effect from *Salviae miltiorrhiza*. Chem Pharmaceut Bull 37: 340-344.

Wu J, Zhong JJ (1999) Production of ginseng and its bioactive components in plant cell culture: current technological and applied aspects. J Biotechnol 68: 89-99.

Yu KW, Hahn EJ, Paek KY (2000) Production of adventitious ginseng roots using bioreactors. Kor J Plant Tissue Culture 27: 309-315.

Zhang YH, Zhong JJ (1997) Hyper-production of ginseng saponin and polysaccharide by high-density cultivation of *Panax notoginseng* cells. Enzyme Microb Technol 21: 59-63.

Zhong JJ, Fujiyama K, Seki T, Yoshida T (1994) A quantitative analysis of shear effects on cell suspension and cell culture of *Perilla frutescens* in bioreactors. Biotechnol Bioeng 44: 649-654.