Production of Ginkgolides and Bilobalide from Optimized the *Ginkgo biloba* Cell Culture

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Abstract The influence of various culture conditions on growth and ginkgolides (GKA and GKB), and bilobalide formation in callus and suspension cultures of *Ginkgo biloba* were investigated. Callus induced from the leaf petioles exhibited distinct morphological and physiological responses. The cell biomass and ginkgolides content varied among the cell lines; brownish callus lines produced high levels of ginkgolides and bilobalide in spite of poor cell growth. Among the culture media used, MS medium showed significant effect on cell growth and ginkgolides production. Low concentration of sucrose (3%) improved cell growth, while higher sucrose levels (5 and 7%) improved ginkgolides production. Cultivation of callus cultures above 28°C dramatically reduced their growth rate; however the cell lines grown at 36°C showed increased levels of bilobalide content. A 2.5-L balloon type bubble bioreactor (BTBB) was successfully developed for the cell growth and ginkgolides production.

Keywords: *Ginkgo biloba*, ginkgolides (GKA and GKB), bilobalide, balloon type bubble bioreactor (BTBB)

INTRODUCTION

Ginkgolides, the active principles of *G. biloba*, have several therapeutic applications, such as antagonists of platelet activating factors, cardiovascular disorders and inflammatory reactions [1]. Ginkgolides are largely present leaves and roots. However, harvest time, age, sex, and geographic origin and agro climatic conditions cause significant effects on ginkgolides content [2]. Since the demand for naturally occurring compounds are rapidly outstripping its supply, search for alternative methods for ginkgolides production has become crucial. Plant cell cultures in bioreactor systems for the production of such secondary metabolites, especially from perennial plants like *G. biloba*, has commercial importance [3].

Attempts have been made to develop cell lines of *G. biloba* using pollen [4], male and female haploid tissues [5], and embryos [6] as explants and the ginkgolides in *in vitro* cultures have been quantified. Carrier et al. [7] produced ginkgolides from suspension and immobilized cultures, but the yield levels were uneconomical. In this study, we are reporting the optimum culture conditions for the growth and of production of ginkgolides and bilobalide by cell cultures of *G. biloba*.

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MATERIALS AND METHODS

Plant Materials

Leaves of *G. biloba* from 4 different male and female plants located in the Kyungpook National University campus, Korea were collected and used *in vitro* cultures.

Callus Cultures

The leaf petioles from *G. biloba* were sterilized in 1% sodium hypochlorite solution with several drops of triton X-100, vigorously shaken for 25 min and rinsed with sterile distilled water several times. To induce callus, 0.7~1.0 cm sterile leaf petioles were placed on MS medium [8] having 3% sucrose, 100 mg/L myo-inositol, and 0.2% (w/v) phytagel, pH 6.0, supplemented with 5~40 μM NAA (α-naphthalene acetic acid) or 2,4-D (2,4-dichlorophenoxy acetic acid). Ten explants were placed on every 9 cm diameter Petri dishes. The calli were subcultured and incubated in total dark or with 16 h photoperiod at 25±1°C for varied period of time.

Cell Suspension Cultures

Suspension cultures of the primary callus were maintained on liquid MS medium containing 20 μM NAA on a rotary shaker at 100 rpm. The biomass was determined

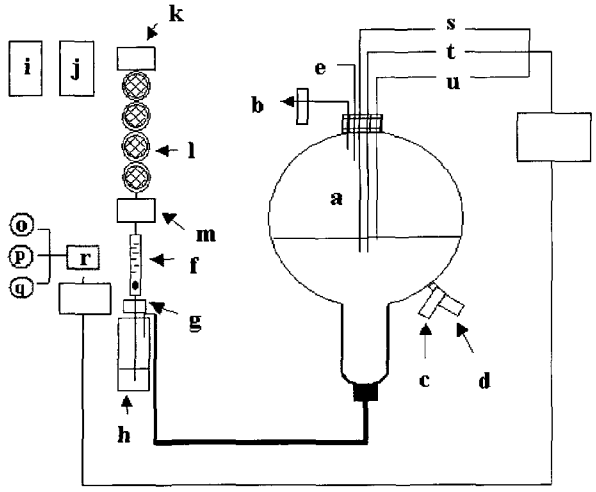

Fig. 1. 5-L sized balloon type bubble bioreactor (BTBB) system used for this study;(a) body of balloon type bubble bioreactor, (b) vent, (c) inoculum port, (d) sampling device, (e) medium exchange port, (f) air flow meter, (g) membrane filter, (h) water column, (i) air compressor, (j) air reservoir, (k) air cooler, (l) filter system, (m) air dryer, (o) O_2 tank, (p) CO_2 tank, (q) N_2 tank, (r) gas mixer.

by measuring sedimented cell volume (SCV), fresh cell weight (FCW) or dry cell weight (DCW). To determine the culture conditions for a high growth rate and ginkgolides accumulation, various basal media like MS, mB5 [9], SH [10] and WPM [11] supplemented with 20 μM NAA, and 100mg/L myo-inositol were used. Sucrose concentrations from 1% to 7% (w/v) were tried to select the concentration for optimal cell growth. The temperature dependence of cell growth and ginkgolides production growing on selected media was determined between 10 to 40°C. Cells with 20% SCV were inoculated into 250 mL Erlenmeyer flask and maintained on a rotary shaker with 100 rpm at 25±1°C in dark condition. The qualitative and quantitative data were recorded for 4 weeks on weekly intervals.

Bioreactor Cultures

The balloon type bubble bioreactor (BTBB) [12] was used to standardize the mass multiplication of the *G. biloba* suspension cultures (Fig. 1). The air flow meter and air filter used in the system were Dwyer (maximum capacity of 2.5 L per min) and Sartorial (6.5 cm in diameter and 0.2 μm pore size) products respectively. The bioreactor vessel and accessories (silicone tube, air inlet and air vent filters, inoculation and harvest ports, etc.) were autoclaved at 121°C for 40 min and dried for 30 min. To confirm that the bioreactor system is free from contamination, pre-run was conducted for 3 to 4 days before initiating cell culture. To start the fermentation, known volume of harvested cells (20% of SCV) and medium (MS medium supplemented with 3% sucrose, 10 μM NAA and 100 mg/L myo-inositol) were transferred into the reservoir flask. The initial cell density in the culture was measured by withdrawing an aliquot of the culture, 30 min after the addition of inoculum.

Extraction and Analysis Ginkgolides and Bilobalide

The *G. biloba* plant tissues and cell cultures were oven dried at 40°C, and powdered in a Wiley mill fitted with 20-mesh sieve. The ginkgolides and bilobalide were extracted and purified by the method described earlier with suitable modifications. 500 mg of the sample was extracted with 50% aqueous acetone (1:20 w/v) with occasional stirring. Filtered through Whatman No.1 filter paper and acetone was removed in vacuum. The aqueous layer was washed with one volume of *n*-hexane, pH 2.0 adjusted 1 N HCl, and extracted with one volume of ethyl acetate. The ethyl acetate layer was evaporated to dryness and the residue was suspended in 10 mL water. The aqueous suspension was extracted with one volume of diethyl ether for five times and the pooled diethyl ether fraction was dehydrated with anhydrous sodium sulfate. The diethyl ether was then evaporated and residue was reconstituted in methanol for HPLC analysis. Ginkgolides and bilobalide contents were quantified by HPLC (TSP, USA) using Lichrosorb R-18 column (10 μm, 3.2 × 250 mm, Merck), methanol-water (40: 60 v/v) as mobile phase at a flow rate of 1.0 mL/min. The elution profile was monitored at 220 nm using UV detector (TSP, 3000HR). GKA, GKB and bilobalide were identified and quantified by comparing the retention times of the various peaks using authentic standards.

RESULTS AND DISCUSSION

G. biloba callus was induced within 7 days and were observed to be soft and friable. The callus comprised cells of varied cell shapes and sizes. The callus was grouped into three types based on the color - white, yellow and brown. These different callus lines demonstrated difference in their growth pattern. Cell growth of whitish callus line was higher than brownish callus. The callus growth was reduced within four to five weeks, followed with increase in color from brown to dark brown. However the contents of ginkgolides and bilobalide recorded higher in the brownish followed by yellowish callus cell lines compare to whitish ones (Fig. 2).

The effective concentration of 2,4-D and NAA required for callus induction was found to be 20 μM for explants derived from both male and female *G. biloba* plants (Table 1). The callus obtained from MS medium supplemented with NAA produced white or yellow colored callus, whereas MS medium with 2,4-D produced brownish ones. Addition of activated charcoal to remove phenolic compounds did not show improvement on callus proliferation (data not shown). However the callus from MS medium supplemented with 20 μM 2,4-D exhibited poor keeping quality for long-term maintenance compared with the callus from MS medium with NAA. Earlier studies also indicate that MS medium with 2 mg/L NAA

Table 1. Effect of different growth regulators on callus induction and growth from the *G. biloba* leaf petioles

Sex	Growth regulators (μM)		Callogenesis (%)	Growth increment mean ± S.E.*		
				<0.2(cm)	0.2-0.5(cm)	>0.5(cm)
Female	NAA	5	63	4.2 ± 0.32	4.4 ± 0.52	0.1 ± 0.03
		10	69	2.6 ± 0.15	8.4 ± 0.73	0.4 ± 0.13
		20	91	2.2 ± 0.30	11.1 ± 0.42	3.4 ± 0.34
		40	76	4.1 ± 0.35	6.7 ± 0.58	1.8 ± 0.23
	2,4-D	5	58	5.4 ± 0.30	2.4 ± 0.27	0.2 ± 0.07
		10	40	2.3 ± 0.31	1.8 ± 0.23	0
		20	70	6.2 ± 0.25	3.9 ± 0.52	0
		40	67	7.7 ± 0.38	2.4 ± 0.31	0.1 ± 0.03
Male	NAA	5	67	3.7 ± 0.28	5.2 ± 0.56	0
		10	64	4.4 ± 0.52	7.2 ± 0.74	0
		20	72	1.6 ± 0.20	12.0 ± 0.97	0.4 ± 0.11
		40	77	4.6 ± 0.34	4.0 ± 0.53	0
	2,4-D	5	56	4.8 ± 0.39	4.0 ± 0.65	0
		10	58	3.7 ± 0.40	3.0 ± 0.49	0
		20	82	6.3 ± 0.67	3.1 ± 0.43	0
		40	53	6.9 ± 0.40	1.6 ± 0.36	0

[1] Values represent means of 10 petioles per plate and 9 plates per treatment ± Standard Error

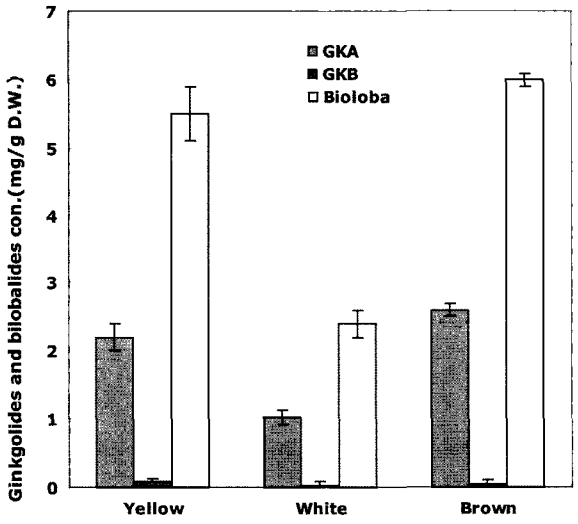

Fig. 2. Variation of ginkgolides (GKA and GKB) and bilobalide (Biloba) content among cell lines. Cell lines were maintained on MS liquid medium containing 20 μM NAA on a rotary shaker at 100 rpm.

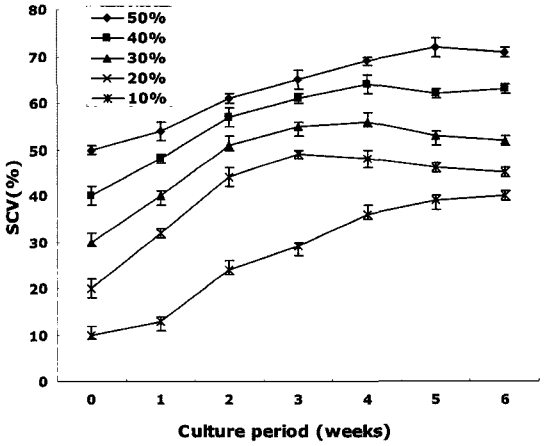

Fig. 3. Effects of inoculum density on cell growth of the *G. biloba*. *G. biloba* calli transferred to MS liquid medium supplemented with 20 μM NAA showed sigmoid growth cycles, with reduced cell growth followed by cell lyses.

and 5 mg/L kinetin in dark [6] and 2 mg/L NAA and 1 mg/L kinetin are the best for callus growth [7]. Our studies indicate that MS medium supplemented with 3% sucrose and 10 μM NAA was good for the callus culturing especially when they have to be maintained for long time in culture media.

G. biloba calli transferred to MS liquid medium supplemented with 20 μM NAA showed sigmoid growth cycles, with reduced cell growth and cell lyses (Fig.3). Initial inoculum of 20% cells was found to be effective and reached stationary phase in about three weeks. The size of the inoculum was critical, influence the survival of cells and maintenance of the vegetative cells, in determining their capacity of secondary metabolites production [13].

Plant cell culture media widely used to support growth are normally not optimum for product formation. Production medium needs to be optimized for the yields of a desired product [14]. Nutritional and growth factors exert influence on the production of secondary metabolite [15]. Various culture media such as MS, MB5, SH, and WPM were used in this study to optimize nutritional requirements for growing *G. biloba* cells (Fig. 4). The results indicate that the cell growth as well as ginkgolides and bilobalide production was significantly higher in MS medium than the other media used in this study.

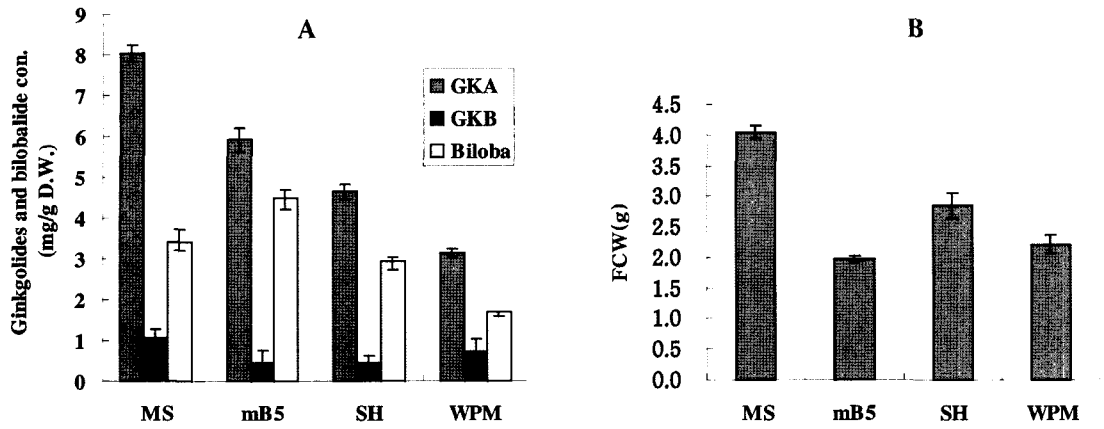

Fig. 4. Effects of culture media on cell growth, ginkgolides (GKA and GKB) and bilobalide (Biloba) production of the *G. biloba*.

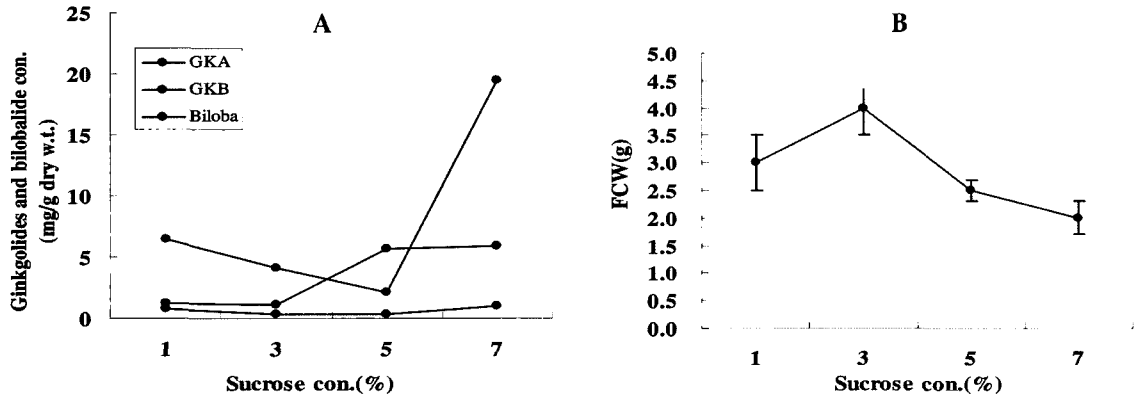

Fig. 5. Effects of sucrose concentration on cell growth, ginkgolides (GKA and GKB) and bilobalide production of the *G. biloba*. Erlenmeyer flask and maintained on a rotary shaker with 100 rpm at 25±1°C in dark condition.

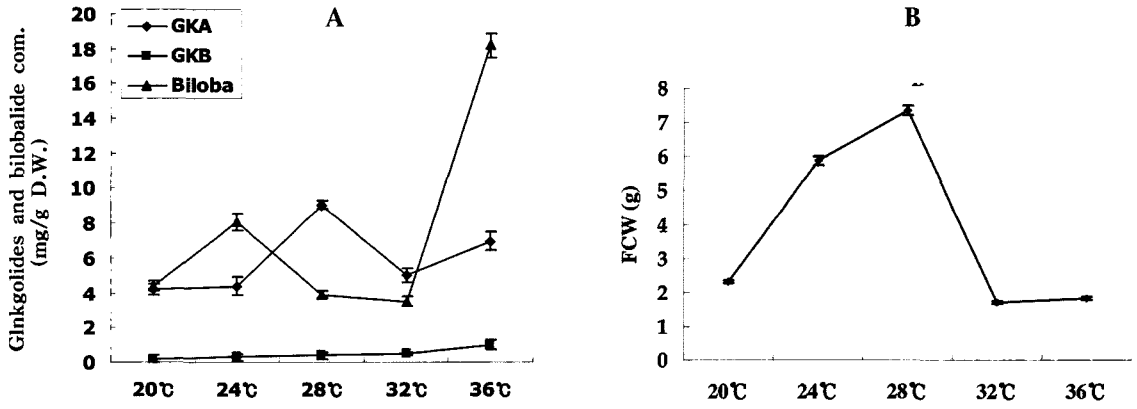

Fig. 6. Effects of temperature on cell growth, ginkgolides (GKA and GKB) and bilobalide production of the *G. biloba*.

Mizukami *et al.* [16] reported that a high sucrose concentration is necessary for high yield of shikonin derivatives in *Lithospermum erythrorhizon*. The present study reveals that 3% sucrose enhanced the cell growth, but the ginkgolides contents were low (Fig. 5). Although the relative cell growth is low in MS medium supplemented with 5 and 7% sucrose, significant increase in the bilobalide and GKA was observed.

The effect of temperatures (20 to 36°C) on the biomass and secondary metabolites production was examined in *G. biloba* cell suspension cultures (Fig. 6). Growing the cells at 25°C was found to be optimum, otherwise rapid decline in the cell growth was observed below or above the optimum temperature. The callus cultures grown at elevated temperatures revealed that 36°C was optimum for GKA and bilobalide production. Earlier studies suggest that such temperature dependent secondary metabolite production cell lines are species specific [17]. The results suggest that the cell cultures can be grown at lower temperatures till sufficient biomass accumulation is achieved, and then can be induced for secondary metabolite production by elevating the temperature to 36°C.

The pattern of cell growth in a 2.5-L balloon type bubble bioreactor used for batch culture of *G. biloba,* was almost similar to that in flask cultures (data not shown). Relatively long lag phase followed by rapid cell lyses at the end of the early stationary phase were characteristic of cell growth patterns. The doubling time of cell growth in BTBB was 12 days with 20% inoculum and 0.1vvm aeration. The ginkgolides and bilobalide production in balloon type bubble bioreactor was on par with the profiles of flask cultures (data not shown). The production ginkgolides was initiated by the cultures 5 days after addition of inoculum and the levels were 6.5 mg/L in GKA, 0.5 mg/L GKB and 3.5 mg/L bilobalide in 4 weeks old cultures, respectively (data not shown). The increase in the production of GKA and bilobalide was substantial upto 20days under the bioreactor condition.

Production of useful secondary metabolites in bioreactor is an attractive alternative to overcome the exploitation of the natural resources. Plant cells are now being cultured in a wide range of bioreactors and the reaction parameters can be selectively regulated for the induction of biomass and secondary metabolites. Further the balloon type of bioreactors used in the present study for growing *Ginkgo* cells hardly had any problems in scaling up process. The results of this study suggest the optimum conditions for mass production of *G. biloba* cell cultures as well as the GKA and bilobalide production. Hence, these results will provide useful information for manipulation of the bioreactor conditions for commercial production of secondary metabolites from *G. biloba* cell cultures.

CONCLUSION

Plant cell culture on a large scale has been shown to be feasible for industrial production of secondary metabolites. Ginkgolides are now obtained from leaves and roots of *G. biloba*. Since the harvest time, age, sex, geographic origin and agro climatic conditions cause significant effects, a major limitation of the commercialization of ginkgolides production. In the present study, culture conditions such as culture media, sucrose concentration, temperature and light have been optimized for cell growth and ginkgolides production. Since the ginkgolides production is influenced by primary inoculum levels and environmental conditions, suitable parameters for scaling up in the bioreactor for culturing the *Ginkgo* cells using a 2.5-L BTBB has been developed for enhancement of cell biomass and ginkgolides production.

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REFERENCES

[1] Van, B. A., H. A. Scheeren, T. Rantio, W. C. Melger, and G. P. Lelyveld (1991) Determination of ginkgolides and bilobalide in Ginkgo biloba leaves and phytopharmaceuticals. *J. Chromatogr.* 51: 375-387.

[2] Webb, D., W. Arias, and E. D. Hostos (1986) Callus formation by *Ginkgo biloba* embryos on hormone free media controlled by closures and media components. pp.121-127. In: R. A. Dixon (ed.). *Plant Cell Culture: A Practical. Approach*, IRL Press. USA.

[3] Morris, P., A. H. Scragg, N. J. Smart, and A. Stafford (1985) Secondary product formation by cell suspension cultures. pp.127-129. In: R. A. Dixon (ed.). *Plant Cell Culture: A Practical Approach*, IRL Press. USA.

[4] Tulecke, W. and A. Rutner (1965) Changes in the amino acid composition of medium and cells of a plant tissue culture during growth in a liquid medium containing arginine. pp.103-116. In: R. A. Dixon (ed.). *Plant Cell Culture: A Practical Approach*, IRL Press. USA.

[5] Tulecke, W. (1964) A haploid tissue culture from the female gametophyte of *Ginkgo biloba. Nature* 203: 94-95.

[6] Yates, W. (1986) Induction of embryogenesis in embryo-derived callus of *Ginkgo biloba*. 5nd ed., pp. 43-48. In: *Plant Tissue Cell Culture*. University of Minnesota, USA.

[7] Carrier, D. J., P. Conlombe, M. Mancini, R. Neufeld, M. Weber, and J. Archambault (1990) *International Congress of Plant Tissue and Cell Culture.* 2nd ed., pp.23-27. Kluwer Academic Publishers, Amsterdam, The Netherlands.

[8] Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant*. 15: 473-497.

[9] Gamberg, O. L., R. A. Miller, and K. Ojima (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell. Res.* 50:148-15.

[10] Schenk, R. U. and A. C. Hildebrandt (1972) Medium and techniques for induction and growth of monocotyledonous plant cell culture. *Can. J. Bot.* 50: 199-204.

[11] Lloyd, G. B. and B. H. McCown (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by the use of shoot top culture. *Comb. Proc. Int. Plant Prop. Soc.* 30: 421-437.

[12] Son, S. H., M. S. Choi, Y. H. Lee, K. B. Choi, S. R. Yun, J. K. Kim, H. J. Kim, O. W. Kwon, E. W. Noh, J. H. Seon, and Y. G Park (2000) Large scale growth and taxane production in cell cultures of *Taxus cuspidata* (Japanese yew) using a novel bioreactor. *Plant Cell Rep.* 19: 628-633.

[13] Nigra, H. M., M. A. Alvarez, and A. M. Giulietti (1990) Effect of carbon and nitrogen sources on growth and solasodine production in batch suspension cultures of *Solanum elaeagnifolium* Cav. *Plant Cell Tiss. Org. Cult.* 21: 55-60.

[14] Fujita, Y., Y. Hara, C. Suga, and T. Moromoto (1981) Production of shikonin derivertives by cell suspension cultures of *Lithospermum erythrorhizon* of shikonin derivertives. *Plant Cell Rep.* 1: 61-66.

[15] Heijden, R. V. R. Verpoorte, H. J. Hoopen, R. V. Heijden, and H. Tenhoopen (1989) Cell and tissue cultures of *Catharanthus roseus* L. *Plant Cell Tiss. Org. Cult.* 18: 231-280.

[16] Mizukami, H., M. Konishima, and M. Tabata (1977) Effects of nutritional factors on shikonin derivertive formation in *Lithospermum erythrorhizon* callus cultures. *Phytochemistry.* 16: 113-118.

[17] Puchan, Z. and S. H. Martin. (1971) The industrial potential of plant cell cultures. *Prog. Ind. Microbiol.* 9: 33-39.

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