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An Optimization of Flavonoid Production from the Suspension Culture of *Scutellaria baicalensis* Georgi Cells

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Flavonoid production by suspended cells of *Scutellaria baicalensis* Georgi was studied and the medium was optimized for cell growth and baicalin production. In SH medium the flavonoid production was not closely associated with the cell growth. A modified SH medium, FPM, was therefore designed for enhanced baicalin production. In FPM, both cell growth and baicalin production were increased by 1.5 times and 1.67 times than in the original SH medium, respectively. The increases could be attributed to the increased metabolic activities involved in the flavonoid biosynthesis as represented by enhanced activities of phenylalanine ammonia-lyase.

Plant cell culture technique often provides an alternative means for large scale production of high-value plant secondary metabolites (10). Although lots of attempts have been made on plant cell suspension culture, economically feasible processes are very rare except the cases of shikonin and berberine (3).

Dedifferentiated plant cells are heterogeneous in genetic composition and the contents of economically important metabolites are often lower than in donor plants. Therefore, it is necessary to develop high yield-cell lines and to design the optimal culture conditions in order to improve the productivity.

Scutellaria baicalensis contains many flavonoids, of which baicalin (5,6-dihydroxyflavone-7-O-glucuronic acid) and wogonin-7-O-glucuronic acid (5-hydroxy-8-methoxy-7-O-glucuronic acid) are kown to be the major components with biological activity for diseases such as inflammation and pyrexia (6, 11). Previously, we reported on the development of a high-yield cell line of *S. baicalensis* for production of baicalin and wogonin-7-O-glucuronic acid as major secondary metabolites (9). In order to increase the production yield of baicalin, an optimal medium composition for cell growth and flavonoid production was studied and the results are reported in this study.

MATERIALS AND METHODS

Cell Line and Suspension Culture

A cell line of *S. baicalensis* Georgi, H3 (9), was maintained in Schenk and Hildebrandt medium (8) in 250 ml Erlenmeyer flasks containing 40 ml medium on a gyratory shaking incubator (80 rpm) at 25°C in the dark, and subcultured every 8 to 10 days with a ten percent inoculum (about 20 g^{FW}/l). Unless specified otherwise, cells were harvested at 12 days of cultivation.

Culture Media

Basal media of Schenk and Hildebrandt (SH), Murashige and Skoog (MS) (7), Gamborg B5 (B5) (4), and White's (13) were used in this study. All media contained 2 mg/l of 2,4-dinitrophenoxyacetic acid (2,4-D), 1 mg/l of kinetin, and 30 g/l of sucrose. The pH was adjusted to 5.8 before autoclaving.

PAL Assay

Phenylalanine ammonia-lyase (PAL) activity was measured by the method of Whetten and Sedroff (12). Protein concentration was determined by the Lowry method (2). The enzyme activity was expressed in specific activity; 1 unit of specific activity represents the conversion of 1 μmol of L-phenylalanine to cinnamic acid per minute by 1 mg of protein.

Analytical Methods

After cells were separated on a stainless steel mesh (pore size: 30 μm), cell fresh weight (FW) was measured and cell dry weight (DW) was then determined by drying the wet cell cake in an oven at 85°C to a constant weight. Sugar concentration was determined by the dinitrosalicylic acid method (1) and the conductivity of the culture medium was measured with a conductance meter (YSI model 35). Flavonoid was extracted with the ten volume of hot water

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(90°C) over the harvested cells for three hours. Concentration of baicalin was determined by using HPLC (Waters with an UV detector, 270 nm) equipped with a stainless steel column (μ-Bondapak™ C_{18}, 3.9×30 mm). The mobile phase was composed of 145 ml of tetrahydrofuran, 125 ml of dioxan, 50 ml of MeOH, 20 ml of AcOH, 2 ml of 5% H_3PO_3, and deionized water to 1 liter, and flow rate was 1 ml/min. Baicalin was separated at the retention time of 9 min. All experiments were triplicated and averaged.

RESULTS AND DISCUSSION

To find a suitable culture medium for cell growth and baicalin production, cells of *S. baicalensis* were cultivated in four different media widely used in plant cell culture, MS, SH, White's and B5. Results are summarized in Table 1. There were considerable differences in cell growth and baicalin production with these media. In White's medium which lacks ammonium nitrogen, cells did not grow appreciably. SH and B5 media were suitable for cell growth and baicalin production, but the highest cell growth and baicalin production were obtained using SH medium. On the basis of the concentration of major nutrients present in the media, a high ratio of NO_3^-/NH_4^+ and high phosphate concentration seemed favorable for both cell growth and baicalin production. Therefore, SH medium was selected as the basal medium and the subsequent optimization studies.

Table 1. Cell growth and baicalin production in different culture media.

Medium	Cell growth (g^{FW}/l)	Baicalin production (g/l)
Murashige and Skoog Total nitrogen, 60 mM (NO_3^-, 40 mM); (NH_4^+, 20 mM); Phosphate, 1.25 mM	195	0.1
Schenk an Hildebrandt Total nitrogen, 26.5 mM (NO_3^-, 24.5 mM); (NH_4^+, 2 mM); Phosphate, 2.6 mM	340	1.8
White's Total nitrogen, 3.3 mM (as NO_3^-); Phosphate, 0.125 mM	45	0.2
Gamborg B5 Total nitrogen, 27.4 mM (NO_3^-, 25.4 mM); (NO_4^+, 2 mM); Phosphate, 1.08 mM	300	1.15

A typical pattern of cell growth and baicalin production in SH medium is shown in Fig. 1. Maximum cell growth and baicalin production were 9.5 g^{DW}/l and 1.8 g/l, respectively. From the relationship between specific growth rate (μ) and specific product formation rate (q_p), it was clear that whereas μ and q_p both increased during the initial growth phase (until day 6), they were uncoupled in the later growth phase (days 6-14), indicating that the synthesis of baicalin in suspension culture was a rather non-growth associated process.

Phytohormone affects cell growth and secondary metabolite production. There have been a number of reports showing the effect of auxins on flavonoid production. In cell suspension cultures of *Epimedium diphyllum* (15) and *Vanconveria hexandra* (16) 2,4-D stimulated the production of flavonol glycosides. However, it was also reported that 2,4-D inhibited the production of other flavonoids such as anthocyanin in the suspension culture of *Vitis* cells (14). Effects of 2,4-D and kinetin on the cell growth and baicalin production in SH medium were therefore examined. As shown in Table 2., the highest production of baicalin was obtained in the medium containing kinetin as a sole phytohormone. The effect of kinetin concentration on the cell growth and baicalin production is shown in Fig. 2. Cell growth increased as kinetin concentration increased up to 5 mg/l, whereas the highest production of baicalin was obtained at kinetin concentration of 0.1 mg/l.

To investigate the effect of carbon sources, cells were cultured in SH media containing 30 g/l of five different sugars such as sucrose, fructose, glucose, lactose, and

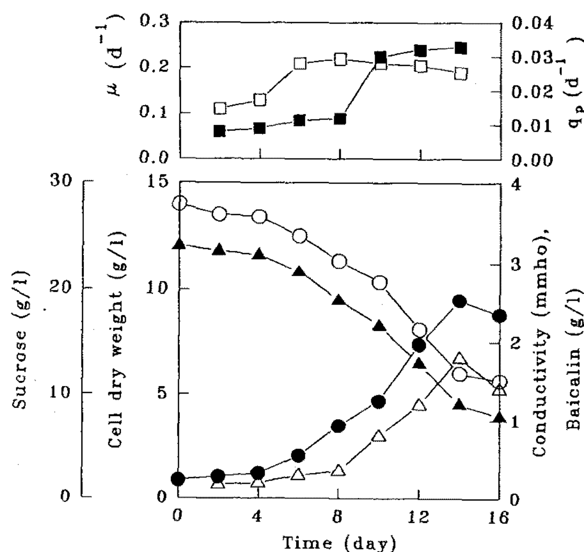

Fig. 1. Time course of cell growth and baicalin production in SH medium.

○, sucrose; ●, cell dry weight; ▲, conductivity; △, baicalin; □, specific growth rate (μ); ■, specific product formation rate (q_p).

Table 2. Effect fo phytohormones on cell growth and baicalin production in SH medium.

Phytohormone	Cell growth (g^{FW}/l)	Baicalin production (g/l)
Phytohormone free	190	1.02
2,4-D, 2 mg/l	320	0.30
Kinetin, 1 mg/l	290	2.01
2,4-D, 2 mg/l + Kinetin 1 mg/l	330	1.30

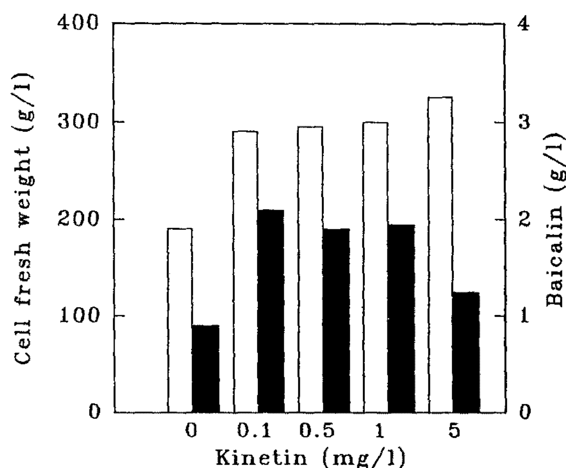

Fig. 2. Effect of kinetin concentration on cell growth and baicalin production.
□, cell fresh weight; ■, baicalin.

Table 3. Effect of carbon sources on the cell growth and baicalin production.

Carbon source	Cell growth (g^{FW}/l)	Baicalin production (g/l)
Sucrose	300	1.9
Fructose	50	0.2
Glucose	330	2.4
Lactose	40	0.2
Galactose	30	0.1

30 g/l of carbon sources was used.

galactose. The results are summarized in Table 3. Sucrose and glucose were effective for the cell growth, but glucose was the most favorable carbon source for baicalin production. When the glucose concentration was varied from 10 g/l to 80 g/l, it was found that 40 g/l of glucose was most effective for baicalin production (Fig. 3).

The effects of inorganic and organic elements on the cell growth and baicalin production were examined in the same manner: the key element was omitted from the basal SH medium and the culture data were compared with the control. Omitting elements except $CoCl_2 \cdot 2H_2O$ and nicotinic acid reduced cell growth and baicalin production (Table 4). The cell cultures were supplied with

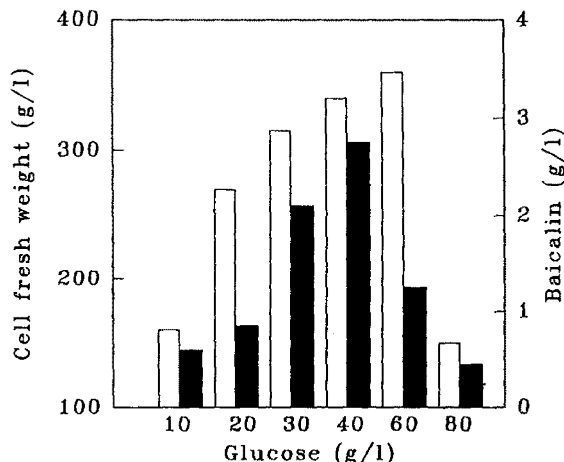

Fig. 3. Effect of glucose concentration on cell growth and baicalin production.
□, cell fresh weight; ■, baicalin.

Table 4. Effect of omitting various inorganic elements from the SH medium on cell growth and baicalin production.

Omitted elements	Cell growth (g^{FW}/l)	Baicalin production (g/l)
Control[a]	330	2.80
-KNO_3	no growth	ND[b]
-$MgSO_4 \cdot 7H_2O$	65	0.14
-$MnSO_4 \cdot H_2O$	300	1.54
-$ZnSO_4 \cdot 7H_2O$	289	0.62
-$CuSO_4 \cdot 5H_2O$	308	2.00
-$CaCl_2 \cdot 2H_2O$	80	0.17
-KI	322	1.93
-$CoCl_2 \cdot 2H_2O$	314	2.93
-$NH_4H_2PO_4$	102	0.79
-H_3BO_3	270	0.07
-$Na_2MoO_4 \cdot 2H_2O$	324	2.71
-$FeSO_4 \cdot 7H_2O$	195	1.06
-Na_2-EDTA	327	1.19
-myo-inositol	90	0.56
-Thiamine·HCl	290	2.50
-Pyridoxine·HCl	320	2.61
-Nicotinic acid	330	2.89

[a]SH basal salt medium containing 40 g/l glucose, 0.1 mg/l kinetin and other SH organic elements. [b]not determined.

various levels of inorganic and organic elements of SH medium, and their optimal concentrations for baicalin production were found to be; 3 g/l of KNO_3, 0.2 g/l of $MgSO_4 \cdot 7H_2O$, 0.1 g/l of $CaCl_2 \cdot 2H_2O$, 0.2 g/l of $NH_4H_2PO_4$, 1 mg/l of $MnSO_4 \cdot H_2O$, 1 mg/l of $ZnSO_4 \cdot 7H_2O$, 5 mg/l of $CuSO_4 \cdot 5H_2O$, 1 mg/l of KI, 1 mg/l of H_3BO_3, 0.1 mg/l of $Na_2MoO_4 \cdot 2H_2O$, 15 mg/l of $FeSO_4 \cdot 7H_2O$, 20 mg/l of Na_2-EDTA, 0.5 g/l of myo-inositol, 1 mg/l of vitamin B_1 and 0.25 mg/l of vitamin B_6 (detailed data not shown).

A modified SH medium for flavonoid production (FPM) was thus designed from the results as described

Table 5. Comparison of modified SH modium for flavonoid production, FPM, with SH medium.

Ingredients (mg/l)	SH	FPM
KNO_3	2500	3000
$MgSO_4 \cdot 7H_2O$	400	200
$MnSO_4 \cdot H_2O$	10	1
$ZnSO_4 \cdot 7H_2O$	1	1
$CuSO_4 \cdot 5H_2O$	0.2	5
$CaCl_2 \cdot 2H_2O$	200	100
KI	1	1
$CoCl_2 \cdot 6H_2O$	0.1	-
$NH_4H_2PO_4$	300	200
H_3BO_3	5	1
$Na_2MoO_4 \cdot 2H_2O$	0.01	0.1
$FeSO_4 \cdot 7H_2O$	15	15
$Na_2 \cdot EDTA$	20	20
Thiamine	5	1
Pyridoxine	0.5	0.25
Nicotinic acid	5	-
myo-Inositol	1000	500
2,4-D	2	-
Kinetine	1	0.1
Sucrose	30000	-
Glucose	-	40000

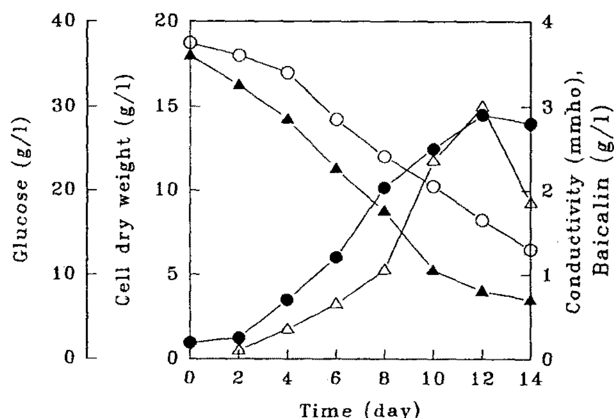

Fig. 4. Time course of cell growth and baicalin production in the modified SH medium, FPM.
○, glucose; ●, cell dry weight; △, baicalin; ▲, conductivity.

above, and summarized in Table 5. The osmotic pressure of FPM was increased 1.7 times (280 milliosmol/kg) over that of the original SH medium (165 milliosmol/kg). The typical cell growth and baicalin production in FPM are shown in Fig. 4. Maximum cell growth and baicalin production obtained after 12 days of culture were 14.5 g^{DW}/l of cell growth and 3 g/l of baicalin, respectively. Production titre of baicalin increased 1.67 times with FPM over the original SH medium. Such an increase in flavonoid productivity with FPM could be attributed to

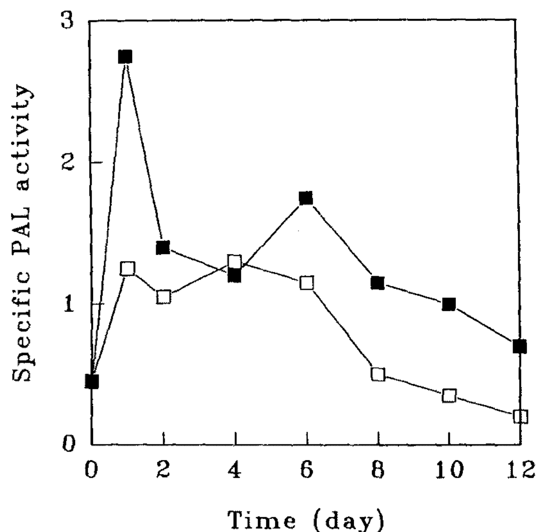

Fig. 5. Time course of phenylalanine ammonia-lyase activity.
□, in SH medium; ■, in FPM.

the increased metabolic activities involved in the flavonoid biosynthesis. In general, the increase in the enzyme activity is directly or indirectly related to the biosynthesis of the metabolites (5). Time courses of the activities of phenylalanine ammonia-lyase (PAL, EC 4.3. 1.5), for example, which catalyses the first step reaction in the biosynthesis of a range of plant secondary metabolites including flavonoids, are shown in Fig. 5. It is noted that PAL activity was maintained in FPM at a higher level throughout the culture period than in the original SH medium. Although there exists no direct evidence that the activity of PAL regulates the cellular baicalin synthesis, it is a strong indication of enhanced cellular metabolism towards increased flavonoid production.

The medium optimized for the baicalin production can be further used for the scale-up study. A large-scale process for baicalin production is under progress.

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