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ORIGINAL ARTICLE

Bioproduction of phenylethanoid glycosides by plant cell culture of *Scrophularia striata* Boiss.: from shake-flasks to bioreactor

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Abstract Phenylethanoid glycosides (PeG) are a class of polyphenols found in some plants that have pharmaceutical effects as anti-inflammatories and anti-oxidants. The presence of PeG (acteoside) in the aerial parts of *Scrophularia striata* Boiss. has been demonstrated. Considerable progress has been made using plant cell cultures to stimulate formation and accumulation of secondary metabolites. The present study optimized phenylethanoid production from shake flasks to bioreactor using a cell culture of *S. striata*. The optimal conditions for production of cell biomass by scale-up to a bioreactor were determined to be a pH of 4.8, air flow rate of 0.5–1.5 l min^{-1}, and mixing speed of 110–170 rpm at 25 ± 1 °C in darkness. Growth parameters and PeG production were measured and compared with the results from the shake flasks. The results showed that cell biomass was high in the bioreactor (15.64 g l^{-1} DW) and in the shake flasks (14.16 g l^{-1} DW). The acteoside content in the bioreactor was 1404.20 μg g^{-1} DW, which is threefold higher than in the shake flasks (459.71 μg g^{-1} DW). The echinacoside concentration in the bioreactor was 1449.39 μg g^{-1}, 1.36-fold lower than in the shake flasks (1973.03 μg g^{-1} DW). This study established an efficient way for production of acteoside, the major PeG, in a bioreactor.

Keywords Acteoside · Bioreactor · Echinacoside · Plant cell culture · *S. striata* Boiss.

Abbreviations

ANOVA	Analysis of variance
DAD	Photodiode array detection
DW	Dry weight
FW	Fresh weight
HPLC	High performance liquid chromatography
PeG	Phenylethanoid glycoside
S. striata	*Scrophularia striata*

Introduction

Secondary metabolites are essential for plant survival, but not necessary for plant growth. PeG is a class of polyphenol compound found in plants (Jia et al. 2009); several hundred compounds of this type have been isolated from medicinal plants. Studies show that these compounds are antibacterial, anticancer, antiviral, antioxidant, anti-inflammatory, immune modulators, and inhibit tyrosinase (Li et al. 2009).

PeG is of interest as a chemical agent and shows potential in pharmaceutical and industrial applications. Acteoside (verbascoside) and echinacoside are PeGs with many biological functions. Acteoside regulates cell apoptosis and is anti-oxidant, anti-inflammatory, and anti-hypertensive (Saimaru and Orihara 2010). Echinacoside is anti-oxidative, a scavenger of nitric oxide radicals, neuroprotective, and anti-hepatotoxic (Jia et al. 2009).

Identification of medicinal plants that stimulate apoptosis can advance production of anticancer drugs. The chemical structures of these compounds are usually

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complex and their production is costly. Plant cell cultures are currently used in medicinal industries as a source of high value secondary metabolites (Suresh et al. 2001). Because biosynthesis of phytochemicals by plant cell culture is independent of environmental conditions, new approaches for cell cultivation have been used for production of important compounds such as taxol and shikonin (Georgiev et al. 2011). Bioreactors have recently been developed for scale-up production of secondary metabolites by plant cell culture (Ouyang et al. 2005a).

The transfer of biosynthetic processes from shake flasks to bioreactors is an essential step in commercialization of plant cell culture-based production. Bioreactors for plant cell culture can monitor and control culture parameters such as pH, dissolved oxygen concentration, temperature, and agitation, making it possible to study cell behavior by determining cell growth, and metabolite production (Chen et al. 2007).

Scrophularia striata Boiss. is a native plant species of the Scrophulariaceae family which grows in Iran and is known by the local name of *tashne dari* in Ilam province. It has been used for many years in traditional medicine to promote wound healing, infectious disease, allergies, rheumatism, and chronic inflammatory disorders (Safavi et al. 2012). Biological functions for *S. striata* include amelioration of anxiety and depression (Babri et al. 2012), and as anti-inflammatory (Shoohani et al. 2010, Azadmehr et al. 2009), anti-tumor (Ardeshiry et al. 2010), antibacterial (Bahrami and Valadi 2010) agents, and repression of nitric oxide (Azadmehr et al. 2009). Monsef-Esfahani et al. (2010) have reported the presence of acteoside as a PeG in the aerial parts of *S. striata*.

The authors have previously reported that cell culture of the plant produces acteoside (Khanpour-Ardestani et al. 2014) and echinacoside (unpublished data). The present work optimized and scaled-up PeG production by plant cell culture of *S. striata* in a shake flask transferred to a bioreactor.

Materials and methods

Growth of cell suspensions in shake flask

S. striata callus was grown on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 2 mg l^{-1} naphthalene acetic acid (NAA), 0.5 mg l^{-1} benzyl adenine (BA), and 30 g l^{-1} sucrose as described by Khanpour-Ardestani et al. (2014). The callus was maintained by subculturing in fresh medium every 15–17 days in a phytotron at 26 ± 1 °C.

To initiate suspension cultures, 2 g of white and friable callus (at 15 days of growth cycle) was inoculated into 50 ml of fresh liquid medium in a 250 ml Erlenmeyer flask. It was subcultured every 15 days and was grown in a shaking incubator (110 rpm, 25 °C) in darkness. After 60 days the cells were homogenized. Cell growth was used to determine the best period for use of the cell suspension. Next, 1 ± 0.05 g fresh cells were inoculated into 50 ml of fresh liquid medium in a 100 ml flask.

Cell growth, cell viability, and PeG (acteoside and echinacoside) content were monitored by subculture of cells harvested from 0 to 25 days at 5 days intervals. Readings were taken from three flasks for each parameter. Cells were separated from the medium by filtration using nylon mesh and their fresh and dry weights were recorded.

Growth of cell suspensions in bioreactor

An FS-01-A05P bioreactor system (Winpact; USA) was used in the experiments and comprised a 10 l double-jacketed vessel providing temperature control, a TruDo dissolved oxygen sensor (PN 608603; Finesse; USA), pH probe (FS-A-PPH03; Switzerland), and Optima air pump (Hiblow HP60; Japan). Before initiating the culture, bioreactors with 5 l of modified MS medium were first sterilized by autoclaving at 121 °C for 20 min and then the *S. striata* fresh cells were inoculated into the vessel of the bioreactor. Inoculation was done under sterile conditions under laminar airflow to avoid contamination. The mixing speed was set at 110–170 rpm, the air flow rate was kept constant at either 0.5 or 1.5 l min^{-1}, and the culture was maintained at 25 ± 1 °C in darkness for 25 days. The pH of the culture medium was controlled at 4.8 by the automatic addition of aq. NaOH (0.5 mol l^{-1}) or aq. HCl (0.5 mol l^{-1}) during cultivation. Aliquots of 15 ml of suspension were removed with the sample tool of the bioreactor at 2 days intervals to follow the growth and PeG content.

Determination of cell biomass

For dry weight determination, cells and medium were separated by filtration under pressure in vacuum and then dried at room temperature under laminar flow.

Cell viability

Cell viability was determined by Evan's blue staining (Rodríguez-Monroy and Galindo 1999). One ml samples were incubated in 0.25 % Evan's blue stain for 3–5 min and then at least 500 cells were counted.

Phenylethanoid glycoside extraction and high performance liquid chromatography

PeG was extracted from 0.2 g of dried cells using 90 % (v/v) methanol aqueous solution (10 ml) at room temperature.

The extract was centrifuged at 5000 rpm for 15 min and the supernatant was collected, air-dried, and re-dissolved in 500 µl high performance liquid chromatography (HPLC) grade methanol. It was then centrifuged at 13,000 rpm prior to HPLC analysis (Khanpour-Ardestani et al. 2014). The isolated PeG was analyzed by HPLC (Agilent Technologies; USA) with a C-18 column (Perfectsil Target ODS-3 (5 µm), 250 × 4.6 mm; Germany). The mobile phases were 0.04 % (v/v) acetic acid aqueous solution (solvent A) and pure acetonitrile (solvent B) (82:18) Components were detected at 330 nm using a DAD detector (Agilent Technologies; USA). All separations were performed at 25 °C and a flow rate of 1 ml min^{-1} with an injection volume of 20 µl.

This assay was based on the method by Vertuani et al. (2011) with some modifications. The compositions of the solvents were water/acetonitrile (95:5; 0.01 M H_3PO_4; solvent A) and acetonitrile/water (95:5; 0.01 M H_3PO_4; solvent B). The amount of solvent A and B used was 83 and 17 ml, respectively. Acteoside and echinacoside standards were purchased from Sigma-Aldrich. Identification of PeG was performed by comparison of retention times and UV spectral peaks of samples with authentic standards (Fig. 3a–e). Quantitative estimation of PeG was based on the peak area of specific concentrations of the sample and the standard.

Statistical analysis

All data was analyzed by analysis of variance (ANOVA) using SPSS 19 software. Duncan's multiple range testing was used to measure statistical differences between treatments and control. A value of $p \leq 0.05$ was considered significant.

Results

Shake flask experiments

The growth kinetics was studied based on fresh and dry weight. The results for the shake flask cultures are shown in Figs. 1d and 2a. They show a lag phase of up to 5 days, exponential phase from days 5 to 15 and decline phase from days 15 to 25. A maximum biomass of 14.16 g l^{-1} DW was obtained at 15 days (Table 1).

Bioreactor experiments

Growth of *S. striata* cultures in the bioreactor were similar to that in shake flasks, except that the growth kinetics in the bioreactor required a longer exponential phase than in the shake flask (from day 5 to 19), making the decline phase from day 19 to 25. The time course of growth is shown in Figs. 1a–c, and 2b. The data showed that a maximum

Fig. 1 **a–c** Cell suspension culture of *S. striata* in: 10 l bioreactor; and **d** in 100 ml shake flask

biomass of 15.64 g l^{-1} DW was obtained at 19 days with an average growth rate of 68 (Table 2).

Cell growth in shake flask versus bioreactor

The experiment for production of cell biomass in scale-up from shake flask to bioreactor used the optimized parameters presented in the Materials and Methods section. The results showed that cell biomass was 15.64 g l^{-1} DW at 19 days in the bioreactor and 14.16 g l^{-1} DW at day 15 in shake flasks (Tables 1, 2). In the bioreactor vessel wall-growth and foaming of the medium was also conspicuous. The cells were viable until the end of culture period.

Phenylethanoid glycoside production

In shake flask

Acteoside and echinacoside accumulation in *S. striata* cells was investigated by HPLC. The peak for standard acteoside occurred at a retention time of 20.08 min and for echinacoside at a retention time of 7.05 min (Fig. 3). The maximum acteoside content was measured at 10 days (329.39 µg g^{-1} DW) and 25 days (459.71 µg g^{-1} DW) and maximum echinacoside content at 15 days (1973.03 µg g^{-1} DW) (Table 3).

In bioreactor

PeG production in the bioreactor is shown in Table 3. The production of acteoside decreased at 15 days and began

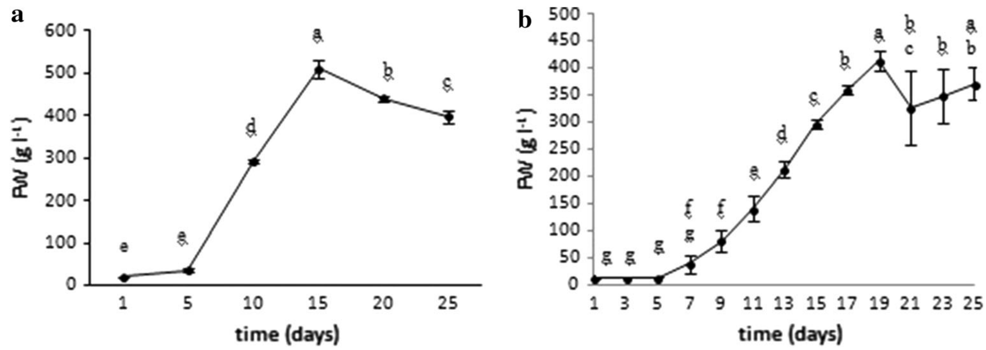

Fig. 2 Time-course of growth of *S. striata* cells cultured in: **a** shake flasks; and **b** bioreactor

Table 1 Growth of *S. striata* cells after 25 days of culture in shake flask

Days	Dry weight (g l^{-1})	Fresh weight (g l^{-1})	Growth rate (X_2 X_1^{-1})
1	4.00 ± 0.00^{f}	20.00 ± 0.00^{e}	–
5	8.33 ± 0.00^{e}	35.78 ± 4.16^{e}	2.07
10	13.16 ± 0.16^{b}	292.50 ± 3.83^{d}	3.29
15	14.16 ± 0.16^{a}	509.44 ± 22.51^{a}	3.54
20	11.50 ± 0.17^{c}	439.78 ± 7.34^{b}	2.87
25	10.31 ± 0.30^{d}	396.67 ± 16.04^{c}	2.58

Values are means of triplicate ± SD; *S. striata* cells were cultured in 50 ml MS medium held in 100 ml shake flasks for 25 days

Different letters indicate significant differences between the means ($p \leq 0.05$)

Table 2 Growth of *S. striata* cells after 25 days of culture in bioreactor

Days	Dry weight (g l^{-1})	Fresh weight (g l^{-1})	Growth rate (X_2 X_1^{-1})
1	0.23 ± 0.13^{g}	12.9. ± 3.01^{g}	–
3	0.37 ± 0.05^{fg}	12.88 ± 0.42^{g}	1.61
5	0.47 ± 0.19^{fg}	13.01 ± 0.25^{g}	2.04
7	1.79 ± 0.90^{f}	38.18 ± 16.94^{fg}	7.78
9	3.99 ± 0.87^{e}	80.32 ± 19.28^{f}	14.35
11	6.56 ± 0.92^{d}	139.42 ± 23.60^{e}	28.52
13	9.35 ± 0.75^{c}	212.00 ± 14.57^{d}	40.65
15	11.47 ± 1.28^{b}	297.08 ± 7.63^{c}	49.87
17	14.57 ± 0.84^{a}	360.83 ± 8.51^{b}	63.34
19	15.64 ± 1.68^{a}	412.80 ± 18.26^{a}	68.00
21	11.53 ± 0.02^{b}	394.20 ± 68.13^{bc}	50.13
23	11.45 ± 0.45^{b}	397.29 ± 49.17^{b}	49.78
25	11.18 ± 0.17^{b}	400 ± 30.21^{ab}	48.60

Values are means of triplicate ± SD; *S. striata* cells were cultured in 5.5 l MS medium held in bioreactor for 25 days

Different letters indicate significant differences between the means ($p \leq 0.05$)

again to increase at 25 days. Onset of echinacoside production began at about 15 days and proceeded until the end of the culture period. PeG content of cells cultured in the bioreactor showed a maximum acteoside content at 10 days (1404.20 μg g^{-1} DW) and maximum echinacoside at 25 days (1449.39 μg g^{-1} DW).

PeG production in shake flask versus bioreactor

PeG production in the shake flask and bioreactor are shown in Table 3. The acteoside content of suspension cells cultured in the bioreactor was about threefold higher than that in the shake flask. The echinacoside content of suspension

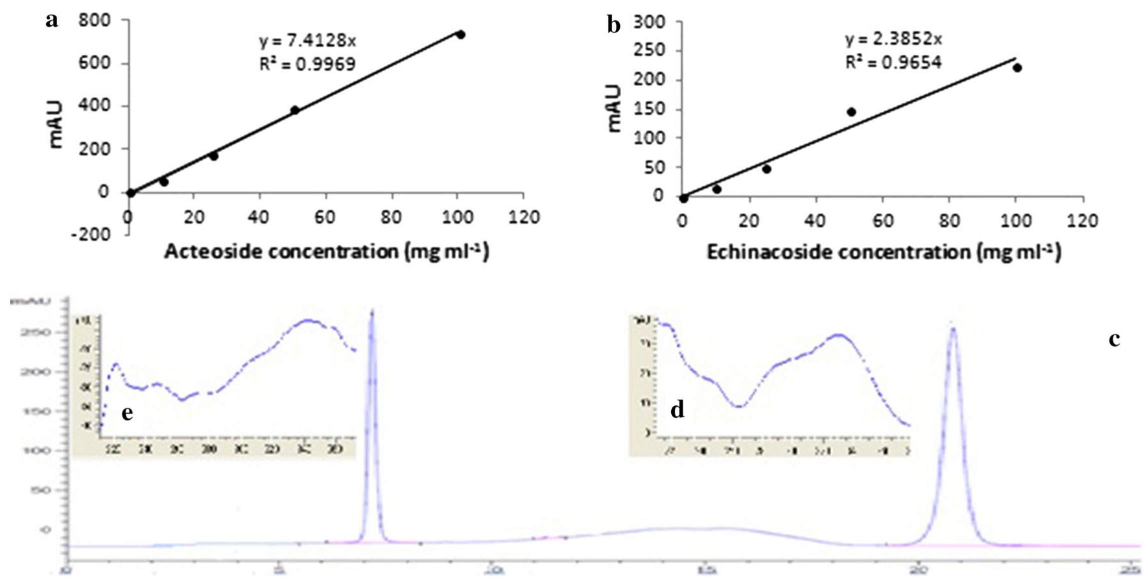

Fig. 3 Standard curves for: **a** acteoside; **b** echinacoside; **c** chromatogram of standards acteoside and echinacoside; **d** spectrum of acteoside; and **e** spectrum of echinacoside

cells in the shake flask was 1.36-fold higher than that in the bioreactor. It is evident that production of acteoside increased when the cell cultures were transferred from the shake flask to the bioreactor, but this trend was reversed for the echinacoside.

Discussion

A bioreactor is the final stage in commercial production of secondary metabolites from plant cell cultures. The present study transferred PeG production by plant cell culture of *S. striata* to a bioreactor. Cell biomass and PeG production were then compared with cells cultured in shake flasks. Georgiev et al. (2011) found that cells transported to the surface of a liquid/gas in a bioreactor caused glass wall growth and foam formation. The present study found that the air flow rate and aeration regimes affected cell growth, metabolism, and PeG production.

The optimal conditions were determined for cell growth and maximum production of acteoside during cultivation. Maximum cell biomass in the bioreactor was 15.64 g l^{-1} DW, which is similar to cell biomass in the shake flasks at 14.16 g l^{-1} DW. The results show that the biomass in the bioreactor peaked at 19 days and in the shake flasks at 15 days. The growth rate of cells in the bioreactor was higher than that in the shake flasks (68 vs. 3.54). Mathew and Jayachandran (2009) reported that low productivity in shake flask cultures could be the result of increased shearing stress that causes partial cell lysis. The high productivity in bioreactors is a result of low cell aggregation with no shear caused by lack of mixing.

The acteoside content in the bioreactor was 1404.20 μg g^{-1} DW, a threefold increase over that in the shake flasks at 459.71 μg g^{-1} DW. The echinacoside content in the bioreactor decreased, but in shake flasks increased (1973.03 μg g^{-1} DW). Estrada-Zuniga et al. (2009) demonstrated that acteoside production is associated with cell growth reaching the highest metabolite production when in the stationary stage.

Georgiev et al. (2011) demonstrated that the aeration regime and air flow rate affected cell growth and secondary metabolite production. Wang et al. (2010) showed that agitation speeds of 100 and 300 r min^{-1} increased cell growth. Terrier et al. (2007) and Sharma et al. (2009) reported that the cell growth profile of legume plant cell cultures were similar for the shake flask and bioreactor.

Ouyang et al. (2005b) transferred a cell culture of *Cistanche deserticola* from shake flasks to a bioreactor and observed that production of PeG decreased and that optimization of culture conditions in the bioreactor improved PeG production. They reported that a relatively high aeration rate for cell growth and PeG production was necessary and improved the supply of O_2 and transfer of nutrients. Chattopadhyay et al. (2002) reported that plant cell cultures of *Podophyllum hexandrum* recorded higher maximum biomass and podophyllotoxin production in shake flasks than in a bioreactor.

The present study determined the optimized conditions for cell suspension cultures of *S. striata* and maximum production of acteoside in a bioreactor. Bioproduction of PeG was then transferred from shake flask to bioreactor. This report provides a new and efficient method of production of PeG by *S. striata* cell culture. The results

Table 3 Comparison of phenylethanoid glycoside production in shake flasks and bioreactor

Product (μg g^{-1} DW)	Type	Days					
		1	5	10	15	20	25
Acteoside	Shake-flask	174.93 ± 11.7[dc]	149.43 ± 11.48[a]	329.39 ± 18.47[b]	286.44 ± 7.01[bc]	249.85 ± 44.09[c]	459.71 ± 71.26[a]
	Bioreactor	174.93 ± 11.79[c]	902.22 ± 2.69[b]	1404.20 ± 34.57[a]	nd	nd	30.68 ± 30.84[d]
Echinacoside	Shake-flask	150.63 ± 19.39[a]	nd	299.64 ± 64.48[d]	1973.03 ± 61.09[a]	1011.81 ± 89.54[b]	527.90 ± 16.67[c]
	Bio reactor	150.63 ± 19.39[a]	nd	nd	539.53 ± 4.59[c]	876.75 ± 1.09[b]	1449.39 ± 23.31[a]

Values are means of triplicate ± SD

nd not detected

Different letters indicate significant differences between the means ($p \leq 0.05$)

suggest that enhancement of PeG by elicitation and precursor feeding of a cell suspension culture of *S. striata* in a bioreactor can be targeted in the future.

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