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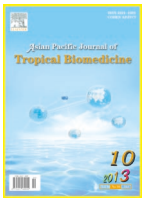

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Production of asiaticoside from centella (*Centella asiatica* L. Urban) cells in bioreactor

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PEER REVIEW

Peer reviewer

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Comments

This is a valuable research work in which authors have demonstrated the suitable conditions for growth of centella cells and their asiaticoside production in bioreactor. The optimization of condition was assessed by optimizing the cell suspension culture, agitation speed, aeration rate, inoculum size *etc*. Quantification of asiaticoside was done by HPLC.
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ABSTRACT

Objective: To investigate the effects of some culture conditions on production of asiaticoside from centella (*Centella asiatica* L. Urban) cells cultured in 5-L bioreactor.
Methods: The centell cell suspension culture was conducted in 5-L bioreactor to investigate the growth and asiaticoside accumulation under various conditions. Asiaticoside content was determined by HPLC analysis.
Results: The results showed that the cell growth and asiaticoside accumulation peaked after 24 d of culture at an agitation speed of 150 r/min and aeration rate of 2.5 L/min. The cell biomass reached a maximum value of 302.45 g fresh weight (31.45 g dry weight) and growth index of 3.03 with inoculum size of 100 g. However, asiaticoside content was the highest (60.08 mg/g dry weight) when culture was initiated with an inoculum size of 50 g.
Conclusions: The present study found the suitable conditions for growth of centella cells and their asiaticoside production in bioreactor.

KEYWORDS
Asiaticoside, Bioreactor, *Centella asiatica*, Cell suspension culture

1. Introduction

Centella (*Centella asiatica* L. Urban) is a small herbaceous annual plant (Apiaceae family) that is distributed in many parts of Asia such as India, Sri Lanka, Indonesia, Malaysia, and Vietnam[1]. Centella has been used for hundreds of years as a traditional medicine to improve wound healing in many Asian countries.

Centella contains many important secondary metabolites, especially asiaticoside and madecassoside belong to triterpenoid[2]. Asiaticoside derivatives can be regarded to be likely candidates for a therapeutic Alzheimer's disease drug because these derivatives have been shown to potentially protect cells against β-amyloid induced cell death[3]. Asiaticoside has also antidepressant activity[4] and increasing granulocyte production to repair wounds and burns[5,6].

There were many reports on asiaticoside and madecassoside production by centella cell suspension, callus, *in vitro* plant or hairy root cultures[7-10]. Some other reports investigated the effects of elicitors (methyl jasmonate, salicylic acid and yeast extract) on centelloside

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and asiaticoside biosynthesis of centella cells[11-14]. However, according to our knowledge no work carried out cell suspension culture of centella in bioreactor.

The objective of the present study was to identify the appropriate culture conditions for centella cells in 5-L bioreactor, the growth profile of the cells and their asiaticoside accumulation.

2. Materials and methods

2.1. Plant materials

In vitro petiole explants were cultured on Murashige and Skoog (MS) medium[15] supplemented with 20 g/L sucrose, 1 mg/L napthaleneacetic acid, 1 mg/L benzylaminopurine and 8 g/L agar for callus production. Friable calli in yellow color were used to establish cell suspension culture.

The medium was adjusted to pH 5.8 before sterilization at 121 °C for 15 min. *In vitro* culture was maintained at a temperature of (25±2) °C under an intensity of about 2 000-3 000 lux with a photoperiod of day light for 10 h.

2.2. Cell suspension culture

Cell suspension cultures were initiated through the agitation of 3 g of callus in 250 mL Erlenmayer flasks containing 50 mL of liquid MS medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid, 1 mg/L kintein and 30 g/L sucrose at a shaking speed of 120 r/min. After 24 d of culture, 3 g of cell biomass were transferred into the fresh medium has similar components and cultured in the same conditions until the obtained homogeneous cell suspension.

Thirty grams of cell biomass collected from shaking culture were transferred into a bioreactor (Biotron, Inc. Korea) containing the same medium with a 5 L working volume and three impellers maintained at an agitation rate of 120 r/min for 30 d. An aeration (2 L/min) was achieved using sterile gas from an air pump added through a flow meter and an air filter. Temperature of the culture maintained at 25 °C was performed by connecting a temperature sensor to the bioreactor. Cells were obtained under sterile conditions every two days during 18 d to determine the biomass in both fresh and dry weights, and to obtain their extracts (Figure 1).

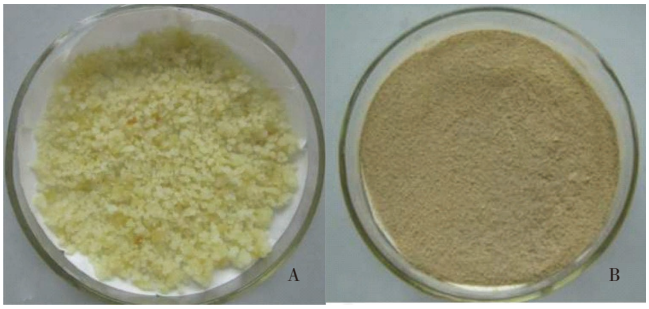

Figure 1. Cell biomass of *Centella asiatica*.
A: Fresh cell biomass, B: Fine powder of cells.

GI (growth index)=Final fresh cell weight/Initial inoculum fresh cell weight

To study effects of culture conditions, centella suspension cells were transferred into 14-L bioreactor in different conditions such as the inoculum size of 30-100 g, agitation speed of 120-200 r/min, and aeration rate of 2-3 L/min to investigate. The culture was incubated under the same conditions as for callus production, except at a light intensity of 500 lux.

2.3. Quantification of asiaticoside

Fresh cell biomass was dried at 50 °C to a constant weight, then ground into a fine powder. One gram of the sample was completely soaked in 10 mL of 90% ethanol for 48 h. The extract was then filtered and concentrated at 70 °C using a vacuum rotary concentrator (Heidolph, Germany). The concentrate was dissolved by 100% ethanol to 10 mL (asiaticoside extract), filtered through Minisart 0.25 μm membranes (Sartorius, Germany), and diluted 5-fold to subject HPLC (high performance liquid chromatography) at ambient temperature, using a LiChrospher 18e column (5 μm, 4 mm×250 mm). HPLC condition was as follows, flow rate: 1 mL/min, run time: 10 min, detector wavelength: 254 nm, stationary phase: silica gel (reverse phase) and mobile phase of ethanol: water (6:4 ratio). Twenty microliters of sample were injected the column using Hamilton syringe.

High performance liquid chromatography analysis was performed on a LC-20A prominence system (Shimadzu, Japan) with LC-20AD pump, SPD-20A UV-VIS detector, SIL-20A HT autosampler and the LC-Solution software (ver. 1.22). All solvents were of analytical grade and were purchased from Sigma and Merck & Co., Inc.

Asiaticoside solution (1 mg/mL in ethanol) from Sigma was used as a standard for determination of the asiaticoside concentration.

2.4. Statistical analysis

Each experiment was repeated three times to calculate the average and analyse one-way ANOVA by Duncan's test ($P<0.05$) using the SAS program (ver.6.12).

3. Results

3.1. Cell suspension culture

Centella cells were cultured in bioreactor with an inoculum size of 30 g, agitation speed of 120 r/min, and aeration rate of 2 L/min. The results on the cell growth after 30 d of culture were presented in Figure 2. The data showed the cell biomass peaked at Day 24 with 116.17 g fresh weight (12.24 g dry weight). The cell growth was then decreased and biomass was only 94.76 g fresh weight (9.01 g dry weight) at Day 30.

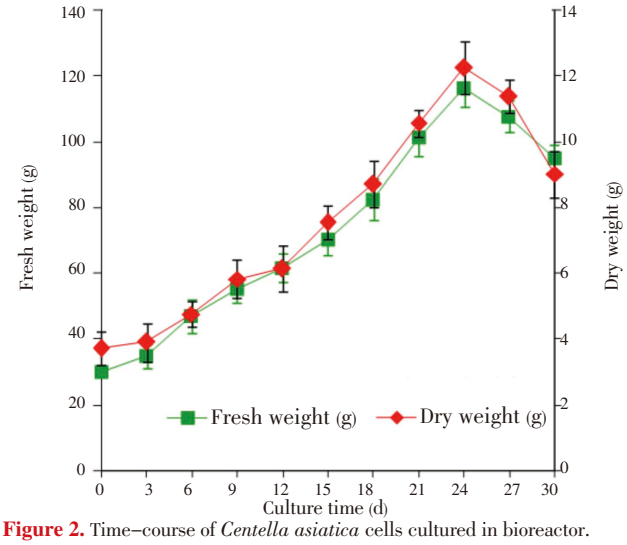

Figure 2. Time-course of *Centella asiatica* cells cultured in bioreactor.

The asiaticoside accumulation of centella cells cultured in bioreactor was also investigated by HPLC analysis based on the chromatogram of standard asiaticoside. Figure 3 indicates that the peak of standard asiaticoside has a retention time of 2.92 min. The HPLC chromatogram of asiaticoside extract from suspension cell has also a peak with equivalent retention time of 2.87 min, and another peak with shorter retention time of 2.58 min (Figure 4). The data in Figure 5 showed asiaticoside content of centella cells increased from Day 3 to Day 24 of culture with a maximum value of 56.21 mg/g dry weight and then it decreased from Day 27-30.

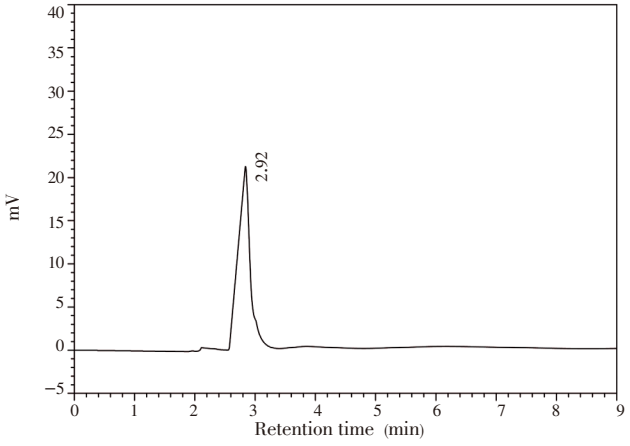

Figure 3. High performance liquid chromatography chromatogram of standard asiaticoside (1 mg/mL).

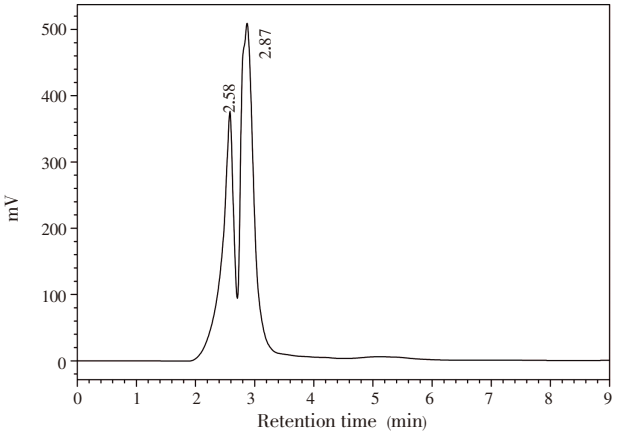

Figure 4. High performance liquid chromatography chromatogram of asiaticoside extracted from 24 day-old centella cells in bioreactor.

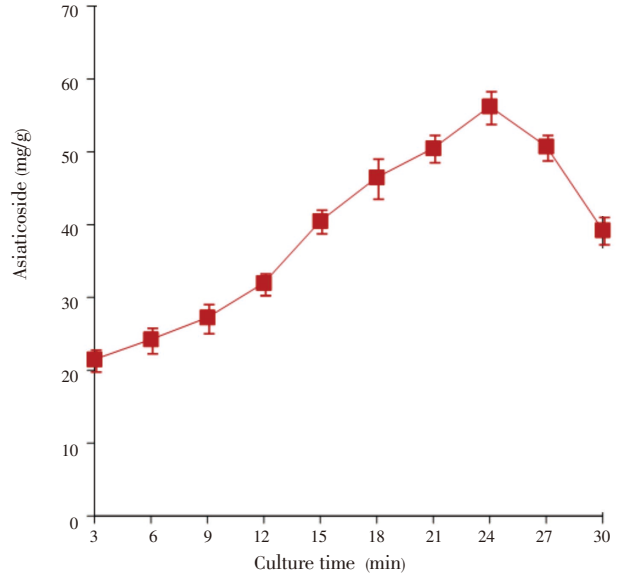

Figure 5. Dynamics of asiaticoside accumulation in a *Centella asiatica* cell culture.

In our previous study, the centella cell biomass reached a maximum value of 9.03 g/50 mL of shaking flask culture with inoculum size of 3 g after 24 d at an agitation speed of 120 r/min. HPLC analysis showed that asiaticoside content in 24-day old suspension cultured cells and *in planta* centella plants were 45.35 mg/g and 10.55 mg/g dry weight, respectively[9].

3.2. Effect of agitation speed

Based on the results of cell growth in bioreactor, we investigated the effects of some culture conditions on the biomass and asiaticoside accumulation after 24 d of culture. The effect of agitation speeds (120-200 r/min) on the growth of centella cells were shown in Table 1. Cells biomass reached a maximum value of 128.09 g fresh weight (13.35 g dry weight) with growth index of 4.27 after 24 d of culture at 150 r/min. The growth was then reduced with increasing agitation speed up to 170-200 r/min. The growth and proliferation of the cells in suspension culture was found to depend on agitation to prevent biomass sedimentation. However, if the agitation speed was too strong, this might breakdown the cell walls[16]. In another study, we investigated the effect of agitation speeds (80-150 r/min) on the growth of centella cells in shaking flask culture. The results showed the growth index reached a maximum value of of 4.29 after 24 d of culture at 120 r/min[9].

Table 1
Effect of agitation on the growth of centella cells after 24 d of culture.

Agitation (r/min)	Fresh cell weight (g)	Dry cell weight (g)	Growth index	Asiaticoside (mg/g)
120	116.17^{bc}	12.24^{ab}	3.87	56.21
150	128.09^{a}	13.35^{a}	4.27	59.43
170	121.52^{b}	13.17^{a}	4.02	54.63
200	107.26^{c}	10.88^{b}	3.58	49.44

Different letters in a column indicate significantly different means (Duncan's test, $P<0.05$).

We have also determined asiaticoside content after 24 d of culture at different agitation speeds from 120–200 r/min. The results showed that at agitation speeds of 170–200 r/min, asiaticoside content was low, while asiaticoside content reached a highest value of 59.43 mg/g dry weight at 150 r/min (Table 1). From these results, an agitation speed of 150 r/min was used for the further experiments.

3.3. Effect of aeration rate

The culture was carried out with inoculum size of 30 g and agitation speed of 150 r/min for 24 d to test the effects of different aeration rates (2–3 L/min) on the cell growth and asiaticoside accumulation. The data in Table 2 indicated that the aeration rate of 2.5 L/min is the most suitable for the biomass production. The cell growth reached a maximum value of 143.36 g fresh weight (15.39 g dry weight) with growth index of 4.78. The results in Table 2 also showed that asiaticoside content peaked at aeration rate of 2.5 L/min (62.14 mg/g dry weight). In lower and higher aeration rates, the cell growth and asiaticoside content were all distinctly low.

Table 2
Effect of aeration on the growth of centella cells after 24 d of culture.

Aeration (L/min)	Fresh cell weight (g)	Dry cell weight (g)	Growth index	Asiaticoside (mg/g)
2.0	128.09^{b}	13.35^{ab}	4.27	59.43
2.5	143.36^{a}	15.39^{a}	4.78	62.14
3.0	141.22^{ab}	15.01^{a}	4.71	60.02

These results suggested that selection of the aeration rate is necessary to attain high production of asiaticoside. Therefore, an agitation speed of 150 r/min and aeration rate of 2.5 L/min were used for next experiment.

3.4. Effect of inoculum size

Inoculum size is one of the important factors that may affect the accumulation of biomass and metabolic products. The effect of the inoculum size on cell growth was investigated by using between 30 and 100 g from the shaking flask culture. The data in table 3 indicated an inoculum size of 100 g resulted in a maximum fresh weight of 302.45 g (31.45 g dry weight) after 24 d of culture more than that obtained using the other inoculum sizes. However, the cell growth index of inoculum is lower than that other inoculums.

Table 3
Effect of inoculum on the growth of centella cells after 24 d of culture.

Inoculum (g)	Fresh cell weight (g)	Dry cell weight (g)	Growth index	Asiaticoside (mg/g)
30	143.36^{c}	15.39^{b}	4.78	62.14
50	170.81^{b}	16.26^{b}	3.42	62.33
100	302.45^{a}	31.45^{a}	3.03	60.08

In our earlier report[9], the effect of inoculum size (1–4 g) on centella cell biomass production was investigated in shaking flask culture. The results showed that 3 g of cells resulted in a maximum fresh weight of 9.03 g (1.34 g dry weight) with growth index of 4.52 after 24 d of culture.

In present work, asiaticoside content is 62.14–62.33 mg/g dry weight with about 1.4-fold higher than that of shaking flask culture (45.35 mg/g dry weight)[9]. Thus, the inoculum size was observed to have a significant influence on the growth of cell suspension culture and their asiaticoside accumulation.

4. Discussion

Many studies have indicated that plant cell suspension culture can produce secondary compounds with content higher than that of plant grown under *in vivo* conditions. For example, Villarreal *et al.* obtained spirostanol saponin of *Solanum chrysotrichum* cells with content of about 14 mg/g, 50-fold higher than that of *in planta* leaves[17]. Cell suspension culture of *Coleus blumei* produced 13%–15% (dry weight) rosmarinic acid after 13 d of culture, 5-fold higher than that of plant grown under *in vivo* condition. Ginsenoside content from cell culture of *Panax ginseng* reached 27% dry weight *vs* 4.5% of *in vivo* growing plant, anthraquinone from cell culture of *Morinda citrafolia* is 18% *vs* 2.2%, and shikonin from cell culture of *Lithospermum erythrorhizon* is 12% *vs* 1.5%[18].

Choi *et al.* determined that the cell biomass of oil palm plant increased to 200% at agitation speeds of 120 and 225 r/min, while at agitation speed of 335 r/min, the biomass increased only 7%[19]. Wang *et al.* found cell biomass of *Glycyrrhiza inflata* reached a highest growth at an agitation speed of 150 r/min. At agitation speeds of 100 and 300 r/min cell growth increased[20].

Lee *et al.* showed that different inoculum sizes (20–120 g) had a significant influence on the growth profile of *Gymnema sylvestre* cells[21]. The growth rate increased significantly with the initial inoculum was 60 g.

In conclusion, the present study found the suitable conditions for growth of centella cells and their asiaticoside production in bioreactor.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This research was supported by the National Foundation for Science and Technology Development (NAFOSTED) of Vietnam (Grant No. 106.16–2012.80).

Comments

Background

The present research is an effort to identify the appropriate culture conditions for centella cells in 5-L bioreactor, the growth profile of the cells and their asiaticoside accumulation. Effects of some culture conditions on production of asiaticoside from centella (*Centella asiatica*

L. Urban) cells cultured in 5-L bioreactor was investigated. Asiaticoside content was determined by HPLC analysis.

Research frontiers

Study are being performed in order to determine cell suspension culture of centella in bioreactor and to investigate the growth and asiaticoside accumulation under various conditions. The centell cell suspension culture was conducted in 5-L bioreactor. Asiaticoside content was determined by HPLC analysis.

Related reports

Centella contains many important secondary metabolites, especially asiaticoside and madecassoside which can be used in Alzheimer's disease and have various pharmacological acivities like antidepressant activity, wound healing activity *ect*. Some other reports investigated on the asiaticoside and madecassoside production by centella cell suspension, callus, *in vitro* plant or hairy root cultures effects of elicitors (methyl jasmonate, salicylic acid and yeast extract) on centelloside and asiaticoside biosynthesis of centella cells. No work carried out cell suspension culture of centella in bioreactor.

Innovations and breakthroughs

The present study found the suitable conditions for growth of centella cells and their asiaticoside production in bioreactor. The asiaticoside accumulation of centella cells cultured in bioreactor was investigated by HPLC analysis based on the chromatogram of standard asiaticoside.

Applications

Centella asiatica L. Urban has been used for hundreds of years as a traditional medicine. Centella contains many important secondary metabolites, especially asiaticoside and madecassoside belong to triterpenoid. The present study found the suitable conditions for growth of centella cells and their asiaticoside production in bioreactor.

Peer review

This is a valuable research work in which authors have demonstrated the suitable conditions for growth of centella cells and their asiaticoside production in bioreactor. The optimization of condition was assessed by optimizing the cell suspension culture, agitation speed, aeration rate, inoculum size *etc*. Quantification of asiaticoside was done by HPLC.

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