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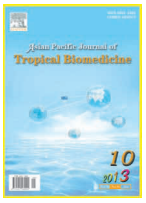

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Production of asiaticoside from centella (*Centella asiatica* L. Urban) cells in bioreactor

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PEER REVIEW

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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).

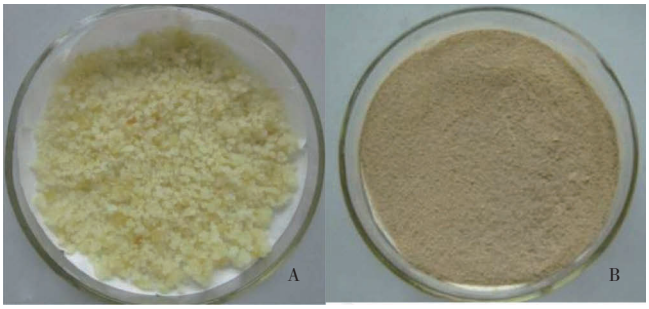

Figure 1. Cell biomass of *Centella asiatica*.
A: Fresh cell biomass, B: Fine powder of cells.

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.