

Effect of Some Factors on the Growth of *Capsicum annuum* L. Cell Suspension Culture and Biotransformation of Hydroquinone to Arbutin

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ABSTRACT

*This study investigates the effect of the type of saltbase and sucrose concentration on the growth of *Capsicum annuum* L. (Solanaceae) cell-suspension culture and the influence of the concentration and method of addition of hydroquinone as well as the time of exposure on the biotransformation of hydroquinone to arbutin. The optimum medium for cell growth is a full-strength Murashige & Skoog liquid media supplemented with 2% (w/v) sucrose, 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.44 μM benzylaminopurine (BAP), pH 6.0 and aerated on a gyratory shaker in a 24-h dark condition. Cell growth reaches a maximum of 4.5 times of the original cell volume between day 17 and 21 of the culture. The highest biotransformation is achieved when 15.57 mM hydroquinone is added into four equally-divided portion of 3.89 mM on each of the four days and the samples are harvested on day 5 after the first hydroquinone addition. HPLC analysis of these samples yields 555.60 $\pm$ 19.31 mg/L of arbutin.*

Key words: *Capsicum annuum*, Arbutin, Hydroquinone, Plant tissue culture, Biotransformation, High-performance liquid chromatography (HPLC)

INTRODUCTION

The use of plant cell cultures as a source of enzymes to biotransform exogenously-supplied chemicals into desired products of higher value has been widely studied in recent years (Yokoyama, 1996; Rao and Ravishankar, 2002). Examples of reactions that were reportedly performed by plant cells including oxidation, reduction, acetylation, esterification, hydroxylation, methylation, isomerization and glycosylation (Giri et al., 2001). These processes are particularly useful for compounds of which the structures are complex or the positions to be modified are very specific and could not be satisfactorily achieved by chemical synthesis or semisynthesis. At present, a number of natural flavors and fragrances as well as phytochemicals used in cosmetics, are conveniently manufactured through biotransformation of plant cell cultures (Kim, 2005; Kondo et al., 2006)

Arbutin (hydroquinone- β -D-glucopyranoside) is a phenolic glucoside found naturally as a secondary metabolite in many plants such as bearberry (*Arctostaphylos uva-ursi*), cranberry (*Vaccinium macrocarpon*), blueberry (*Vaccinium corymbosum*), bergenia (*Bergenia crassifolia*) and pears (*Pyrus communis*). Arbutin possesses an ability to inhibit tyrosinase enzyme and has been widely employed as a whitening agent in many cosmetic preparations. Compared to its aglycone counterpart and biosynthetic precursor, hydroquinone (Figure 1), arbutin offers more or equally-effective whitening activity with much less toxicity and side effects. Because arbutin is also much more expensive than hydroquinone, alternative routes have been investigated for the production of arbutin. These include chemical synthesis (Huang et al., 2004) and biotransformation, using plant cell cultures. Although the production of arbutin through biotransformation of hydroquinone has been successful and documented since 1976 (Tabata et al., 1976; Inomata et al., 1991), the search for new, high level arbutin-producing plant-based systems remains a widely-studied topic as indicated by a number of reports on hydroquinone-arbutin biotransformation in recent years (Casas et al., 1998; Duskova et al., 1999; Zhao et al., 2001; Skrzypczak-Pietraszek et al., 2005). In addition to the plant species, several factors were reported to play important roles in the production of arbutin by the plant cell biotransformation system. Examples included the types of plant growth regulators (Duskova et al., 2001), the difference in cell morphology or cell lines (Inomata et al., 1994), the culture stage of the cultivated cells and the manner of hydroquinone addition (Inomata et al., 1991), the density of the cells in the culture (Suzuki et al., 1987), chemical structure of the precursors (Mizukami et al., 1987; Duskova et al., 1999) and the addition of antioxidant (Yokoyama, 1991; Casas et al., 1998).

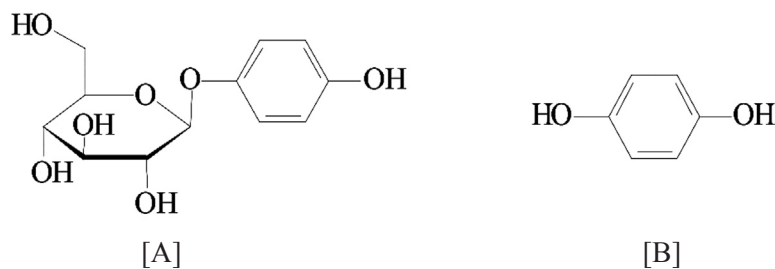


Figure 1. Chemical structures of arbutin [A] and hydroquinone [B].

Our group has recently reported the ability of cell-suspension cultures of *Capsicum annuum* L. (Solanaceae), *Datura fastuosa* L. (Solanaceae), and *Solanum aculeatissimum* Jacque. (Solanaceae) to biotransform hydroquinone into arbutin (Kittipongpatana et al., 2006). Of the three plants studied, cell-suspension cultures of *C. annuum* exhibited the highest potential for further development as calluses could be easily initiated, the growth was rapid and the texture or morphology was suitable for establishment as cell-suspension culture. *C. annuum* cell culture yielded the highest arbutin content in the biotransformation experiment. Most of the arbutin produced was released into the culture medium, thus allowing for simple retrieval

of the compound for purification and analysis.

This study aims to optimize the growth of *C. annuum* cell cultures and to study the arbutin production by varying the medium composition, hydroquinone concentration, method of addition and time of exposure.

MATERIALS AND METHODS

Materials

Chemicals

Arbutin standard (>98%), Murashige and Skoog (M&S) saltbase (Murashige and Skoog, 1962) and Gamborg B-5 (GB5) saltbase (Gamborg et al., 1968) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydroquinone was supplied by Fischer Chemicals (USA). Other medium components and plant growth regulators (PGRs) were of tissue culture grade or equivalent. All solvents used for chromatographic purposes were of HPLC grade. Other solvents were of reagent grade or equivalent.

Plant Materials

The seeds of *Capsicum annuum* L. were obtained from a commercial source. A voucher specimen has been deposited in the herbarium of the Faculty of Pharmacy, Chiang Mai University. The seeds were surface-sterilized with 20% (v/v) sodium hypochlorite (Clorox®) solution with Tween® 80 as wetting agent for 15 to 20 min. After rinsing with sterile distilled water, seeds were germinated on hormone-free medium, containing one-quarter strength M&S salt base and 2% (w/v) sucrose. The seedlings were grown in sterile vessels at 25°C with a 16/8-h light/dark cycle (light intensity 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PPFD, 100% cool-white fluorescent).

Methods

Initiation of callus and establishment of cell- suspension culture

Sterile leaf or stem explants were excised and placed in test tubes containing M&S medium (2% w/v sucrose), supplemented with 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D). These test tubes were maintained at 25°C with a 16/8-hour light/dark cycle (light intensity 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$). After two weeks, calluses formed on the explants were transferred onto a growth-promoting medium, in which the appropriate PGRs were previously determined to be a combination of 4.52 μM 2,4-D and 0.44 μM benzylaminopurine (BAP) (Kittipongpatana et al., 2006). Calluses were subcultured onto fresh medium every three weeks. Cell-suspension cultures were established by inoculating friable calluses into a 1-L Erlenmyer flask containing M&S liquid medium and the PGR combination. The flask was placed on a shaker, gyrating at 120 rpm, for three days to yield homogenous cells. These cells were regularly subcultured every 17 days and maintained in a 500 mL Erlenmyer flask containing 100 mL of M&S liquid medium and the PGR combination. For the experiments, each of a 200 mL culture bottle containing 35 mL of M&S liquid medium and the PGR combination was inoculated with 1 g FW of homogenous, newly-subcultured

cells.

Effect of medium composition and culture condition on the growth of cell-suspension culture

The type of saltbases (M&S vs. GB5), the concentration of sucrose (2, 5, 8 and 10% w/v), the pH of the media (4.7, 5.2, 6.0 and 7.0) and the presence or absence of light during culture period (16/8-h light/dark vs. 24 hours dark) were investigated. These factors were individually and sequentially tested while keeping other components constant to obtain a growth-promoting medium for *C. annuum* cell-suspension cultures. Each test was done in triplicate. The growth of the cultures was determined, using settled cell volume (SCV) technique, on day 3 and day 7 of the week for 24 days. A growth curve was established for each experiment by plotting SCV vs. day of growth.

Effect of HQ concentration, method of addition and time of exposure on the production of arbutin

All *C. annuum* cell-suspension cultures used in the following experiments were grown in a growth-promoting medium for 14 days before use. The aseptic addition of hydroquinone into the culture bottles was achieved by filtration through a 0.2 μm membrane filter. Each experiment was done in triplicate.

- *Hydroquinone concentration*

Three concentrations of hydroquinone were tested. One mL of 15, 30 and 60 mg/mL was added into the cultures which was equivalent to 3.89, 7.78 and 15.57 mM, respectively.

- *Method of hydroquinone addition*

Hydroquinone was added into the cultures as a single portion on day 1, two equally-divided portions on day 1 and 2 or four equally-divided portions on day 1, 2, 3 and 4.

For each experiment, the cell-suspension samples were harvested on day 2, 5 and 7 after the addition of hydroquinone, except for the experiment on method of hydroquinone addition in which the samples were collected on day 5 and 7 after the first addition of hydroquinone.

HPLC Analysis of Arbutin

The harvested cells and the culture media were separated by filtering through a paper filter. The liquid media were then filtered through a 0.45 μm syringe filter and were placed in HPLC sample vials for analysis. HPLC was performed using a method described by Kittipongpatana et al., (2006). In brief, samples were separated on a Hewlett Packard HP 1100 LC system equipped with an Apollo C-18 (4.6 x 150 mm, 5 μm particle diameter, 100 Å average pore size) column. The mobile phase was methanol: water (90:10). The flow rate was 0.9 mL/min and the detection for both arbutin and hydroquinone was done by UV detection at 280 nm.

RESULTS AND DISCUSSION

Initiation of callus and establishment of cell-suspension culture

Light brown, friable calluses appeared from the explants after 7-10 days on the initiation medium. The transfer of the callus onto a fresh medium which contained a growth-promoting combination of PGRs resulted in a rapid growth of callus cultures. By the end of the third week, a subculture was required to prevent the calluses from turning dark brown and stop growing. The pooling of calluses from several bottles into a 1-L flask containing liquid M&S medium and gyrating on a shaker at 120 rpm for a few days was found to be an effective way to establish homogenous cell suspension for the experiments.

Effect of medium composition and culture condition on the growth of cell-suspension culture

• *The type of saltbases*

C. annuum cells grown on the M&S-based medium showed significantly higher growth than those grown on the GB5-based medium during a 24-day growth determination by SCV technique (Figure 2). The highest growth was reached on day 21 when the SCV was 23.75 ± 5.42 mL and the viscosity of the culture increased significantly because of increased cell population. The cells in the GB5-based medium showed very little growth during the study period.

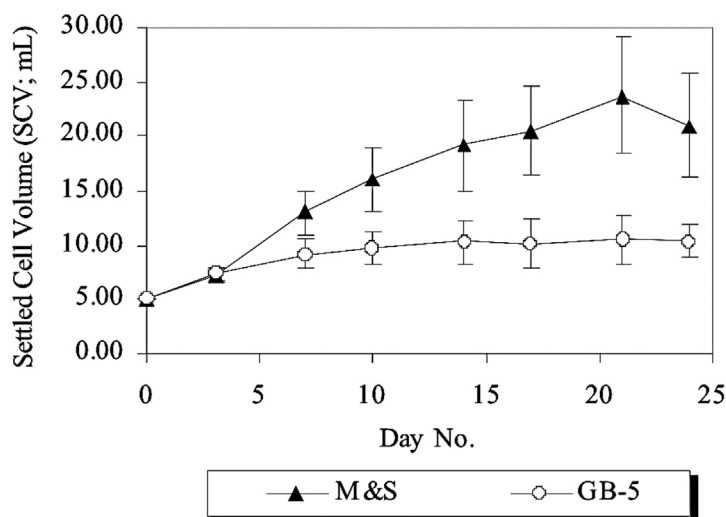


Figure 2. Time-course growth study of cell suspensions of *C. annuum* cultured in M&S or GB5 liquid media containing 2% sucrose, 4.52 μ M 2,4-D, 0.44 μ M BAP (n=5, error bars = $\pm$ SD).

• *The concentration of sugar*

The media that contained sucrose at 2% w/v (5.55 mM) and 5% w/v (13.88 mM) promoted higher growth than did the 8% w/v and 10% w/v concentrations. The

growth of cell cultures in the media with 2% and 5% sucrose were not significantly different (Figure 3), which indicated that a low concentration of sucrose, as low as 2% w/v, was adequate for supporting cell growth. The lower growth observed at higher sugar concentrations could be due to the higher osmotic pressure of the media that caused the dehydration of the cells and interfered with cell growth.

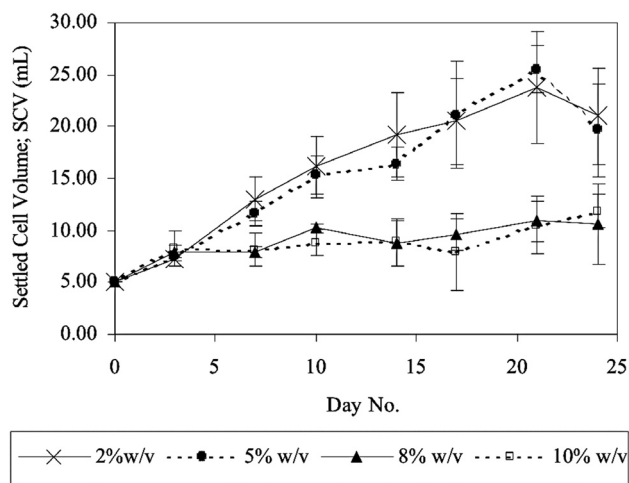


Figure 3. Time-course growth study of cell suspensions of *C. annuum* cultured in M&S liquid media containing 2, 5, 8 or 10% sucrose, 4.52 μ M 2,4-D, 0.44 μ M BAP ($n=5$, error bars = $\pm$ SD).

• *The pH of the media*

The variation in the pH of the culture media in the range between 4.7-7.0 did not affect the growth of the cells during the first two weeks. However, the growth between day 17 to day 24 of the cells cultured in the media pH 6.0 was significantly higher than those grown in the media pH 4.7 and 7.0 (Figure 4). The media with pH less than 4.7 and greater than 7.0 did not support the growth of *C. annuum* cells (data not shown).

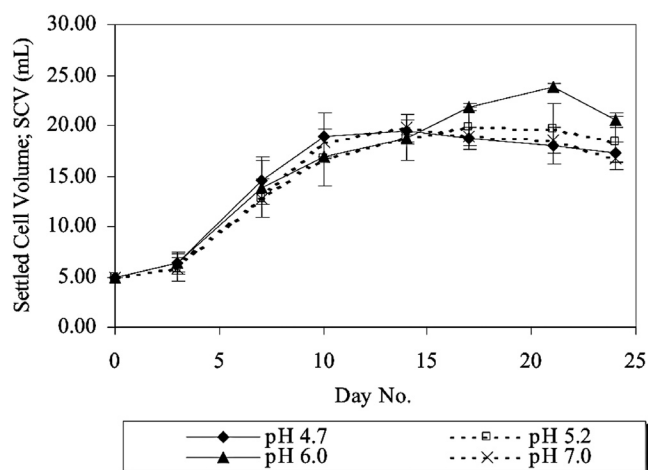


Figure 4. Time-course growth study of cell suspensions of *C. annuum* cultured in M&S liquid media containing 2% sucrose, 4.52 μM 2,4-D, 0.44 μM BAP, and adjusted pH to 4.7, 5.2, 6.0 or 7.0 ($n=5$, error bars = $\pm\text{SD}$).

• *The presence or absence of light during culture period*

In the absence of light, the cell cultures showed significantly higher growth, after 7 days, than those grown under the 16-h of light (300 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PPFD, 100% cool-white fluorescent) (Figure 5). However, the appearance of the cell cultures (size of cells, color, turbidity) was not different.

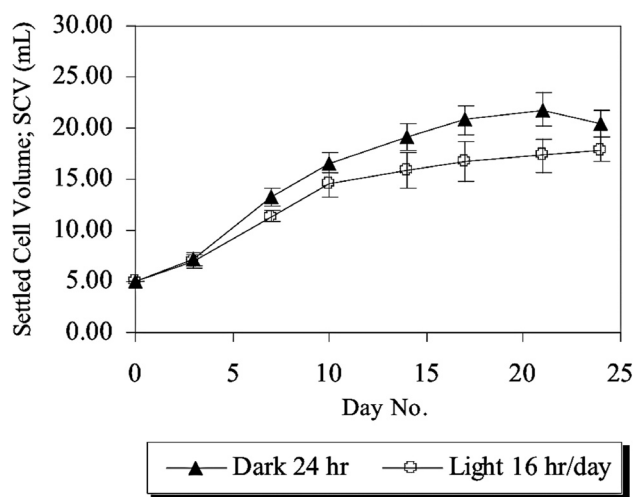


Figure 5. Time-course growth study of cell suspensions of *C. annuum* cultured in M&S liquid media containing 2% sucrose, 4.52 μM 2,4-D, 0.44 μM BAP, pH 6.0, and maintained in the dark (24 h) or light/dark (16/8 h) ($n=5$, error bars = $\pm\text{SD}$).

Overall, the compositions of liquid medium that effectively promoted the growth of *C. annuum* cell suspension cultures in this study were M&S saltbase supplemented with 2% w/v sucrose, 4.52 μM 2,4-D, 0.44 μM BAP, pH 6.0, and cultured on shaker in the absence of light. The stationary phase of cell growth in this medium was reached after 21 days, probably due to the aeration limitation caused by the increase in the culture viscosity and in the cell population. The highest SCV of cell suspension cultures in this medium was approximately 4.5 times of the initial SCV, after which a subculture was required. This medium was used in the biotransformation experiments.

Effect of HQ concentration, method of addition and time of exposure on the production of arbutin

• *Hydroquinone concentration*

The samples collected on day 5 after the addition of 15.57 mM HQ yielded the highest arbutin amount at 287.41 ± 29.14 mg/L. This amount was significantly higher than those added with 7.78 and 3.89 mM HQ, in which the arbutin yields were 186.08 ± 11.30 and 70.55 ± 9.98 mg/L, respectively. For the samples collected on day 7 after HQ addition, the highest arbutin content was from the 7.78 mM HQ group (126.47 ± 16.10 mg/L), while the samples added with 3.89 and 15.57 mM produced 91.51 ± 13.52 and 51.53 ± 4.28 mg/L, respectively (Figure 6). The results suggested that while *C. annuum* cell-suspension culture system required a relatively high concentration of substrate HQ to “push” the reaction forward to produce arbutin, the exposure time between the cells and the added hydroquinone was also important. The optimum time of exposure for maximum arbutin production was found to be between 2 and 5 days, in which the difference in arbutin production was not significant. The gradual decrease of the pH of the media due to the presence of excess hydroquinone and its light-induced degradation product, quinone, could play a crucial role in the hydrolysis of the produced arbutin which resulted in the observed decrease in the arbutin contents after day 5.

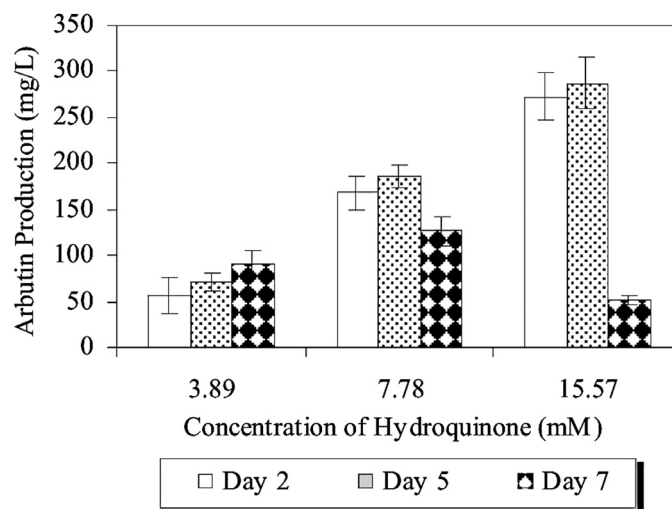


Figure 6. Arbutin production (mg/L) in *C. annuum* cell suspension cultures added with different concentrations of hydroquinone and harvested on different days after hydroquinone addition ($n=5$, error bars = $\pm$ SD). Arbutin analysis was carried out using HPLC.

• *Method of hydroquinone addition*

The concentration of 15.57 mM hydroquinone was selected from the previous experiment and was added into the cultures as a single portion (15.57 mM*1) on day 1, two equally-divided portions (7.78 mM*2) on day 1 and 2, or four equally-divided portions (3.89 mM*4) on day 1, 2, 3 and 4. The results showed that the addition of a concentration of hydroquinone in divided multiple portions clearly improved the production of arbutin over a single portion addition (Figure 7). The addition of 3.89 mM HQ per day for 4 days yielded the highest amount of arbutin at 555.60 ± 19.31 mg/L when harvested the media on day 5 after the first HQ addition. This amount, which is equivalent to 111.12 mg/L/day was significantly higher than those from the cultures which were added with 7.78 mM per day for 2 days and a single day with 15.57 mM, in which the arbutin amounts produced over 5 days were 357.88 ± 63.15 and 287.41 ± 29.14 mg/L, respectively. The arbutin production of the cultures harvested on day 7 followed the same trend ($3.89*4 > 7.78*2 > 15.57*1$) but the amounts were lower than those harvested on day 5. Since hydroquinone is known to be cytotoxic (Terasaka et al., 2005), the addition of a high concentration in a single portion is more likely to cause sudden cell death, which resulted in a low effectiveness of biotransformation. Similar to the strategy employed by Inomata et al., (1991), the addition of hydroquinone in divided, multiple portions caused less cell death per each addition, allowing viable cells to perform biotransformation more effectively and produced more arbutin.

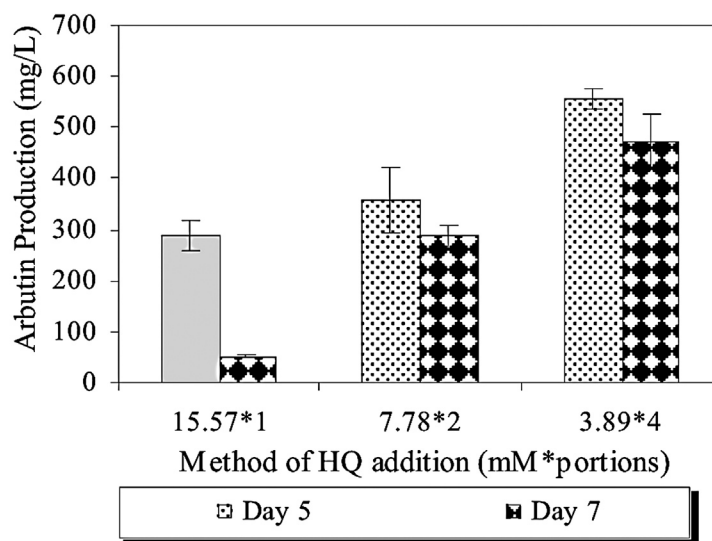


Figure 7. Arbutin production (mg/L) in *C. annuum* cell suspension cultures added with 15.57 mM hydroquinone by different methods and harvested on day 5 or 7 after the first hydroquinone addition ($n=5$, error bars = $\pm$ SD).

CONCLUSIONS

A new arbutin-producing biotransformation system was developed based on the cell-suspension culture of *C. annuum*, using hydroquinone as a precursor. The type of the saltbase, concentration of sucrose and pH of the medium played significant roles in the growth of the cells while the biotransformation ability was affected by the concentration of hydroquinone, time of cell-hydroquinone exposure and method of hydroquinone addition. The optimized compositions and condition for promoting the growth were Murashige & Skoog (M&S) liquid media (1X) supplemented with 2% (w/v) sucrose, 4.52 μ M 2,4-dichlorophenoxyacetic acid (2,4-D), 0.44 μ M benzylaminopurine (BAP), pH 6.0 and aerated on a gyratory shaker in a 24-h dark condition. A maximum amount of arbutin was obtained when 15.57 mM hydroquinone was added as 3.89 mM/day on each of the four days and the samples were harvested on day 5 after the first hydroquinone addition. Because of the relatively rapid growth and strong glycosylating activity, *C. annuum* cell-suspension cultures should be further studied to develop an arbutin production system.

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