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Production of adventitious root biomass and secondary metabolites of *Hypericum perforatum* L. in a balloon type airlift reactor

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ABSTRACT

The effects of inoculum density, aeration volume and culture period on accumulation of biomass and secondary metabolites in adventitious roots of *Hypericum perforatum* in balloon type airlift bioreactors (3 l capacity) were investigated. The greatest increment of biomass as well as metabolite content occurred at an inoculum density of 3 g l^{-1} and an aeration volume of 0.1 vvm. After 6 weeks of culture, an approximately 50-fold increase in biomass was recorded containing 60.11 mg g^{-1} dry weight (DW) of phenolics, 42.7 mg g^{-1} DW of flavonoids and 0.80 mg g^{-1} DW of chlorogenic acid. Liquid chromatography–mass spectroscopy/mass spectroscopy demonstrated that the presence of quercetin and hyperoside in adventitious roots at a level of 1.33 and 14.01 μg g^{-1} DW, respectively after 6 weeks of culture. The results suggest scale-up of adventitious root culture of *H. perforatum* for the production of chlorogenic acid, quercetin and hyperoside is feasible.

1. Introduction

Hypericum perforatum L. (St. John's wort) is an important traditional medicinal plant native to Europe that is grown worldwide for commercial purposes. It has gained international popularity mainly for the treatment of depression and as a dietary supplement (Miller, 1998). *H. perforatum* extracts contain secondary metabolites with documented biological activity including phenolics, a broad range of flavonoids, naphthodianthrones and phloroglucinols (Barnes et al., 2001). Among the flavonoids, hyperoside and quercetin showed antidepressant, antiphlogistic and antioxidant properties (Linde, 2009). In addition, chlorogenic acid from this plant was an effective peroxyl radical-scavenger (Silva et al., 2008). The efficacy of medical preparations of *H. perforatum* is based on the whole mixture of metabolites, rather than the presence of single constituent. However, the quality of these phyto-chemicals in field-grown plants may be affected by physical and chemical factors, as well as biological processes and environmental factors (Sirvent and Gibson, 2002; Mosaleeyanon et al., 2005; Zobayed et al., 2005; Couceiro et al., 2006).

Cell or organ cultures have emerged as a valuable route for biosynthesizing phytochemicals, and bioreactor-based systems have been developed for the production of ginsenosides (Yu et al., 2000; Kim et al., 2004; Thanh et al., 2005; Jeong et al., 2008), eleutherosides (Park et al., 2005; Shohael et al., 2005), phenolics (Wu et al., 2007) and alkaloids (Min et al., 2007). It is crucial to optimize parameters in cultures since the production of biomass and secondary metabolites can be greatly affected by factors such as, inoculum density, medium components, oxygen transfer and mixing, and other physico-chemical factors (Kim et al., 2002; Jeong et al., 2009a,b). Especially, in bioreactor cultures, aeration volume and inoculum density are key factors to maximize biomass and secondary metabolites but no studies have been conducted to optimize these factors in *H. perforatum* cultures. In this study, the effects of inoculum density, aeration volume and culture period on the accumulation of biomass and metabolites in bioreactor cultures of *H. perforatum* adventitious roots were determined and the antioxidant response of cultured roots in bioreactor liquid cultures was estimated.

2. Methods

2.1. Plant material and maintenance of adventitious root cultures

Adventitious roots of *H. perforatum* were induced and maintained according to the procedures described by Cui et al. (2010a,b).

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2.2. Optimization of the inoculum density

All the experiments were carried out in balloon type airlift bioreactors of 3 l capacity (Fig. 1). To determine the optimal inoculum density, different levels of the inoculants (1.5, 3.0, 6.0, 9.0, and 12.0 g l^{-1} FW) were added to separate bioreactors with 2 l of half strength modified Murashige and Skoog (1962) medium with an ammonium to nitrate ratio of 5:25 mM supplemented with 1.0 mg l^{-1} IBA, 0.1 mg l^{-1} kinetin, 3% (w/v) of sucrose. Bioreactors were aerated with filtered air at 0.1 vvm and maintained in the dark at 22 ± 1 °C for 5 weeks.

2.3. Optimization of the aeration volume

The medium was aerated at 0.05, 0.1, 0.2, and 0.3 vvm for 5 weeks or 0.05–0.3 vvm (increased at 9-d interval) to determine the optimum air flow. In the aeration experiments, bioreactors were inoculated with adventitious roots at a concentration of 3 g l^{-1} and the cultures were maintained in the dark at 22 ± 1 °C for 5 weeks.

2.4. Growth kinetics

To determine the growth pattern of adventitious roots in bioreactors, the bioreactors were maintained in the dark at 22 ± 1 °C for 7 weeks. The bioreactor cultures were then initiated by inoculation with adventitious roots at a density of 3 g l^{-1} and were aerated at 0.1 vvm. Fourteen bioreactors were maintained to establish the growth kinetics of the adventitious roots. The medium (spent medium) was sampled at weekly intervals from two bioreactors in each treatment group, with the experiment in that were sampled being terminated at the time of sampling.

2.5. Determination of the root weight, growth ratio

The root fresh weight (FW), dry weight (DW) and growth ratio were measured using a previously described method (Cui et al., 2010a).

2.6. Determination of the specific growth rate of adventitious roots

The specific growth rate (μ) of the adventitious roots is defined as:

$$\mu = 1/X \cdot dX/dt$$

where X is the adventitious root weight (g l^{-1}), t is the time (day), and μ is the specific growth rate (per day). The doubling time (Td) of the adventitious roots is defined as $Td = \ln 2/\mu = 0.693/\mu$.

2.7. Preparation of root extract and determination of total phenolics, flavonoid, and chlrogenic acid

Ground-dried roots (0.5 g) were refluxed with 15 ml of 80% methanol at 80 °C for 1 h. After centrifugation (Union 5KR; Hanil, Inchun, Korea) at 4000×g for 10 min, the supernatant solution was filtered under vacuum into a volumetric flask. The residue was re-extracted in the same way and the final volume of the solution was set at 30 ml. The concentration of the total phenolics, flavonoids, and chlorogenic acid in the roots were determined using the methods described by Cui et al. (2010a).

2.8. Preparation of root extract and identification and quantitation of hyperoside and quercetin

Two gram of ground-dried roots were extracted in 20 ml of acetonitrile and 4 ml of 0.1 N hydrochloric acid at room temperature for one day using a gyratory shaker. Crude extract was filtered through filter paper (Advantec 110 mm, Toyo Rosihi Kaisha Ltd., Japan) and was concentrated by using a vacuum rotary evaporator (Tokyo Rikakikai Co., Japan) in a water bath at 50 °C. The residues were re-dissolved in 5 ml of 80% aqueous methanol, and filtered through a 0.2 μm membrane filter (Gelman, USA), and used for the HPLC analysis (Chung et al., 2011). Mass spectra of hyperoside and quercetin were acquired with a Thermo LTQ linear iontrap mass spectrometer with an electrospray ion source (ESI) (Thermo electron corporation LTQ XL, San Jose, CA). The instrument was operated in the negative ion mode, scanning m/z. The identification and quantitation of hyperoside (Sigma chemical Co., St. Louis, MO, USA) and quercetin (Sigma chemical Co., St. Louis, MO, USA) were achieved using an HPLC system with an XTerra C_{18} column (particle size 5.0 μm, 150 mm × 2.0 mm). The mobile phase was (A) water (0.1% formic acid) and (B) methanol. The gradient elution was modified as follows: initial 90% A for 15 min; 30% A for the next 1 min; 5% A for the next 4 min; after which to the mobile phase was returned to its initial conditions with a flow rate 0.2 ml min^{-1} (Table 1). Retention times for hyperoside and quercetin were 17.27 min and 19.53 min, respectively.

2.9. Determination of total polysaccharides content

After the extraction of adventitious roots (1 g) with 80% methanol, the sediment was collected and desiccated in an oven at 60 °C. The sediment of 0.2 g was resuspended in 5 ml 5% (v/v) sulphuric acid and placed in a boiling water for 2 h. After acidic hydrolysis, the liquid–solid mixture was diluted to 100 ml with distilled water. After filtering through filter paper (Advantec 110 mm, Toyo Rosihi Kaisha Ltd., Japan), the polysaccharide in the supernatant was assayed according to the carbazole reaction method (Zhu et al., 1991). A sample of 0.2 ml taken from the above supernatant was mixed with 6 ml concentrated sulphuric acid, held in a boiling water

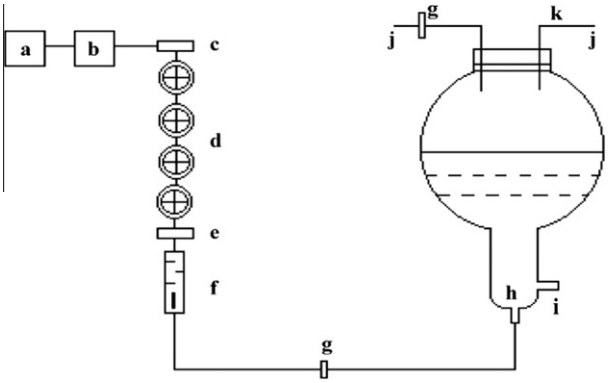

Fig. 1. Schematic diagram of an airlift bioreactor. (a) Air compressor, (b) air reservoir, (c) air cooling device, (d) air filter system, (e) air dryer, (f) air flow meter, (g) membrane filter, (h) glass sparger, (i) medium feeding port, (j) vent, (k) prefilter.

Table 1
LC gradient elution conditions for the separation of quercetin and hyperoside.

Time (min)	H_2O (0.1% formic acid) (%)	Methanol (%)
0.00	90	10
15.00	30	70
16.00	5	95
20.00	5	95
20.10	90	10
35.00	90	10