Industrial Crops and Products 49 (2013) 675–681

Contents lists available at SciVerse ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Identification of triterpenoids and flavonoids, step-wise aeration treatment as well as antioxidant capacity of *Glycyrrhiza uralensis* Fisch. cell

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ARTICLE INFO

Article history:
Received 19 February 2013
Received in revised form 15 May 2013
Accepted 18 May 2013

Keywords:
Glycyrrhiza uralensis Fisch.
Suspension cell
Triterpenoid saponin
Flavonoids
Aeration volume
Antioxidant

ABSTRACT

In this study, suspension cells of *Glycyrrhiza uralensis* Fisch. have been successfully established. Liquiritin, licorice glycoside B, licorice saponin J_2, glycyrrhizic acid and licorice saponin B_2 were identified from *G. uralensis* cells on the basis of LC–MS^n analysis. Step-wise aeration treatment was conducted using a 5 l balloon-type bubble bioreactor (BTBB) to enhance cell density and metabolite production. The results showed that step-wise aeration of 0.6–0.4 vvm was optimal for the accumulation of triterpenoid saponins (2.58 mg l^{-1}) and flavonoids (24.33 mg l^{-1}) in *G. uralensis* cells. Growth kinetics was also conducted with cells at aeration volume of 0.6–0.4 vvm. This study also revealed that *G. uralensis* cells possessed a similar radical scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) test as native licorice had.

1. Introduction

Licorice, the roots and stolons of some Glycyrrhiza species, is one of the oldest medicinal plants that have been used by human beings. Glycyrrhizic acid, namely a kind of triterpenoid saponin, is the most important pharmacologically active component in it (Zhang et al., 2009). Glycyrrhizic acid has significant effects similar to adrenal cortical hormone and can be used in clinical trials for anti-inflammatory, anti-aging, decompression, enhancing body immunity, improving physiological function and restraining cancer cells growth, showing really curative effect (Woo et al., 2001). Flavonoids, secondary metabolites of the plant, are especially used for anti-oxidative, whitening and dispelling freckle (Valeria and Maria, 2005; Ohad et al., 2003). In recent years, licorice has been increasingly used as a healthy additive formulated into a variety of commercial products, including drugs, foods, drinks and cosmetics, which are marketed in Asian as well as in many other countries around the world (Zhang et al., 2011).

However, natural sources of wild licorice are very limited because of the low bud ratio of the seeds and destructive exploitation of people (Zhang et al., 2001). The current supply of licorice mainly depends on field cultivation, which is an extremely long-lasting and labor-intensive process. Plant cell culture offers an alternative for obtaining valuable chemicals, especially plant-specific bioactive secondary metabolites. However, low productivity mainly caused by slow growth rate and poor secondary metabolite content coupled with high production cost limits the commercial application of plant cell culture technology (Zhang and Zhong, 2004). To enhance productivity, bioreactor culture technology should be optimized and evaluated for the commercial-scale production of *G. uralensis* secondary metabolites.

In the cultivation of plant cells as well as organ cultures, the bioreactor culture system has comparative advantages over classical tissue culture because the culture conditions in the bioreactor can be optimized by real-time manipulation of temperature, pH, concentration of oxygen, carbon dioxide and nutrients in the

Abbreviations: MS, Murashige and Skoog; 2,4-D, 2,4-dichlorophenoxyacetic acid; KT, kinetin; NAA, naphthalene acetic acid; 6-BA, 6-benzylaminopurine; t_R, retention times; BTBB, balloon-type bubble bioreactor; SOUR, specific oxygen uptake rate; OUR, oxygen uptake rate; EC, electrical conductivity; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

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http://dx.doi.org/10.1016/j.indcrop.2013.05.021

medium (Sivakumar et al., 2005). Aeration volume, which regulates the accumulation of biomass and secondary metabolite, is one of the most important components affecting these bioreactor cultures because it is closely related to the agitation of explants and dissolved oxygen in the media (Shohael et al., 2005; Lee et al., 2006).

Until now, there are no reports regarding the identification of triterpenoids and flavonoids in *G. uralensis* cells. In this study, compositions of triterpenoids and flavonoids in *G. uralensis* cells were investigated. To provide a theoretical reference for commercial-scale culture of *G. uralensis* cells that could be more productive in commercial healthy products, we conducted studies on step-wise aeration treatment of *G. uralensis* cells. In addition, radical scavenging activity of the *G. uralensis* cells on 1,1-diphenyl-2-picrylhydrazyl (DPPH) test was also discussed for real application.

2. Materials and methods

2.1. Plant material

Native roots and seeds of *G. uralensis* harvested in 2010 were supplied by Beijing Institute of Shizhen Chinese Herbal Medicine.

2.2. Callus induction and proliferation

Seeds surface were sterilized with 70% EtOH for 30 s, immersed in 2% NaClO solution for 30 min and rinsed 3 times with sterile distilled water. Disinfected seeds were inoculated into Murashige and Skoog (MS) (1962) solid medium containing 3% sucrose. Each conical flask (250 ml) was cultured at $23 \pm 2\,^{\circ}C$ in light. After 1 week of culture, seedlings were observed. The middle tissue of hypocotyls were cut into sections of 5 mm and inoculated into MS solid media containing 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.7 mg l^{-1} kinetin (KT) and 3% sucrose. Each Petri dish (9 cm in diameter and 1.5 cm in height) was sealed with a wrap and cultured at $23 \pm 2\,^{\circ}C$ in dark. After 4 weeks of culture, calli were induced, which proliferated in the same media and hormones as well as under the same conditions at intervals of 4 weeks.

2.3. Shake flask culture of suspension cells

Suspension cells were initiated from callus and cultivated in 1 l conical flasks with 400 ml MS medium supplemented with 1.0 mg l^{-1} 2,4-D, 1.0 mg l^{-1} naphthalene acetic acid (NAA), 0.2 mg l^{-1} 6-benzylaminopurine (6-BA) and 3% (w/v) sucrose. Suspension cells were cultured on a rotary shaker (120 rpm) at $23 \pm 2\,^{\circ}C$. Subculture was routinely conducted at intervals of 3 weeks.

2.4. Optimization of the aeration volume

Bioreactor cultures were initiated by inoculating 200 g fresh weight of cells into a 5 l BTBB containing 3 l MS liquid medium supplemented with 1.0 mg l^{-1} 2,4-D, 1.0 mg l^{-1} NAA, 0.2 mg l^{-1} 6-BA and 30 g l^{-1} sucrose, in which the aeration volume was set at 0.2, 0.4, 0.6, 0.8 or 1.0 vvm. Samples were taken on day 20 to determine the dry weight, triterpenoid saponins and flavonoids content. Each experiment was repeated at least 3 times.

2.5. Dynamic change of growth and specific oxygen uptaker rate (SOUR)

Cells were inoculated into 5 l BTBB, and the air volume was adjusted to constant flow rate of 0.6 vvm during BTBB cultivation. Culture conditions were the same as above. Samples were taken on days 0, 5, 10, 15 and 20 to determine the dry weight and SOUR. Each experiment was repeated at least 3 times.

2.6. Step-wise aeration treatment

Bioreactor cultures were divided into 3 groups based on their aeration volume:

- 0.6 vvm group (from 0 days to 20 days).
- 0.4 vvm (from 0 days to 5 days)–0.6 vvm (from 5 days to 15 days)–0.4 vvm (from 15 days to 20 days) group.
- 0.6 vvm (from 0 days to 10 days)–0.4 vvm (from 10 days to 20 days) group.

Culture conditions were the same as above. Samples were taken on day 20 to determine the dry weight, triterpenoid saponins and flavonoids content. Each experiment was repeated at least 3 times.

2.7. Growth kinetics of G. uralensis cells

Cells were inoculated into 5 l BTBB, and the air volume was adjusted to flow rate of 0.6–0.4 vvm during BTBB cultivation. Culture conditions were the same as above. Samples were taken on days 0, 5, 10, 15 and 20 to determine the dry weight, triterpenoid saponins, flavonoids, SOUR and electrical conductivity (EC). Each experiment was repeated at least 3 times.

2.8. Determination of biomass

Cells were rinsed with tap water before oven-drying. Dry weight was recorded after cells were dried to a constant weight at $50\,^{\circ}C$ for 1 day. The growth rate was calculated as (harvested dry weight – inoculated dry weight)/inoculated dry weight.

2.9. Sample preparation

Dried, pulverized samples (1.0 g) were extracted twice with 25 ml 50% ethanol containing 0.3% ammonia water for 1 h at $65\,^{\circ}C$. Following the filtration, the extract was evaporated to dryness and then dissolved in 1 ml of 50% ethanol.

2.10. HPLC–MS and ESI-MS^n

Samples were analyzed using an Agilent HPLC–MS system containing a surveyor autosampling system, interfaced to an ion-trap mass spectrometer *via* an electrospray ion source. Source setting used for the ionization of triterpenoids and flavonoids were: nebulizer gas flow, 30.00 psi; dry gas flow, 8.00 l/min; electrospray voltage of the ion source, 3000 V; capillary temperature, $350\,^{\circ}C$; capillary exit, −136 V; and skimmer, 40 V. Nitrogen (>99.99%) and He (>99.99%) were used as sheath and damping gas, respectively. The full scan of ions ranging from m/z 100 to 1000 in the negative ion mode and the positive ion mode was carried out. The fragment ions were obtained using collision energy of 35% for both MS^2 and MS^3 experiments. Analyses were conducted at ambient temperature and the data were operated on the Xcalibur software.

Chromatographic separations were performed on a Kromasil C_{18} (4.6 mm × 250 mm, 5 μm) column. The column was maintained at $35\,^{\circ}C$ and eluents used were: A-acetonitrile and B-0.04% formic acid. Gradient elution profile was 0 → 10 min, A: 10% → 15%; 10 → 25 min, A: 15% → 20%; 25 → 100 min, A: 20% → 60%. The flow rate of mobile phase was 1.0 ml min^{-1}. 20 μl of sample was injected into the HPLC and the peaks were monitored at 254 nm.