Biotechnol Lett (2014) 36:1727–1733
DOI 10.1007/s10529-014-1534-1

ORIGINAL RESEARCH PAPER

Pilot-scale culture of somatic embryos of *Eleutherococcus senticosus* in airlift bioreactors for the production of eleutherosides

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Received: 31 January 2014 / Accepted: 9 April 2014 / Published online: 4 May 2014

Abstract
Purpose of work To establish pilot scale bioreactor cultures of somatic embryos of Siberian ginseng for the production of biomass and eleutherosides. Somatic embryos of *Eleutherococcus senticosus* were cultured in airlift bioreactors using Murashige and Skoog medium with 30 g sucrose l^{-1} for the production of biomass and eleutherosides. Various parameters including the type of bioreactor, aeration volume, and inoculum density were optimized for 3 l capacity bioreactors. Balloon-type airlift bioreactors, utilizing a variable aeration volume of 0.1–0.3 vvm and an inoculum of 5 g l^{-1}, were suitable for biomass and eleutheroside production. In 500 l balloon-type airlift bioreactors, 11.3 g dry biomass l^{-1}, 220 μg eleutheroside B l^{-1}, 413 μg eleutheroside E l^{-1}, and 262 μg eleutheroside E1 l^{-1} were produced.

Keywords Airlift bioreactors · Eleutherosides · Ginseng · Pilot-scale culture · Siberian ginseng · Somatic embryos

Introduction

Eleutherococcus senticosus (Rupr. & Maxim.) Maxim (*Acanthopanax senticosus*), popularly known as 'Siberian ginseng', is an important medicinal plant distributed in southeast Russia, north eastern China, Korea, and Japan (Davydov and Krikorian 2000). It is used in traditional Chinese medicine as an adaptogen and to strengthen the spleen and kidneys (Huang et al. 2011). The natural products isolated from *E. senticosus* (eleutherosides) possess diverse biological activities, including antibacterial, anticancer, anti-inflammatory, anti-gout, anti-hepatitis, antihyperglycemic, anti-leishmania, antioxidant, haemostatic, immunostimulatory, and hypocholesterolemic effects (Lee and Shin 2002). The term eleutheroside is somewhat non-specific, and has been applied to compounds of different chemical classes. For example, the eleutherosides E (syringaresinol diglucoside) and E1 (episyringaresinol 4-*O*-β-D-glucopyranoside) are syringaresinol-based macromolecules, while eleutheroside B (syringin) and other

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Fig. 1 Suspension cultures of somatic embryos of *Eleutherococcus senticosus*: **a** Embryogenic cells in MS liquid medium supplemented with 30 g sucrose l^{-1} and 1 mg 2,4-dichlorophenoxyacetic acid l^{-1}. **b** Embryogenic suspension in 3-l capacity balloon-type airlift bioreactor containing 2-l MS medium with 30 g sucrose l^{-1}. **c** Embryogenic suspension in 500 l balloon-type airlift bioreactor. **d** Biomass harvested from 500 l balloon bioreactor after 30 days of culture

phenylpropane glucosides are synthesized by the shikimate and phenylpropanoid pathways (Dewick 2002). Among the eleutherosides, eleutherosides B, E, and E1 have major pharmacological activities (Lee and Shin 2002) (Fig. 1).

Plant cell and organ cultures are useful for the production of bioactive compounds, and bioreactor-based systems have been developed specifically for the production of ginsenosides (Paek et al. 2009) and phenolics (Wu et al. 2007). Currently, our research efforts are focused on developing bioreactor methodologies for the production of eleutherosides, as well as Siberian ginseng biomass, from the suspension cultures of somatic embryos. The dried biomass of somatic embryos of Siberian ginseng is used by the pharmaceutical industry as raw material for the preparation of various Siberian ginseng-based products. In the present study, we used large-scale (500 l) drum- and balloon-type airlift bioreactors for the production of biomass in the form of somatic embryos.

Materials and methods

Induction of embryos and establishment of suspension cultures in shake flasks

Embryogenic callus of *E. senticosus* (Rupr. & Maxim.) Maxim was induced by using root explants on Murashige and Skoog (1962) medium (MS) supplemented with 30 g sucrose l^{-1}, 1 mg 2,4-dichlorophenoxyacetic acid (2,4-D) l^{-1}, and 2.3 g Gelrite l^{-1} (Shohael 2006). Embryogenic cells were maintained in 100 ml (in 250 ml shake-flasks) MS liquid medium supplemented with 30 g sucrose l^{-1} and 1 mg 2,4-D l^{-1} by sub-culturing once every 3 weeks. Cultures were incubated in the dark at a

temperature of 25 °C, on a shaker at 100 rpm. All the macro and microelements, vitamins, growth regulators, sucrose, and Gelrite were procured from Duchefa Biochemie (Haarlem, The Netherlands).

Bioreactor cultures and optimization of culture conditions

The various models of bioreactors employed were as follows: Balloon (model balloon-type 3 l), bulb (model bulb-type 3 l), cone (model cone-type 3 l), and cylinder (model cylinder-type 3 l). All were supplied by Samsung Science Co. Ltd. (Jangsa dong 58, Jongrogu, Seoul, Republic of Korea). Cultures were established in 2 l MS medium supplemented with 30 g sucrose l^{-1} using 3 l balloon, bulb, cone, and cylinder bioreactors, to verify the effects of bioreactor configuration on biomass and metabolite accumulation. Cells at 5 g l^{-1} were used as the inoculum, while the aeration volume in the bioreactors was automatically adjusted to 0.1 vvm (air volume per culture volume per min) with air flow meters (RMA series; Dwyer Instruments Inc., Michigan, USA). In another set of experiments, bioreactor cultures were established as described above, but here the cultures were aerated with volumes of 0.05, 0.1, 0.2, and 0.3 vvm constant air supplies, or with an air supply that was increased from 0.05–0.3 vvm every 6 days to verify the most appropriate aeration volume for the accumulation of biomass and eleutherosides. In a further set of experiments, the cultures were established using different inoculum densities (1, 3, 5, 7, and 10 g cells l^{-1}), to test the effects of inoculum density on the accumulation of biomass and eleutherosides. In these experiments, the aeration rate was controlled at 0.05 vvm and increased to 0.1, 0.2, and 0.3 vvm after 6, 12, and 18 days, respectively. All bioreactors were maintained at 25 ± 1 °C in darkness.

After establishing the effects of the various parameters (bioreactor configuration, aeration volume, and inoculum density) on biomass and eleutheroside accumulation in 3 l bioreactors, experiments were then conducted in pilot-scale balloon- and drum-type (500 l) bioreactors for cultivation of somatic embryos of Siberian ginseng. These bioreactors have a sparger positioned at the bottom that generates air bubbles of less than 0.5 µm diameter. The aeration rate was controlled at 0.05 vvm, and subsequently increased to 0.1, 0.2, and 0.3 after 6, 12, and 18 days, respectively. The same medium mentioned above was used with an inoculum of 5 g l^{-1}. All bioreactors were maintained at 25 ± 1 °C in darkness. After 30 days, growth parameters (fresh weight, dry weight, and growth ratio) and eleutheroside content were assessed.

Determination of cell biomass

After 30 days culture, the embryos were separated from the medium by filtering through a stainless steel sieve. The fresh biomass was measured after blotting away the surface water. The dry weight was recorded after drying the filtered biomass at 60 °C for 24 h. The growth ratio was calculated as follows: [harvested dry weight (g) − inoculated dry weight (g)/inoculated dry weight (g)].

Estimation of eleutheroside content

The extraction and analysis of eleutherosides were carried out as per the protocol of Apers et al. (2005). The eleutherosides were quantified by HPLC using a Symmetry C18 column (4.6 mm × 250 mm, Waters, USA). Eleutherosides were separated using water/acetonitrile as mobile phase, with a linear gradient of 10 % (v/v) acetonitrile from 0 to 5 min, 20 % (v/v) acetonitrile from 5 to 20 min and 40 % (v/v) acetonitrile from 20 to 35 min, followed by re-equilibration with 5 % (v/v) acetonitrile for 5 min. The flow rate was 0.8 ml min^{-1}. Eleutherosides were detected at 216 nm. Standards were obtained from ChromaDex, Laguna Hills, CA, USA.

Statistical analysis

All experiments were carried out in a randomized design, and data were collected from three replicates. Mean values were subjected to Duncan's multiple range test using SPSS software (version 9.0).

Results and discussion

Effect of bioreactor configuration on the production of embryogenic biomass and eleutherosides

Bioreactors of various types (balloon, bulb, cone, and cylinder) were tested, and the data on optimal O2 transfer coefficient values (K_La) of each bioreactor, biomass, and eleutheroside accumulation are

Table 1 Suspension culture of somatic embryos of Siberian ginseng: effect of type of bioreactors on biomass accumulation and production of eleutherosides after culturing for 30 days

Bioreactor type	Initial K_La (h^{-1})	Biomass (g l^{-1})			Eleutheroside (μg l^{-1})		
		Fresh weight	Dry weight	Ratio	B	E	E1
Balloon	6.98 a	102.3 ± 1.2 a	11.3 ± 0.5 a	17	327 ± 4 a	533 ± 9 a	388 ± 9 a
Bulb	6.95 a	98.6 ± 1.5 a	11.3 ± 0.6 a	16.9	194 ± 9 c	550 ± 10 a	299 ± 9 b
Cone	5.69 b	85 ± 1.8 b	9.8 ± 1.0 b	14.5	223 ± 5 b	418 ± 9 b	198 ± 9 c
Cylinder	5.25 c	73.8 ± 0.9 c	8.2 ± 0.7 c	12	164 ± 6 b	291 ± 5 c	136 ± 4 d

Embryogenic cells (5 g cells l^{-1}) were cultured in 3 l bioreactors containing 2 l MS medium supplemented with 30 g sucrose l^{-1}. Data represent mean values of three replicates with standard error. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$. Growth ratio is the quotient of the dry of biomass harvested divided by the dry weight of the inoculum

presented in Table 1. Maximal quantities of 11.3 g dry biomass l^{-1} and eleutherosides B (327 μg l^{-1}) and E1 (388 μg l^{-1}) were recorded with the balloon-type airlift bioreactors. However, eleutheroside E (550 μg l^{-1}) accumulation was maximized in the bulb-type airlift bioreactor. The enhanced accumulation of biomass and secondary metabolites in balloon-type bubble bioreactors might be due to their optimal K_La values (6.98; Table 1).

Biomass and metabolite accumulation of suspension cultures often depends on the K_La value (Georgiev et al. 2013). The same report considers K_La to be one of the most important factors during the scale-up process, given that an insufficient K_La value leads to a decrease in growth of cultured cells/organs, and reduces productivity of secondary metabolites (Georgiev et al. 2013). In suspension cultures of *Catharnathus roseus* (Leckie et al. 1991) and *Perilla frutescens* (Zhong et al. 1993), the initial K_La values affected the accumulation of both alkaloids and anthocyanins significantly. In the current studies, the balloon-type (K_La values of 6.98) and bulb-type airlift bioreactors (K_La values of 6.95) were suitable for both biomass and eleutheroside accumulation. Schlatmann et al. (1997) are of the opinion that the gaseous phase and recirculation of gas is more important than mere aeration of cultures. In suspension cultures of *C. roseus,* they have demonstrated the enhanced accumulation of alkaloid biosynthesis by recirculation of the gas in 3 l turbine-stirred bioreactors.

Effect of aeration volume on production of embryogenic biomass and eleutherosides

Aeration volume is another factor affecting biomass and metabolite accumulation in bioreactor cultures (Jeong et al. 2009a; Paek et al. 2009). Aeration in bioreactors aids culture development by thoroughly mixing the medium and facilitating nutrient availability to the cultured cells/organs. In the present study, constant aeration volumes of 0.05, 0.1, 0.2, and 0.3 vvm were tested, as was an increasing aeration volume ranging from 0.05 to 0.3 vvm, incremented every 6 days. The results are presented in Table 2. The maximal biomass (11.3 g dry biomass l^{-1}) and eleutheroside contents were accumulated using the incrementally increasing aeration volume (0.05–0.3 vvm) protocol. The positive effects of increasing aeration might be due to more effective oxygenation in the bioreactors with increased air supply. These results are in agreement with previous data demonstrating that incremental increases of the air supply over time are preferable for optimal production of biomass and secondary metabolite accumulation (Jeong et al. 2009a).

Effect of inoculum density on production of embryogenic biomass and eleutherosides

An additional component that could alter the accumulation of biomass and the production of bioactive compounds by in vitro cultures is the inoculum density (Cui et al. 2014; Jeong et al. 2009b). For instance, a high inoculum stimulated root growth, but inhibited biosynthesis of phenolics and flavonoids, in adventitious root cultures of *E. angustifolia* (Wu et al. 2006). In *S. parviflora,* a high inoculum density was found to inhibit scopolamine production (Min et al. 2007). In strawberry cell suspension cultures, cell growth and the biosynthesis of anthocyanin were significantly affected by inoculum density (Sakurai et al. 1996). Here, we found the inoculum (5 g cells l^{-1}) to have a

Table 2 Suspension culture of somatic embryos of Siberian ginseng: effect of aeration volume on biomass accumulation and production of eleutherosides after culturing for 30 days

Aeration volume (vvm)	Initial K_La (h^{-1})	Biomass (g l^{-1})			Eleutheroside (μg l^{-1})		
		Fresh weight	Dry weight	Ratio	B	E	E1
0.05	4.95	97.7 ± 1.0 a	11.1 ± 0.6 a	16.6	205 ± 3 c	456 ± 8 b	194 ± 9 d
0.1	7.84	96.1 ± 0.9 a	10.1 ± 0.7 a	15	257 ± 4 a	442 ± 6 b	267 ± 14 b
0.2	11.42	90.7 ± 1.2 b	10 ± 0.8 a	14.9	210 ± 4 b	428 ± 8 b	214 ± 12 c
0.3	16.81	78 ± 0.9 c	8.3 ± 0.6 b	12.2	190 ± 4 b	313 ± 6 c	183 ± 7 c
0.05/0.1/0.2/0.3[a]	5–16.58	99.2 ± 0.8 a	11.3 ± 0.8 a	16.5	290 ± 4 a	593 ± 10 a	341 ± 9 a

Embryogenic cells (5 g cells l^{-1}) were cultured in 3 l bioreactors containing 2 l MS medium supplemented with 30 g sucrose l^{-1}. Data represent mean values of three replicates with standard error. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$. Growth ratio is the quotient of the dry of biomass harvested divided by the dry weight of the inoculum

[a] Aeration volume was increased one step after every 6th day

Table 3 Suspension culture of somatic embryos of Siberian ginseng: effect of inoculum density on biomass accumulation and production of eleutherosides after culturing for 30 days

Inoculum density (g l^{-1} of cells)	Biomass (g l^{-1})			Eleutheroside (μg l^{-1})		
	Fresh weight	Dry weight	Ratio	B	E	E1
1	97.7 ± 1.4a	10.8 ± 0.9 a	19.4	139 ± 6 c	538 ± 10 a	287 ± 10 c
3	98.1 ± 1.2 a	11.0 ± 0.8 a	19.8	225 ± 4 b	526 ± 12 b	277 ± 8 c
5	103.7 ± 1.2 a	11.5 ± 0.9 a	20.8	243 ± 5 a	580 ± 12 a	377 ± 10 a
7	98.0 ± 1.0 a	10.3 ± 1.0 b	18.5	229 ± 8 a	434 ± 8 c	307 ± 6 b
9	94.2 ± 1.2 b	9.3 ± 0.6 c	16.6	169 ± 5 b	242 ± 5 d	274 ± 5 b

Embryogenic cells were cultured in 3 l bioreactors containing 2 l MS medium supplemented with 30 g sucrose l^{-1}. Data represent mean values of three replicates with standard error. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$. Growth ratio is the quotient of the dry of biomass harvested divided by the dry weight of the inoculum

profound effect on embryogenic biomass accumulation and eleutheroside production (Table 3). Maximal biomass (11.5 g dry biomass l^{-1}) and eleutheroside production was reached when 5 g cells $l^{-1}$of inoculum was fed into the bioreactors. Higher inocula density (7 and 9 g l^{-1}) was not only not beneficial, it was also responsible for decreasing the accumulated biomass and eleutherosides.

Pilot-scale bioreactor cultures

Based on the above data using 3 l bioreactor cultures, we established and quantified pilot-scale bioreactor cultures (using 500 l airlift drum- and balloon-type bioreactors). About 5.2 kg (10.4 g dry biomass l^{-1}) and 5.7 kg (11.3 g dry biomass l^{-1}) of dry biomass was produced in the 500 l drum- and balloon-type airlift bioreactors, respectively, after 30 days of culture (Table 4). Accumulated eleutheroside content in the pilot-scale cultures included 220 μg eleutheroside B l^{-1}, 413 μg eleutheroside E l^{-1} and 262 μg eleutheroside E1 l^{-1}. Interestingly, a decreased production of indole alkaloids was noted during the scale-up of *C. roseus* cell cultures (Zhao and Verpoorte 2007). On the other hand, a simultaneous increase in biomass and metabolite accumulation was found upon scale-up of adventitious root cultures of ginseng (Choi et al. 2000). The current study demonstrated a pattern similar to that of ginseng, with concurrent increases in biomass and metabolite production after scale-up to the pilot-scale cultures.

Table 4 Growth and metabolite production of Siberian ginseng somatic embryos in pilot-scale airlift bioreactors

Bioreactor type and volume	Biomass yield (kg)		Eleutherosides (μg l^{-1})		
	Fresh weight	Dry weight	B	E	E1
500 l horizontal drum-type airlift bioreactor	55.6 ± 0.7 b	5.2 ± 0.2 b	187 ± 1 b	364 ± 4 b	231 ± 3 b
500 l balloon-type airlift bioreactor	63 ± 0.8 a	5.7 ± 0.3 a	220 ± 6 a	413 ± 6 a	262 ± 5 a

Embryogenic cells (5 g cells l^{-1}) were cultured in MS medium supplemented with 30 g sucrose l^{-1}. Data represent mean values of three replicates with standard error. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

Conclusion

The pilot-scale culturing of *E. senticosus* somatic embryos was achieved for the first time, using 500 l airlift bioreactor cultures, and doing so without the loss of production of biomass and eleutherosides when compared to small-scale bioreactors. These data will be useful for the application of embryogenic cultures for the production of eleutherosides on a large scale.

Acknowledgments This study was conducted in 2013 by grants supporting Chungbuk National University. Dr. H. N. Murthy is thankful to the Ministry of Education, Science, and Technology, Republic of Korea, for the award of the Brain Pool Fellowship (131S-4-3-0523). This study was also supported by the Ministry of Science, ICT, and Planning (MSIP).

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