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Production of eleutherosides, total phenolics and total flavonoids from somatic embryos of Siberian ginseng affected by different aeration volume in bioreactor

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Abstract

Physical culture condition plays crucial roles in mass production of somatic embryos in bioreactor. The effects of supplied aeration volume in a balloon type bubble bioreactor (BTBB) to produce somatic embryos were investigated in this study. Best response in case of fresh weight (FW), dry weight (DW) and growth ratio (GR) were observed when input aeration volume were changed every 10 days as follows 0.05/0.1/0.2/0.3 vvm rather than constant aeration volume. Maximum FW 99.28 g/l and DW 11.35 g/l were found in above aeration volume although the highest growth ratio was noticed when 0.05 vvm constant aeration volume was applied. Highest amount of total eleutherosides (108.51 µg/g DW) were produced while aeration volume was changed in every 10 days but higher chlorogenic acid was found while constant aeration volume was 0.01 vvm (1.05 mg/g DW). Pattern of total phenolics and total flavonoids production also revealed that, maximum amount of those metabolities were produced when input air was changed every 10 days. The results indicated that input aeration volume has a potential role on somatic embryos production and secondary metabolites accumulation in bioreactor.

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Introduction

Siberian ginseng (*Eleutherococcus senticosus* Maxim) is an endangered species due to over harvesting from natural habitat. It is a woody shrub available only in northeast Asia (Lee, 1979). Seed propagation in this species is difficult (Isoda and Shoji, 1994), so that micro-propagation through somatic embryogenesis is now widely using for efficient plantlets production (Shohael *et al*., 2005). The active ingredients in Siberian ginseng, called eleutherosides, are thought to stimulate the immune system (Brekhman and Dardymov, 1969). These nutracutical properties help to scavenge free radicals, reduce stress, to improve the immune system, to fight cancer and aging, to reduce other effects of radiation, to improve adrenal function (Eschbach *et al.,* 2000; Schmolz *et al.,* 2001; Gaffney *et al.,* 2001).

Micropropagation via direct and indirect somatic embryogenesis in Siberian gineng (Gui *et al.,* 1991; Choi *et al.,* 1999) and embryogenic callus and cell suspension culture has been reported in *E. sessiliflorus* (Shohael *et al.,* 2005). Somatic embryogenesis has also been studied as a model system to understand the physiological, biochemical and genetic events during their development (Zimmerman, 1993; Tao *et al*., 2012). Somatic embryogenesis from embryogenic callus or cell suspension in another means of plant regeneration (Choi *et al.,* 1999). Moreover extraction of secondary metabolites from somatic embryos also a potential alternative than extraction from whole plant or plant parts (Shohael *et al.,* 2005). Somatic embryogenesis also offer a potential system for large-scale plant propagation in automated bioreactor (Peak and Chakrabarty, 2003; Paek *et al.,* 2005). Due to intensive labor conventional micropropagation limits commercial application. Application of bioreactor system can reduce the labor requirements, which is linked to scale up of cultures. Somatic embryos culture in bioreactor may have lower costs and better results in more manageable culture condition, rapid growth and higher productivity (Yang *et al.,* 2010). The successful production of somatic embryos through bioreactors has been reported in a number of species (Paek *et al.,* 2005), but still many improvements are required for commercial somatic embryos production. The use of plant tissue culture for secondary product biosynthesis, particularly in plants of pharmaceutical importance, holds promise for the controlled production of these important plant constituents (Lindsey and Yeoman, 1983).

Reduced productivity during plant cell culture scale up has often reported (Schlatmann *et al.,* 1993). Various factors affect the productivity during culture such as shear stress, oxygen supply and gas composition. Plant cells are sensitive to shear stress for their large volume and aggregating phenomenon (Pan *et al.,* 2000). A conventional stirrer tank bioreactor can produce a high shear region, but in an airlift bioreactor provides gentle shear environment. Gas exchange between the gas and liquid medium is another important factor that affects the scale up of plant cells. In bioreactor forced aeration is needed to supply oxygen and to improve fluid mixing (Pan *et al.,* 2000). Gaseous substances were proven to be important for the cell growth and synthesis of secondary metabolites in plant cell culture (Smart and Fowler, 1981). In a shake flask there is no forced aeration and gaseous metabolites can be accumulated in its headspace, so that dissolved gases concentrations may be quite different between shake flask and bioreactor. For different culture system effects of gaseous compounds also different. Aeration in liquid culture has function as an oxygen supply. Oxygen transfer usually limits the function of biological system. Due to limited oxygen supply, cell growth and metabolites production also reduced (Zhong, 2010). The airflow supplied into the airlift bioreactor play two important functions to the cultured explants, one is supply of O_2 for metabolic activities, and the other is agitating. The cells and organs in bioreactors were strongly subjected to three forces: agitation-based laminar forces, turbulent forces, and bubble-based forces (Namdev and Dunlop, 1995).

In order to establish successful bioreactor system for somatic embryos production, various parameters were needed to study for optimization. There was not much information available on effect of aeration volume on somatic embryos growth and secondary metabolites production in bioreactor. In this study we described the effect of supplied aeration volume on Siberian ginseng's somatic embryos and metabolites production in airlift bioreactor. Obtained data provides a better understanding of the growth kinetics of somatic embryos and their production of secondary metabolites in bioreactors with the effect of input aeration volume treatments.

Materials and methods

Somatic embryogenesis from leaf explants and culture conditions:

In vitro seedlings of *E. senticosus* were maintained in MS medium (Murashige and Skoog, 1962) without plant growth regulators (PGRs). Young leaves were cut into 5 x 5 mm pieces and used as an explant source. Leaf explants were placed on semisolid MS basal medium supplemented with 1.0 mg l^{-1} 2,4-D with 3 % sucrose and 0.2 % gelrite for callusing and somatic embryogenesis. The medium was adjusted to pH 5.8 prior to addition of gelrite and sterilized at 121 ^{o}C for 15 min and distributed into 15 x 140 mm Petridishes (15 ml of medium). Cultures were maintained in the dark at 25 ^{o}C and evaluated for somatic embryogenesis after 12 weeks. For proliferation of embryogenic cultures on solidified medium, the embryogenic calli were cultured on the same medium as described above and subcultured on every 15 days interval.

Bioreactor culture for somatic embryogenesis:

A balloon type bubble bioreactor (BTBB) (Paek *et al.,* 2005; Shohael *et al.,* 2006) was used for the entire regeneration phase using embryogenic suspensions. Ten grams of embryogenic aggregates were placed in the 3L bioreactor along with 2L of full strength of MS medium (Shohael *et al.,* 2013) without any PGRs. The pH of the medium was adjusted to 5.8 before autoclaving (at 121 ^{o}C and 1.2 kg cm^{-2} pressure for 32 min). The volume of input air was adjusted to five different concentrations as follow 0.05, 0.1, 0.2 and 0.3 vvm (aeration volume/culture volume, min). Another concentration was 0.05/0.1/0.2/0.3 vvm increased every 10 days. A sterilized dissolve oxygen (DO) electrode was placed in the bioreactor to measure the dissolved oxygen. The initial volumetric oxygen transfer coefficient (k_La) was determined using a dynamic gassing-in and gassing-out method (Pan *et al.,* 2000). All the bioreactors were maintained at 25 ^{o}C in dark.

Measurement of growth

After finished the culture period, embryos were harvested and washed several times with distilled water then soaked in soft tissue paper to remove water and measured fresh weight. For dry weight, the embryos were dried at 60^{o}C in to an oven drier until completely dry and then measured.

Determination of Eleutherosides and chlorogenic acids

Oven dried embryos were powdered in to the blender. One gm of fine powder was extracted with 60% aqueous methanol (2x50 ml) for 30 min each at 60 ^{o}C separately, and filtered through filter paper (Advantec, Toyo, Japan). The combined extract, evaporated to dryness in vacou, and washed with diethyl ether. The insoluble fraction was dissolve in water and extracted with n-butanol (water saturated). The organic phase was evaporated to dryness, dissolved in (5 ml) of HPLC grade methanol and filtered through 0.45 μm PVDF (Gelman, USA) filter. Eleutherosides and Chlorogenic acids were quantified by HPLC according to procedures reported by Patrick *et al.,* 1978; Shohael *et al.,* 2006 with use of a liquid chromatograph system (Waters 2690 separation modules, Waters, USA) and a photodiode array detector (Waters 996 photodiode array detector, Waters, USA) on Symmetry^{R} C 18 (4.6 x 250 mm) column (Waters, USA). Eleutherosides were analyzed by using a flow rate of 0.8 ml/min with water and acetonitrile as the mobile phase with a linear gradient of 10 % acetonitrile for 0-5 min, 20 % acetonitrile for further 20 min, 40 % for another 15 min and reequilibration with 5 %

acetonitrile for 5 min. Quantitation was based on ultraviolet absorption at 216 nm. The peak areas corresponding to eleutherosides from the samples, with the same retention time as authentic eleutherosides B, E and E1 and Chlorogenic acid (ChromaDex, Inc.USA) were integrated by comparison with an external standard calibration curve.

Determination of total phenolic and total flavonoid contents:

The content of total phenolic in plant methanolic extracts was analyzed following the modification of Folin–Ciocalteu method (Folin and Ciocalteu, 1927). Hundred microliter of methanolic extractswere mixedwith 2.5 ml deionizedwater, followed by addition of 0.1 ml (2N) Folin–Ciocalteu reagent. They were mixed well and allowed to stand for 6 min before 0.5 ml of a 20%sodium carbonate solutionwas added. The color developed after 30 min at room temperature and the absorbance was measured at 760 nm. Total flavonoid content was determined following Shohael *et al.,* 2013. Briefly, 0.25 ml of the methanolic plant extract or (+)-catechin standard solution was mixed with 1.25 ml of distilled water, followed by addition of 0.75 ml of 5% sodium nitrite solution. After 6 min, 0.150 ml of 10% aluminum chloride solution was added and the mixture was allowed to stand 5 min and then 0.5 ml of 1M sodium hydroxide was added. The mixture was brought to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately at 510 nm. The data were calculated using gallic acid and (+)-catechin for total phenolic and flavonoid contents, respectively.

Statistics

Statistical analyses were performed according to SAS system (Version 6.21, SAS Institute Inc., Cary, NC). Statistical significance between mean values was assessed using Duncan's multiple range test. A probability of $P < 0.05$ was considered significant.

Results and discussion

Effect of aeration volumes on somatic embryos growth and their accumulation of secondary metabolites:

Somatic embryos of Siberian ginseng were successfully cultured in a BTBB bioreactor. After ending the culture period (40-45 days) FW, DW and metabolites production were analyzed (Table 1 & 2 and Fig. 1). Those data showed that input air volumes were affected growth and yield of somatic embryos. Maximum FW (99.28 g/l) and DW (11.35 g/l) were obtained when supplied aeration volume was changed every 10 days as follows 0.05/0.1/0.2/0.3 vvm. Growth rate was gradually decreased with the increase of aeration volume (Table 1). Similar result was obtained from ginseng adventitious root culture (Kim, 2002) and ginseng cell culture (Thanh *et al.*, 2006). Initial k_La value were increased (4.95-16.81) with the increase of aeration volume from 0.05~0.3 vvm, that affects the cell growth. Obtained results indicated that the somatic embryos growth was highly affected by input aeration volume. According to Pan (Pan *et al.,* 2000) k_La had a significant effect of cell growth and secondary metabolites accumulation by means of oxygen supply. Air flow supply in the bioreactor determines the degree of aeration and agitation as well as prevents settling of the embryo biomass, thus also affecting the growth and proliferation of embryos. Aeration volume change every 10 days allows the degree of aeration and proper agitation with the increase of embryo biomass; slowly increase of aeration volume with the increase of biomass also reduced the extra stress on embryos. Adequate aeration also ensured the continuous oxygen supply to the bioreactor. Oxygen requirements also vary from species to species and it affects metabolic activity and energy supply to the plant cell (Yesil-Celiktas *et al.,* 2010). The level of oxygen supply in liquid culture in bioreactors can be regulated by agitation or stirring methodologies and intensities as well as aeration techniques and gas flow rates, which affect bubble size, mixing and circulation times, gas hold-up values and mass transfer coefficients (Vandar-Sukan, 1985, 1986; Takayama and Akita,

1989). Effect of oxygen partial pressure on somatic embryos growth and development has been reported (Kurata and Shimazu 2006), in Cyclamen (Hohe et al., 1999) Coffe (De Feria *et al.,* 2003). Similar our findings high aeration rates reduced the biomass growth also reported by Ziv, 2000.

Table 1. Effect of different aeration volume on k_La coefficient and growth of somatic embryos of *E. senticosus* in bioreactors.

Aeration volume (vvm)	Initial k_La (h^{-1})	Biomass (g/l)			Growth ratio[z]
		FW	DW	% DW	
0.05	4.95	97.74 a[y]	11.13 a	11.38	16.66
0.10	7.84	96.10 a	10.10 a	10.50	15.03
0.20	11.42	90.71 b	10.06 a	11.09	14.96
0.30	16.81	78.06 c	8.35 b	10.69	12.25
0.05/0.1/0.2/0.3[x]	5.0-16.58	99.28 a	11.35 a	10.97	15.42

[z]Growth rate is the quotients of the dry weight after culture and the dry weight of the inoculum size.

[y]Mean separation within column by Duncan's multiple range test at $p \leq 0.05$.

[x]Aeration volume increased at 10day intervals.

Table 2. Content of eleutherosides and chlorogenic acid in somatic embryos of *E. senticosus* as affected by different aeration volume in bioreactors.

Aeration volume (vvm)	Eleutherosides (µg/g DW)				Chlorogenic acid (mg/gDW)
	B	E	E_1	Total	
0.05	18.55 c[z]	41.15 b	17.51 d	77.21 d	0.91 b
0.10	25.56 a	43.83 b	26.55 b	95.94 b	1.05 a
0.20	21.01 b	42.81 b	21.40 c	85.22 c	0.78 c
0.30	23.05 b	37.80 c	22.11 c	82.96 c	0.83 c
0.05/0.1/0.2/0.3[y]	25.70 a	52.55 a	30.25 a	108.51 a	1.00 a

[z]Mean separation within column by Duncan's multiple range test at $p \leq 0.05$.

[y]Aeration volume increased at 10day intervals.

Eleutherosides and other metabolites contents were also significantly enhanced by the change of aeration volumes (Table 2). The highest eleutheroside accumulation (108.51µg g/l DW) was obtained by increasing the aeration volume at 0.05/0.1/0.2/0.3 vvm with 10-day intervals. Higher chlorogenic acid accumulation was found in gradually changing aeration volume in every 10 days and a constant aeration volume 0.10vvm (1.0mg/l & 1.05mg/L respectively). Lower and also higher aeration volume also affects the eleutherosides and chlorogenic acid accumulation in somatic embryos (Table 2). In case of total phenolics and flavonoids accumulation the similar phenomenon also noticed (Fig. 1). The ginseng adventitious roots cultured in different bioreactors were influenced by different air agitating environments. It has been confirmed that agitation in the bioreactor can affect the cell aggregation and secondary metabolite production in plant cell suspension cultures (Su, 1995).

The initial K_La coefficient was very low at low aeration volume and was greatly enhanced with an increase of aeration volume. At aeration volume 0.05/0.1/0.2/0.3 vvm with increase 10-day intervals, the highest k_La coefficient was achieved at 5 ~ 16.6/h. It seems that a relatively high aeration volume was beneficial to speed up the oxygen transfer in bioreactor culture. As also reported, oxygen supply is significantly affecting secondary metabolite formation in plant cell cultures (Gao and Lee, 1992; Zhong *et al.,* 1993; Schlatmann *et al.,* 1994). For example, in suspension cultures of *Catharanthus roseus* and *Perilla frutescens*, (Leckie *et al.,* 1991; Zhong *et al.,* 1993), initial k_La coefficient affected the accumulation of alkaloids and anthocyanin pigments in each case.

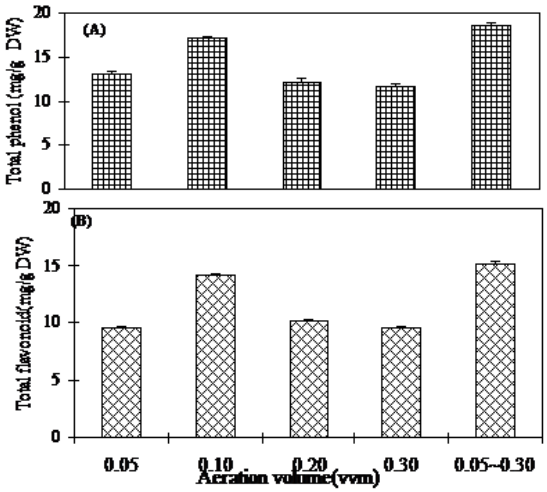

Fig. 1. Contents of total phenol (A) and total flavonoid (B) in somatic embryos of *E. senticosus* as affected by different aeration volume in bioreactors.

Conclusions

Intensive aeration, mixing and medium agitation causes shearing damage, breakdown of cell walls in the growing cells in a bioreactor. Cell wall breakdown produces cell debris and releases polysaccharides into the medium that also causes cell death. Dead cells and other debris also create foaming, those caused the aggregation of cells and those aggregates gradually deposited in the bioreactor walls and also top of the medium. Medium turns become viscous due to secretion of different metabolic residues in to the medium from growing biomass. For those reason growths become decreased. To ensure proper amount of oxygen circulation to the growing biomass, higher aeration rates also required. Production of somatic embryos in bioreactor is a stable convenient and easy to scale up method. Optimum aeration is very important factor for growth and secondary metabolites production.

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