TYPES AND DESIGNS OF BIOREACTORS FOR HAIRY ROOT CULTURE

YONG-EUI CHOI[1], YOON-SOO KIM[2] AND KEE-YOEUP PAEK[3]
[1]Department of Forestry, College of Forest Sciences, Kangwon National University, Chunchon 200-701, Kangwon-do, Korea – Fax: 82-33-252-8310 – Email: yechoi@kangwon.ac.kr
[2]Korea Ginseng Institute, Chung-Ang University, Ansung-shi, Kyunggi-do, Korea – Fax: 82-31-676-6544 – Email: yoosoony@hanmail.net
[3]Research Centre for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju 361-763, Korea- Fax: 82-43-272-5369 – Email: packky@chungbuk.ac.kr

1. Introduction

Plants synthesize a wide range of secondary metabolites such as alkaloids, anthocyanins, flavonoids, quinins, lignans, steroids, and terpenoids, which play a major role in the adaptation of plants to their environment. The secondary metabolites have been used as food additives, drugs, dyes, flavours, fragrances, and insecticides. Such chemicals are extracted and purified from naturally grown plants. However, production of secondary metabolites from plants is not always satisfactory. It is often restricted to a limited species or genus, and geographically to a specific region. Many important medicinal plants were endangered by overexploitation. Some plants are difficult to cultivate and grow very slowly or are endangered in their natural habitats. The biotechnological approach by utilizing plant cell and organ culture system can offer an opportunity to produce the secondary metabolites. Plant materials via *in vitro* culture are produced with high uniformity regardless of geographical and seasonal limitations and environmental factors. However, there are many problems in the production of metabolites by plant cell and organ culture technology due to the high cost to natural counterparts, and the low yield of metabolites in cultured plant cells. Although there are many efforts for establishing the cell and organ culture systems, application in the commercial production of pharmaceuticals is limited to a few examples only. Production of shikonin from the cell culture of *Lithospermum erythrorhizon* [1,2], taxol from *Taxus baccata* [3] and berberine from *Coptis japonica* [4] was reached for the application for industrialization. The main problem using cell suspension culture is a low product yield and instability of the cell lines [5].

The secondary metabolites can be produced by developed organ and plantlets [6,7]. An alternative method for the production of plant materials for secondary metabolite production is the culture of shoots, roots, or whole plants. However, the organ culture tends to grow slowly and renders the difficulty of the large-scale cultivation compared

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to cell culture. *Agrobacterium rhizogenes*-transformed hairy roots synthesize the same component as does the roots of the intact plants and have a fast growth property in hormone-free medium. Many efforts have been made to commercialize the plant metabolites via a bioreactor culture of hairy roots. The bioreactor for microorganism fermentation (stirred tank bioreactor) is unsuitable for the mass production of hairy roots because of strong shear stress. Therefore, various types of bioreactor systems were designed and evolved to enhance the productivity and the bioprocess. Among them, airlift, bubble column, and liquid-dispersed bioreactor are largely adopted for the hairy root culture because of the low shear stress and the simplicity of their design and construction. Significant progress has been made in biotechnology and bioprocess for the large-scale culture of hairy roots. In this chapter, we focus on the recent technology covering the bioreactor culture systems, such as the shape of bioreactor, aeration condition, and introduce the large-scale production of ginseng hairy-like roots for commercialization.

2. Advantage of hairy root cultures

Normally, adventitious root cultures need an exogenous phytohormone supply and grow very slowly. Hairy roots can be produced by transformation with the soil bacterium *Agrobacterium rhizogenes*, resulting in the so-called hairy roots disease [8]. Long-term genetic and biosynthetic stability was noted from this type of culture [9,10]. In addition, they produce similar secondary metabolites to the normal roots and much higher levels than do cell cultures [6,11,12]. Therefore, hairy roots can offer a valuable source of root-derived secondary metabolites that are useful as pharmaceuticals, cosmetics, and food additives. Transformed roots of many plant species have been widely studied for the *in vitro* production of secondary metabolites [13,14].

Another interesting strategy of hairy root cultures is the genetic engineering of secondary metabolism by introducing useful genes. Enhanced production of alkaloid nicotine by the introduction of ornithine decarboxylase into *Nicotiana rustica* was reported [15]. The hairy roots of *Atropa belladona* overexpressing hyoscyamine 6-beta-hydroxylase (H6H) gene isolated from *Hyoscyamus niger* produced high amounts of scopolamine [16]. In *Hyoscyamus niger* hairy root cultures, overexpression of genes encoding both putrescine N-methyltransferase (PMT) and the downstream enzyme hyoscyamine-6-beta-hydroxylase (H6H) resulted in the enhanced scopolamine biosynthesis [17]. Hairy root cultures of *Datura metel* overexpressing the SAM N-methyltransferase (PMT) gene encodes for putrescine, which accumulated higher amounts of tropane alkaloids (hyoscyamine and scopolamine) than do the control hairy roots [18]. The transgenic hairy roots by introducing the genes regulating secondary metabolism will provide an effective approach for efficient and large-scale commercial production of secondary metabolite production.

3. Induction of hairy roots

Hairy roots are induced from the transfer and integration of the genes of Ri plasmid of *Agrobacterium rhizogenes* [8]. Integration of a DNA segment (T-DNA) of Ri-plasmid

into the host plant genome results in the active proliferation of hairy roots [8]. The Ri plasmids are grouped into two main classes: agropine and mannopine type strains [19]. The agropine type strains contain both the TL (about 15-20 kb) and TR (about 8-20 kb) region in their Ri plasmid are more virulent than mannopine strains, and are therefore more often used for the establishment of hairy root cultures [20]. *Agrobacterium rhizogenes* A4 type (A4, ATCC, 15834, 1855, TR105, etc) can synthesize both agropine and mannopine. *Agrobacterium rhizogenes* 8196 type (TR7, TR101, etc.) synthesize the mannopine only.

The *vir* region comprises about 35 kb in the Ri plasmid, and encodes six transcriptional loci: *vir* A, B, C, D, E, and G, which have important functions in gene transfer. Transcription of the *vir* region is induced by various phenolic compounds such as acetosyringone [21]. Acetosyringone or related compounds have been reported to increase the frequency of *Agrobacterium* mediated transformations in a number of plant species [22], especially for recalcitrant monocotyledonous plant species [23]. Various sugars also act synergistically with acetosyringone to induce a high level of *vir* gene expression [24,25].

In the agropine Ri plasmid T-DNA is referred to as left T-DNA (T_L-DNA) and right T-DNA (T_R-DNA) [26]. Genes involved in agropine and auxin syntheses are located in the T_R DNA region. Genes of Ri T_L-DNA such as *rolA, rolB, rolC* and *rolD* stimulate hairy root differentiations under the influence of endogenous auxin synthesis [27]. T-DNA analysis in hairy roots reveals that TL and TR-DNAs exist in random manners either as distinct inserts, or as a single and continuous insert including the region between TL and TR on pRi 15834 [28]. Sequencing of genomic DNA/T-DNA junctions in hairy roots reveals that genomic DNA at the cleavage sites are usually intact, whereas donor T-DNA ends are often resected, as are found in random T-DNA inserts. Batra *et al*. [29] reported that growth and terpenoid indole alkaloid production in *Catharanthus roseus* hairy root clones is related to left and right-termini-linked Ri T-DNA gene integration. Therefore, each hairy root line shows different morphology and growth pattern together with different biosynthetic capability of secondary metabolites.

4. Large-scale culture of hairy roots

Generally, the hairy root culture in bioreactors is focused on both secondary metabolites production via the biomass growth of root tissues. Growth of hairy roots and production of secondary metabolites is controlled by the genetic characteristics of plant species, and they are strongly influenced by physical and chemical culture conditions such as the types of culture vessels, composition and concentration of macro and micro-element, concentration of carbon sources, pH, light, and temperature etc. In hairy root culture systems, biomass growth is achieved due to a series of two characteristic growths: the lateral root primordium formation on parent root segments and their elongation [30]. In comparison to a cell suspension culture, the growth of hairy roots in liquid medium results in the packed root mass playing an inhibitory role in fluid flow and limiting oxygen availability [31]. In addition, the roots hairs play a detrimental role for the growth in a liquid environment because they induce the stagnation of fluid flow and limit the availability of oxygen [31]. Therefore, the morphological character of hairy roots and oxygen supply are primary factors for designing and optimizing the culture

condition of hairy roots [32,33]. To achieve successfully a scale-up, reactor types and assessments of reactor performance must be considered to minimize the problems, which will be encountered during the scale-up. In the case of the Erlenmeyer flask culture, it is very difficult to modify the culture environment within flasks and is used for only small-scale culture due to the limited air supply. A bioreactor fitted with controllers for air supply, pH, temperature etc. is mainly utilized for the large-scale culture of hairy roots. Various configurations of hairy root bioreactors such as the stirred tank, airlift, bubble column, liquid-dispersed bioreactor have been designed for hairy root cultures [14,34]. Therefore, we introduce the cultures of well-known bioreactors for the production of hairy roots and recent advances on the bioreactor culture technology for large-scale production of hairy roots.

4.1. STIRRED TANK REACTOR

In this type of bioreactor, mortar-derived impeller or turbine blades regulate aeration and medium currency. This reactor is widely adopted for microorganism, fermentation and plant cell culture. Temperature, pH, amount of dissolved oxygen, and nutrient concentration can be better controlled within this reactor than in other type of reactors. In general, the impellers used in this reactor produce a high-shear stress compared to other types [35-37]. For hairy roots culture, the impeller must be operated with restricted power input and speed to minimize the shear stress. Ways of improving impeller performance by modifying internal reactor geometry have been designed [38-40]. In the hairy root culture of *Catharanthus trichophyllus,* hairy root line cultures in stirred bioreactor showed a similar alkaloid composition to normal root [41]. The cultivation of *Swertia chirata* hairy roots in a 2-L stirred-tank bioreactor was successful only with a stainless-steel mesh fitted inside the culture vessel for immobilization of the roots [42]. In the *Panax ginseng* hairy root culture, the growth of roots in a stirred bioreactor in which stainless-steel mesh fitted in culture vessel was about three times as high as in the flask cultivation [43].

4.2. AIRLIFT BIOREACTORS

In the airlift bioreactor, both liquid currency and aeration are driven by externally supplied air. This reactor is advantageous for the culture of plant cells and organs those are sensitive to shear stress. However, this reactor is not suitable for high-density culture because of insufficient mixing process inside the reactor. In 2.5-L hairy root culture of *Pueraria phaseoloides*, puerarin accumulation is 200 times as much as in a 250 ml shake flask culture [44]. In the hairy root culture of *Astragalus membranaceus,* both the dry weight of hairy roots and astragaloside IV from a 30-L airlift bioreactor were higher than the yields from a 10-L bioreactor [45]. In the *Panax ginseng* hairy root culture, the growth of roots in both the bubble column and the stirred bioreactor was about three times as high as in the flask cultivation [46]. Hairy roots growth was about 55-fold of inoculums after 39 d in a 5-L airlift bioreactor and about 38-fold of inoculums after 40 d in a 19-L airlift bioreactor [43].

4.3. BUBBLE COLUMN REACTOR

The bubble column reactor is one of simplest types of reactors and is easy to scale-up. Its disadvantage is the undefined flow pattern inside the reactor resulting into non-uniform mixing. Like an airlift bioreactor, the bubbles in a bubble column create less shear stress compared to other stirred types, so that it is useful for organized structures such as hairy roots. In this case, the bubbling rate needs to be gradually increased with the growth of hairy roots. However, at a high tissue density level, the bubble column has been observed to reduce growth performance [47]. In hairy root culture of *Solanum tuberosum* in a 15-L bubble column, stagnation and channelling of gas through the bed of growing roots exists, however, the gas-liquid interface is not the dominant resistance factor to oxygen mass transfer, and the oxygen uptake of growing tips increase with the oxygen tension of the medium [48]. The growth and production of hyoscyamine and scopolamine in the culture of hairy roots of *Datura metel* was enhanced by the treatment of permeabilizing agent Tween 20 in an airlift bioreactor with root anchorage [49]. In hairy root cultures of *Hyoscyamus muticus* accumulated tissue mass in submerged air-sparged reactors was 31% of gyratory shake-flask controls [50]. They reported that impaired oxygen transfer due to channelling and stagnation of the liquid phase are the apparent causes of poor growth [50]. Inclusion of polyurethane foam in the vessel of air-sparged bioreactor reduces the entrapping of gas by hairy roots, which improve biomass and alkaloid production [51]. In *Artemisia annua* hairy root culture, the bubble column reactor was superior to mist reactors for the biomass concentration [52,53]. Souret *et al*. [53] examined the difference between the two types of bioreactors, a mist reactor and a bubble column reactor. Mist reactors produce significantly more artemisinin, while bubble column reactors produce greater biomass. The roots grown in shake flasks contain a negligible amount of artemisinin. The high-density culture of red beet hairy roots was obtained by a radial flow reactor, which consists of a cylindrical vessel with a radial flow of medium [54].

4.4. LIQUID-DISPERSED BIOREACTOR

The reactors used for hairy root culture can be classified as either liquid-phase or gas-phase. Liquid-dispersed reactor is advantageous both for sufficient oxygen supply to roots and for a low shear stress environment compared with reactors in which the roots remained submerged in a liquid medium [50]. In liquid-dispersed reactors, roots are exposed to ambient air, or gas mixture, and the nutrient liquid, which is dispersed as spray or mist onto the top of the root bed [52,55]. The sprayed liquid and mist are drained from the bottom of the bioreactor to a reservoir and is re-circulated. The degree of distribution of liquid varies according to the mechanism of liquid delivery at the top of the reactor chamber. Various types of liquid-dispersed reactors are developed for the hairy root culture. Mist or nutrient mist [56-59], droplet [52,59], trickle-bed or tricking film [57,60], and drip-tube [61] are reported. In these bioreactors, certain types of configurations to internal support of roots such as glass beads, rasching rings, steel wire scaffolding, polyurethane foam, horizontal mesh trays, and cylindrical stainless steel mesh are invented [52,57,59-61]. *Cichorium intybus* hairy roots grown in an acoustic mist bioreactor produce nearly twice as much aesculin as compared to roots grown in bubble column and nutrient sprinkle bioreactors [62]. *Artemisia annua* hairy roots

grown in nutrient mist reactors produce nearly three times as much artemisinin as roots grown in bubble column reactors [63], and the authors suggest that higher levels of artemisinin in roots grown in the mist reactors are due to a response to the increased osmotic strength of the medium within the mist reactor, the medium becomes concentrated due to water evaporation [63]. In contrast to artemisinin accumulation in *Artemisia annua* hairy roots, the mist reactor accumulates lower biomass than does the bubble column reactor due to insufficient nutrient availability [52].

5. Commercial production of *Panax ginseng* roots via balloon type bioreactor

Panax ginseng has been used for important Oriental medicine since ancient time, owing to its tonic properties. The ginseng root contains terperpenoid saponins, referred to as ginsenosides. Cultivation of ginseng requires at least more than four years under shade condition and also requires the careful control of disease. Cell and organ culture technology have been developed for the alternative production of ginseng raw materials and secondary metabolites. The ginseng cell culture has been applied to the production of useful secondary metabolites [64,65]. Hormone-independent embryogenic cells are induced and cultivated via a bioreactor [66,67]. The cell suspensions produced from pilot scale culture have been commercialized into various ginseng tea and tonic beverages by Nitto Denko Co., Japan. [68].

Hairy roots provide an efficient way of biomass production due to fast growth and displays high biosynthetic capabilities that are comparable to those of natural roots [6, 11,12]. There are many publications on the hairy root culture of ginseng [43,69]. However, hairy roots are still not well utilized for the production of health food and need further analysis for the safety of proteins and compounds expressed by introduced genes of T-DNA. Recently, hairy-like adventitious roots culture without transformation with *Agrobacterium rhizogenes* was reported [70,71]. Induction and growth of hairy-like adventitious roots is achieved from initial root explants by exogenous auxin supply, which is direct motive for the mass production of ginseng roots for commercial scale. Son *et al*. [71] designed a balloon-type bubble bioreactor (BTBB) (Figures 1, 2A), which is superior for biomass growth than the bubble column bioreactor, and stirred tank bioreactor in cell culture of *Taxus cuspidata* [72], *Beta vulgaris* hairy roots [73], ginseng hairy root [74] and adventitious root culture [75]. The fresh weight of ginseng hairy-like adventitious root culture in 20-L BTBB was three-times higher than that of the stirred tank bioreactor [71]. The maximum biomass production of 2.2 kg fresh weight in 20-L bioreactor was obtained after 42 days after inoculation of 240 g [76]. In mountain ginseng cell line maintained by CBN Biotech Co., Korea, biomass growth of ginseng roots is reached to 30-fold of inoculums after 42 days of culture (Table 1).

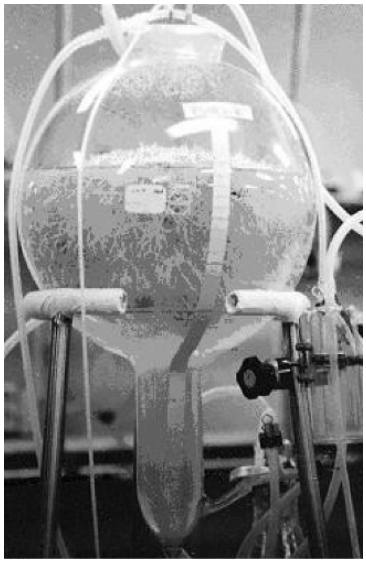

Figure 1. Actively growing ginseng hairy roots in 20-L balloon-type bubble bioreactor after 42 days of culture. Photograph provided by Son SH of VitroSys Co., Korea.

Table 1. Growth and saponin accumulation of adventitious ginseng roots after 42 days of culture in 5, 20, 500 and 1,000-L balloon-type bubble bioreactors.

Working volume (L)	Inoculums (g)	Fresh Wt. (g)	Dry Wt. (g)	Saponin content (mg/g^{-1} Dry Wt.)
4	20	520	48	5.6
18	90	2,294	212	5.8
500	2,500	58,500	5,800	6.0
1000	50,000	108,000	120,000	33.5*

* Methyl-jasmonate (100 μM) treatment 7 days before harvest.

The pilot-scale 500 and 1000-L stainless bioreactor was designed according to the BTBB type (Figure 2B). This reactor is comprised of a main body, air bubbling device, steam generator for sterilization, air inlet, air vent system, and various control systems for checking the temperature, oxygen, pH, and pipeline systems for transferring steam, air, medium, and root masses (Figure 3). Additional equipments such as a distilled water reservoir, medium mixer, medium sterilizer, and inoculation bioreactor are necessary.

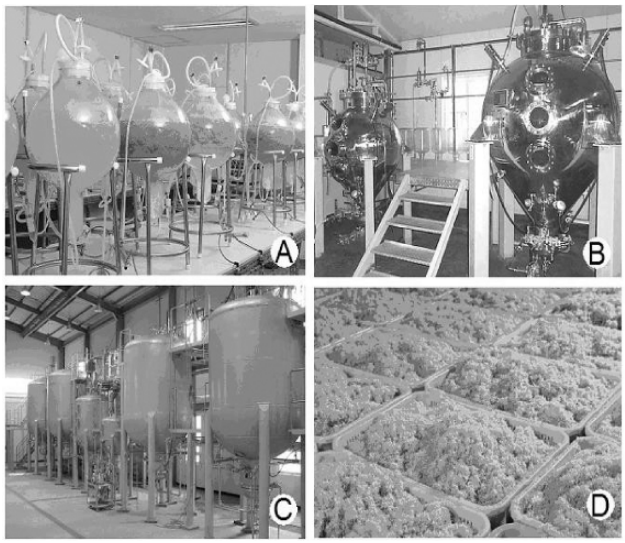

Figure 2. Scale-up of hairy-like adventitious roots of Panax ginseng. (A) 20-L balloon-type bubble bioreactors. (B) 500 and 1000-L pilot-scale balloon-type bubble bioreactors. (C) 10,000-L pilot-scale balloon-type bubble bioreactors for the commercial production of ginseng roots. (D) Harvested ginseng roots from a 10,000-L pilot-scale balloon-type bubble bioreactor. Photograph provided by Paek KY of CBN Biotech Co., Korea.

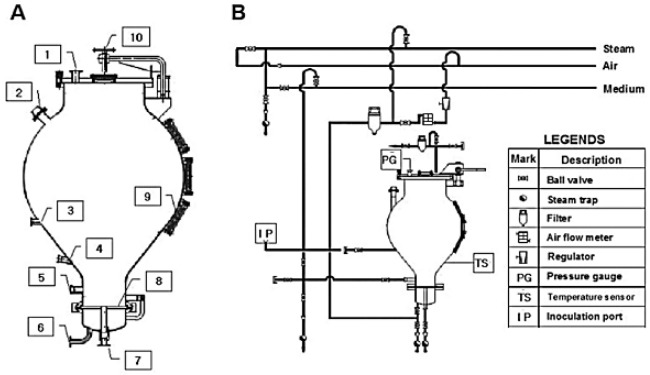

Figure 3. Schematic diagram of a balloon type bioreactor (A) and steam, air, and medium flow (B) in pilot scale culture (1,000 L). 1, ventilation port; 2, light glass; 3, dissolved oxygen probe port; 4, pH probe port; 5, inoculation port; 6, air inlet; 7, medium drain port; 8, stainless sparger; 9, sight glass; 10, screwed lid opener.

Before transfer to large-scale tanks, root tissues are homogenized into approximately one cm length size and are moved via an air compressor though the inter-connector between the inoculation reactor and the main tanks. The increase of the fresh weight of ginseng roots was more than 30-fold after 40 days of culture in both bioreactors. The

biomass increase in this bioreactor was similar to the ginseng hairy root culture [43,69]. There is no serious problem with the stagnation of fluid flow and limit oxygen due to the actively growing root mass. Based on the pilot-scale balloon-type bioreactor, production of ginseng roots via 10,000-L bioreactor was practically attempted for the commercial production (Figure 2C). In Korea, three companies produce the ginseng roots commercially using pilot-scale bioreactor (10,000 to 20,000-L) and the basic design follows the balloon-type bubble bioreactor. The root materials are processed into various types of health foods and food ingredients (Figure 2D).

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