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Comparative Evaluation of Modified Bioreactors for Enhancement of Growth and Secondary Metabolite Biosynthesis Using *Panax ginseng* Hairy Roots

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Abstract Hairy root cultures have demonstrated great promise in terms of their biosynthetic capability toward the production of secondary metabolites, but continue to constitute a major challenge with regard to large-scale cultures. In order to assess the possibility of conducting mass production of biomass, and the extraction of useful metabolites from *Panax ginseng*. *P. ginseng* hairy roots, transformed by *Rhizobium rhizogenes* KCTC 2744, were used in bioreactors of different types and sizes. The most effective mass production of hairy roots was achieved in several differently sized air bubble bioreactors compared to all other bioreactor types. Hairy root growth was enhanced by aeration, and the production increased with increasing aeration rate in a 1 L bioreactor culture. It was determined that the hairy root growth rate could be substantially enhanced by increases in the aeration rate upto 0.5 vvm, but at aeration rates above 0.5 vvm, only slight promotions in growth rates were observed. In 20 L air bubble bioreactors, with a variety of inoculum sizes, the hairy roots exhibited the most robust growth rates with an inoculum size of 0.1% (w/v), within the range 0.1 to 0.7% (w/v). The specific growth rates of the hairy roots decreased with increases in the inoculum size.

Keywords: *Panax ginseng*, hairy root, bioreactor, scale-up, secondary metabolite

INTRODUCTION

Plant-derived natural products have been, and will continue to be, valuable sources for a host of useful compounds. Many natural products can be obtained by direct extraction from plants. This method, however, is known to cause serious ecological problems. The large-scale production of valuable materials, by virtue of field-grown plants and original habitats, has been limited, primarily, by a variety of environmental factors, including low growth rates, restricted cultivation areas, climate dependency, pests, plant diseases, labor shortages and the overall time-consuming nature of the tasks inherent to the pursuit [1-3].

Despite the enormous potential for the derivation of useful products from plants, the mass production of useful metabolites from plant cells and tissues or organ cultures grown in bioreactors, has been largely unsuccessful in commercial applications thus far. Notable exceptions include the commercial production of shikonin, berberine and ginseng cells in Japan, and pilot-scale trials for the production of sanguinarine, rosmarinic acid, digoxin, geraniol and immunologically active polysaccharides, which are currently underway in the USA, Canada, and Germany [4-6].

Hairy roots induced by *Rhizobium rhizogenes* constitute a valuable and promising source of root-derived phytochemicals, which have proven useful as pharmaceuticals, cosmetics, pigments and food additives. These transformed hairy roots are able to synthesize similar, or even greater, amounts of several metabolites to those produced by the original plants [1]. Hairy roots are characterized by their high growth rates, high metabolite productivity and inherent genetic stability [7,8]. The transformed hairy roots of many plant species have been intensively studied, with an eye toward the *in vitro* production of secondary metabolites [9]. In the near future, this approach may become a reality, allowing for the commercial production of a multitude of useful compounds from a plant-derived transgenic hairy root culture system [5].

Several bioreactors have been designed for the large-scale production of hairy roots [8,10]. Bioreactors used for hairy root cultures tend to be relatively complex, due

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to the necessity for unique configurations, which can compensate for the heterogeneous, cohesive, structured and entangled nature of fibrous roots [5,11].

Despite the many recent improvements in our understanding of plant tissue cultures in bioreactors, this technology has met with even less commercial success than observed for cell suspension cultures for the production of secondary metabolites. However, plant tissue cultures remain a promising goal, and many such projects are currently under development [12].

Ginseng plants appear to exert many beneficial bioactive effects on human health, including haemostatic qualities, as well as abilities to promote blood circulation, relieve pain, cure bleeding wounds and trauma, relieve stress, and improve the immune function. A great deal of chemical, biochemical and pharmacological research has been carried out on ginseng plants to date [13,14]. The major compounds involved in pharmaceutical interactions in ginseng have previously been isolated and identified. These include ginseng saponin (ginsenosides), polysaccharides, antioxidants, peptides, fatty acids, alcohols, vitamins and phenolic compounds [13,15]. In recent years, ginseng polysaccharides have been considered as potentially useful compounds, exerting important pharmacological effects, and apparently possessing immune stimulation, antitumor and anti-hepatitis, mitogenic and hypoglycemic properties [8].

In this study, a transformed hairy root culture system, induced from *Panax ginseng* C.A. Meyer, which is widely known to have substantial amounts of active ingredients and a great deal of efficacy, was developed for the production of useful metabolites. This system was then applied to variant bioreactors, on a relatively large scale, in order to conduct a trial of the mass production of *P. ginseng* hairy root biomass and confirm the possibility of useful metabolites biosynthesis.

MATERIALS AND METHODS

Hairy Roots and Culture

The hairy roots of *P. ginseng* C.A. Meyer were initiated and maintained as described previously [16]. In all experiments, the hairy roots were cultivated in liquid hormone-free 1/2 MS medium, containing 30 g/L sucrose. The pH of the medium was adjusted to 5.8 with 2 M NaOH, and autoclaved at 121°C for 15 min before use. The cultures were incubated at 23 ± 1°C, in darkness, in a 250 mL Erlenmeyer flask, on a rotary shaking incubator, operated at 80 rpm.

Experimental Procedures

Effect of Air Flow Rate

In order to determine the effects of airflow rate, a 1 L bioreactor (working volume of 800 mL) was used. This bioreactor had a height/diameter ratio of 1.4. Bubble bioreactors have no internal mechanical moving parts. About 3.2 g (0.4% (w/v)) of hairy roots were inoculated into the bioreactor, and filtered air supplied at flow rates of 0.02, 0.05, 0.2, 0.5, or 1.0 vvm to the bottom of the reactor.

Air Bubble Bioreactors

3, 5, 12, and 20 L column bioreactors, with working volumes of 2.5, 4, 10, and 16 L, were used as our experimental bioreactors. These bioreactors had height/diameter ratios of 7.14, 1.41, 0.83, and 1.48, respectively. Each bioreactor was inoculated with a various inoculum size. The supplied aeration rate was about 0.5 vvm to the bottom of the reactor using a sparger.

Horizontal Air Bubble Bioreactor

A 20 L horizontal air bubble bioreactor (w.v. of 16 L; height/diameter ratio of 0.67) was also used. As with other air bubble bioreactors, it had no internal mechanical moving parts. Hairy roots were inoculated at different inoculum sizes into the bioreactor, and filtered air supplied at a rate of 0.5 vvm, with three spargers, to the bottom of the reactor.

Analytical Methods

In order to determine the biomass weight, the hairy roots were harvested, rinsed with distilled water and the extra water eliminated. The treated hairy roots were then measured for fresh and dry weights. The dry weight was measured gravimetrically after drying the roots for 24 h at 60°C. In the medium, the reducing sugar was measured colorimetrically by the 3,5-dinitrosalicylic acid (DNS) method [17], using a spectrophotometer (DR/4800, HACH, CO, USA). A standard curve was constructed for glucose. The total sugars were measured via the phenol-sulfuric acid method, using a spectrophotometer, with sucrose used as the standard. The conductivity (expressed in milli Siemens; mS) measurements of the culture medium were conducted using a conductivity meter, Model CM-20E (TOA Electronics, Kobe, Japan; cell constant k = 1.013), at a constant temperature of 25°C.

Measurement of Sulfite Oxidation

Sulfite is oxidized to sulfate in a zero-order reaction, in the presence of Cu^{2+} as a catalyst. Due to the rapid reaction, the actual dissolved oxygen (DO) concentration in the medium approaches zero. The oxygen transfer rate (OTR) is identical to that of oxygen consumption, which is half that of sulfate formation. 800 mL of 0.2 mol/L Na_2SO_3 and 1.344 mL of 0.63 mol/L $CuSO_4$ solutions were added to a 1 L bioreactor [18]. The OTR was determined at 23°C. A 5 mL sulfate solution sample, taken from the flask at different intervals, was mixed with 5 mL of 0.2 M KI-I_2 solution. Excess I_2 was then titrated with 0.1 mol/L standard thiosulfate ($Na_2S_2O_3$) solution. The molar change rate of the $Na_2S_2O_3$ solution used for the titration of two consecutive samples, which equals one fourth of the OTR [18].

Analysis of Metabolite Content

In order to quantify the total ginseng saponin, 100 mg

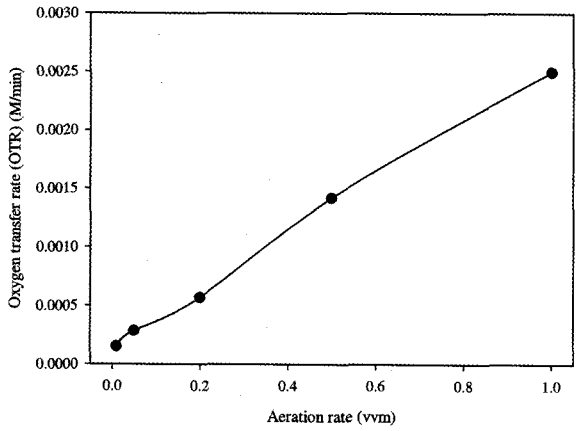

Fig. 1. Effects of aeration rate on the oxygen transfer rate in a 1 L bioreactor. The OTR (M/min) was measured at 25°C, with a working volume of 800 mL.

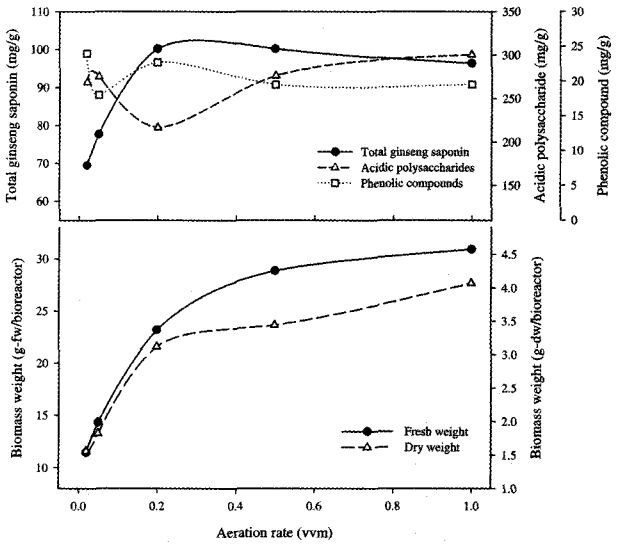

Fig. 2. Effect of aeration rate on hairy roots culture in a 1 L bioreactor.

of powdered dry hairy roots were soaked in 5 mL of n-BuOH saturated with distilled water, stored at 4°C for 24 h, sonicated in an ultrasonic cleaning bath for 60 min, and centrifuged twice at 10,000 rpm for 10 min. The collected supernatants were then used for the analysis of the total amount of ginseng saponin. The total ginseng saponin was measured via the Vanillin-H_2SO_4 colorimetric method [16]. In order to determine the amount of acidic polysaccharides and phenolic compounds, 100 mg of powdered dry hairy roots were soaked in 5 mL of 70% MeOH, sonicated for 30 min, and centrifuged twice for 10 min at 10,000 rpm. The collected supernatants were then used for the analysis of acidic polysaccharides and phenolic compounds, via the carbazole reaction and Folin-Denis methods, respectively [16].

RESULTS AND DISCUSSION

As plant cells/tissues produce unique and useful metabolites, their production in large-scale bioreactors would clearly be useful. Shake flasks, which provide relatively high biomass yields, and are also associated with efficient metabolite production, are considered both the simplest and smallest type of bioreactor, with a great deal of published research on hairy root cultures having been carried out with these instruments [19]. Hairy roots from several plant species have been investigated for their efficacy in scale-up bioreactor studies. However, due to the structural features and metabolite biosynthesis characteristics inherent to hairy root cultures, they require different bioreactor types than those used for plant cell cultures [9]. In this study, we used different bioreactor configurations, according to the idiosyncrasies of the particular hairy root cultures used.

Effect of Air Flow Rate

A non-uniform distribution of hairy roots in the culture space induces severe technological problems, including insufficient mass transfer of oxygen and nutrients, which can, in turn, result in a reduction of the secondary metabolite production, or even cell necrosis or autolysis. The extent to which oxygen and nutrients are transferred through the hairy root culture may be improved, to some degree, by increasing the agitation and/or aeration rates in the bioreactor. However, there are limits to how much these parameters can be increased, as root tissue can be easily damaged by excessive shear stresses [19,20]. In batch cultures, oxygen constitutes the only nutrient, which must be supplied continuously in order to meet the demands of the cells, as the solubility of oxygen in aqueous systems is relatively low (a maximum of 0.27 mmol/L at 25°C) [21].

Oxygen transfer rates were measured in a 1 L bioreactor, with an 800 mL working volume, at different aeration rates (0.01 to 1.0 vvm). The results for the OTR are shown in Fig. 1. The OTR was found to improve with increases in the aeration rate. In order to determine the effects of aeration rate on the growth of hairy roots, as well as the metabolite production in the bioreactors, a 1 L bioreactor, with an 800 mL working volume, was used in this experiment. About 3.2 g (fresh wt) (0.4% (w/v) inoculum) of hairy roots were inoculated into the bioreactor, and filtered air supplied at rates of 0.02, 0.05, 0.2, 0.5, and 1.0 vvm to the bottom of the reactor. The hairy root growth was enhanced with increases in the aeration rate in the 1 L bioreactor culture, as shown in Fig. 2. With increasing aeration rates upto 0.5 vvm, the growth rates of the hairy roots were sharply enhanced. However, at aeration rates above 0.5 vvm, only minor growth promotion was observed. These results indicate that the aeration rate range should be optimized in order to ensure optimal growth and metabolite production in the plant cell cultures. The total ginseng saponin content of the hairy roots was found to increase with increasing in the aeration rate, but the levels of acidic polysaccharides

Table 1. Comparison of growth kinetics and metabolite productions for *P. ginseng* hairy roots in bioreactors

Bioreactor type	Working volume (mL)	Inoculum size (g-fw)	Culture time (day)	Growth rate (d^{-1})	Total ginseng saponin (mg/g)	Acidic polysaccharide (mg/g)	Phenolic compound (mg/g)
Air bubble	800	3.2	36	0.451	–	–	–
	2,000	8.0	17	0.349	81.3	119.3	14.1
	4,000	16.0	35	0.534	91.6	348.3	15.6
	10,000	20.0	37	0.414	76.3	294.3	12.0
	16,000	32.0	36	0.371	85.8	254.9	11.5
20 L horizontal	16,000	32.0	35	0.407	87.3	270.4	14.7
bubble	16,000	64.0	41	0.181	68.1	202.0	15.2

and phenolic compounds were not affected by the aeration rates, except at a rate of 0.2 vvm. Kreis and Reinhard [22] explained that the influence of a dissolved oxygen level of 50% allowed for an alkaloid yield of around 3 g/L of culture after 20 days growth in an airlift bioreactor. Higher aeration rates produced dramatic decreases in the alkaloid production. Smart and Fowler [23] cultivated *Catharanthus roseus* in an airlift reactor, using initial aeration rates in the range 0.33 to 1.33 vvm. Increases in the aeration rate up to 0.66 vvm resulted in 40~70% increases in cell yields, but the cell yields dropped by 38% at an aeration rate of 1.32 vvm compared to those at 0.66 vvm.

Characteristics of Hairy Roots in Air Bubble Bioreactors

In bubble bioreactors, aeration and mixing are achieved by air sparging. As in an airlift bioreactor, the bubbles create less shear stress in a bubble column, which makes this method useful for hairy root cultures. Bubble column bioreactors are structurally very simple, so this type of bioreactor requires little initial investment and maintenance capital, with a low probability of contamination [5]. Table 1 summarizes the results of the hairy root growth characteristics and metabolite productions in various air bubble bioreactors. The growth rates obtained from several bioreactors were found to be higher than those obtained from flask cultures.

A 2.5 L balloon-type air bubble bioreactor, with a 2 L working volume, was also used. Aeration and mixing were achieved by air bubbling. An approximate 0.4% (w/v) hairy root inoculum was inoculated into the bioreactor, and filtered air supplied at a rate of 0.5 vvm to the bottom of the reactor. Over 17 days of cultivation, the hairy roots initially grew from 8 g-fw to about 47.5 g-fw (5.36 g-dw), exhibiting a growth rate of 0.473 (0.349) per day, on a dry wt (fresh wt) basis. The floating of hairy roots in the upper part of the medium, due to air bubbles, during the culture period was the principal cause of this low growth rate, but the culture space was diminished by the shape of the bioreactor.

The total ginseng saponin content of the hairy roots in this type of bioreactor was determined to be 81.3 mg/g, on a dry wt basis. The acidic polysaccharide and phenolic compound contents were 119.3 and 14.1 mg/g, respectively, on a dry wt basis.

In a 1 L air bubble bioreactor, with a 1.4 height/diameter ratio, *P. ginseng* hairy roots exhibited a growth rate of 0.458 (0.464) per day, on a dry wt (fresh wt) basis. After 35 days, the total biomass weight of the hairy roots had undergone a 16.3-fold increase relative to the inoculated amount. When approximately 0.4% (w/v) of hairy root was inoculated into a 5 L air bubble bioreactor, the hairy roots initially grew from 16 g-fw (about 1.33 g-dw) to about 299 g-fw (21.6 g-dw) over 35 days of cultivation, exhibiting a growth rate of 0.463 (0.534) per day, on a dry wt (fresh wt) basis. The total ginseng saponin content of the hairy roots in this bioreactor type was 91.6 mg/g, on a dry wt basis. Acidic polysaccharides and phenolic compound contents were 348.3 and 15.6 mg/g, respectively, on a dry wt basis.

In this particular bioreactor trial, we used a 12 L bioreactor, with a 10 L working volume and H/D = 0.83. An approximate 0.2% (w/v) inoculum of hairy root was inoculated into the bioreactor, and filtered air supplied at a rate of 0.5 vvm to the bottom of the reactor. Over 37 days of cultivation, the hairy roots initially grew from 20 g-fw to about 306 g-fw (32.9 g-dw), exhibiting a growth rate of 0.534 (0.414) per day, on a dry wt (fresh wt) basis. The total ginseng saponin content of the hairy roots in this type of bioreactor was 76.3 mg/g, on a dry wt basis. Acidic polysaccharide and phenolic compound contents were 294.3 and 12.0 mg/g, respectively, on a dry wt basis.

Fig. 3 shows the time course of the growth and nutrition consumption of *P. ginseng* hairy roots in the 20 L air bubble bioreactor (w.v. of 16 L, H/D = 1.48), supplied with a 0.2% (w/v) inoculum. After 36 days of cultivation with this inoculum, the weight of the hairy roots increased by about 13-fold, on a fresh wt basis (18-fold on a dry wt basis), exhibiting a growth rate of 0.34 d^{-1}. These results demonstrate the possibility for the mass cultivation of *P. ginseng* hairy roots using a bioreactor. Kim *et al.* [22] reported that hairy roots grew to about 37 times (2.7 to 101 g dw) their original amount in a 20 L airlift bioreactor, using a *Phytolacca esculeuta* hairy root culture. Fig. 3B shows the change in fresh wt/dry wt

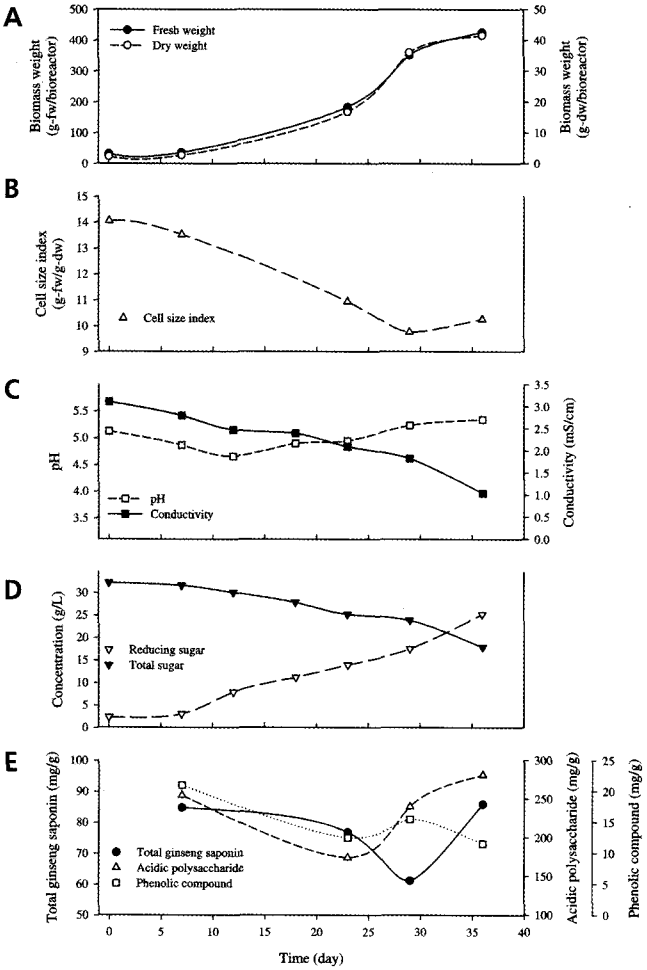

Fig. 3. Time course of the growth, nutrient consumption and metabolite production of hairy roots in a 20 L air bubble bioreactor, with a 0.2% inoculum.

(FW/DW) ratios of the hairy roots during the culture period. The water content decreased in the first 29 days, but thereafter subsequently increased. The FW/DW ratio of the hairy roots increased with decreasing osmotic pressure, which occurred due to nutrient consumption. As shown in Fig. 3C, the medium pH consistently dropped to 4.65 after the first 12 days, but subsequently gradually increased, to 5.34. The conductivity of the medium was observed to decrease inversely with increasing biomass, decreasing from 3.11 to 1.04 mS/cm during the culture period. Decreases in the conductivity of the medium were determined to reflect the amounts of electrolytic or inorganic nutrients consumed by the cells [16]. The total sugar concentration was also observed to decrease inversely to increasing biomass, as shown in Fig. 3D. Sucrose was initially hydrolyzed, but continuously consumed afterwards, from 32.1 to 17.85 g/L during the culture periods. The reduction in the sugar concentration accelerated consistently throughout the experimental periods. As shown in Fig. 3E, the total ginseng saponin content of the hairy roots in this type of bioreactor decreased after 28 days. This result can be explained by noting that, at that period, the hairy root growth was higher than at any other period. Therefore, less ginseng saponin was synthesized compared to the other compounds, as secondary metabolites are generally synthesized during the non-growth phase. Acidic polysaccharide and phenolic compound contents were observed to increase more robustly than those of the total ginseng saponin.

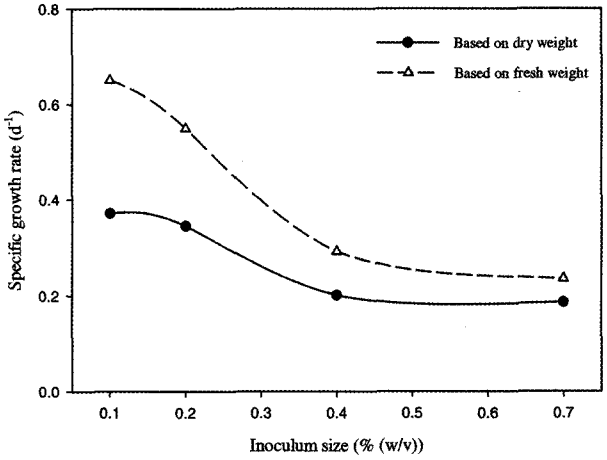

Fig. 4. Effects of inoculum size on the specific growth rate of hairy roots in a 20 L air bubble bioreactor.

Effect of Inoculum Size on the Growth of Hairy Roots in the 20 L Air Bubble Bioreactor

In order to characterize the effects of inoculum size on the hairy root growth in the 20 L bioreactor, the hairy roots were inoculated in sizes of 0.1 to 0.7% (w/v). Fig. 4 shows the specific growth rates of the *P. ginseng* hairy roots for the different inoculum sizes. Hairy roots exhibited the highest growth rates with a 0.1% (w/v) inoculum ratio. The specific growth rates of the hairy roots were demonstrated to decrease with increasing inoculum size. The inoculum ratios and final cell weights were not proportional to increases in the inoculum size, but were limited by the substrate, dissolved oxygen and limited space in the bioreactor. Carvalho and Curtis [24] reported that, with a root culture of *Hyoscyamus muticus*, growth rates decreased, from 0.43 to 0.24 d^{-1}, due to an increase in the inoculum size, within the range of 0.1 to 4 g-fw/L.

20 L Horizontal Air Bubble Bioreactor

In a 20 L horizontal bioreactor (H/D = 0.41; w.v. of 16 L), free of internal moving parts, about an 0.2% (w/v) hairy root inoculum was introduced into the bioreactor, and filtered air supplied at a rate of 0.5 vvm, with three spargers, to the bottom of the reactor. Over 35 days of cultivation, hairy roots initially grew from 32 g-fw to about 456 g-fw (45.3 g-dw), exhibiting a growth rate of 0.484 (0.407) per day, on a dry wt (fresh wt) basis. The total ginseng saponin content of the hairy roots was de-

termined to be 87.3 mg/g, on a dry wt basis. Acidic polysaccharide and phenolic compound contents were found to be 270.4 and 14.7 mg/g, respectively, on a dry wt basis. When an approximately 0.4% (w/v) inoculum of hairy roots was introduced to the bioreactor, the hairy roots initially grew from 64 g-fw to about 476 g-fw (41.85 g-dw) within a 41-day culture period, exhibiting a growth rate of 0.191 (0.181) per day, on a dry wt (fresh wt) basis. Under these conditions, the total ginseng saponin content of the hairy roots was determined to be 68.1 mg/g, on a dry wt basis. Acidic polysaccharide and phenolic compound contents were 202 and 15.2 mg/g, respectively, on a dry wt basis.

CONCLUSIONS

In order to assess the possibility of mass producing biomass, and obtain useful metabolites from *P. ginseng* hairy roots, several types and sizes of bioreactors were evaluated. Hairy root growth increased with increasing aeration rate in 1 L bioreactor cultures. The hairy root growth rates were markedly improved by increases in the rate of aeration up to 0.5 vvm. In air bubble bioreactors with different sizes, the mass production of hairy roots proved more effective than in any other bioreactor type, with the exception of a stage-separated air bubble bioreactor. In the 20 L air bubble bioreactor, inoculated with a variety of hairy root inoculum sizes, the hairy roots exhibited the highest growth rate with an inoculum size of 0.1% (w/v), within the range 0.1 to 0.7% (w/v). The specific growth rates of the hairy roots were reduced by increasing inoculum size. The inoculum ratio and final biomass weight were not proportional to increases in the inoculum size. The levels of useful metabolite production from *P. ginseng* hairy roots in the various bioreactors were similar, or even greater, than those previously obtained from flask cultures.

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