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Comparison of Growth Characteristics of *Panax ginseng* Hairy Roots in Various Bioreactors

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Abstract

This study investigated the effects of flask-to-liquid volume ratio on the growth of *Panax ginseng* hairy root, transformed by *Agrobacterium rhizogenes*, in flask cultures and compared the characteristics of various bioreactors for scale-up. The flask-to-liquid volume ratio was optimum at 1.5 mL of air/mL of medium in flask cultures, and hairy root growth was not affected above the optimum ratio. In 500-mL flask culture, hairy root showed two growth phases. After the first exponential growth, specific growth rate decreased. The growth characteristics of *P. ginseng* hairy root in various bioreactors were investigated. Hairy root growth was about 55-fold of inoculum after 39 d in a 5-L bioreactor and about 38-fold of inoculum after 40 d in a 19-L bioreactor. Carbon yield was higher in a 19-L bioreactor than in others, but it did not show any linear relationship to the growth rate of hairy roots in bioreactors.

Index Entries: *Panax ginseng*; transformed hairy root; ginseng polysaccharide; bubble bioreactor; flask-to-liquid volume ratio.

Introduction

Plants are the potential source of a large number of important biochemical constituents such as pharmaceuticals, food additives, pigments, flavors, fragrances, and fine chemicals. Large-scale plant cell and tissue

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cultures for producing useful products have been considered as an attractive alternative to whole-plant extraction for obtaining valuable chemicals *(1–3)*. Large-scale culture of plant cell and tissue has been hampered by a low growth rate, sensitivity to shear stress, surface adhesion, cell aggregation, and contamination *(4)*.

Transformed root cultures provide a promising alternative for biotechnological exploitation and a method for the constant and standardized production of useful metabolites of plant cells. Hairy roots are induced by the genetic transformation of plant cells by *Agrobacterium rhizogenes* and are characterized by a high growth rate; high secondary metabolite productivity; and inherent genetic stability, reflected in stable growth and reproduction *(5)*. Several bioreactor designs for large-scale production of hairy roots have been demonstrated *(6,7)*. Bioreactors used for hairy root culture are more complex owing to continuous growth of hairy root. They must have a unique configuration to compensate for the heterogeneous, cohesive, structured, and entangled nature of fibrous roots *(4)*. In flask and bioreactor cultures, oxygen transfer to hairy roots submerged in medium has three external mass transfer resistances: the gas-liquid, liquid-liquid, and liquid-solid resistances. In shake-flask cultures, the important parameters affecting flask properties are flask size, shaking speed, closure type, and medium volume *(8)*.

Panax ginseng C.A. Meyer, which belongs to the Araliaceae family, is one of the most famous oriental medicinal plants and is mainly distributed in Korean peninsula and China. Field cultivation of ginseng plant is a time-consuming and labor-intensive process. It takes 4–6 yr from seeding to final harvesting and requires much care because the growth is subjected to several conditions such as soil properties, climate, pathogens, pets, and inplant diseases *(6)*.

Ginseng plants have many beneficial bioactive effects on human health, such as their hemostatic qualities and abilities to promote blood circulation, relieve pain, cure bleeding wounds and trauma, relieve stress, and improve immune functions *(6,9,10)*. Many chemical, biochemical, and pharmacologic studies of ginseng plant have been conducted. The major compounds of pharmaceutical interaction in ginseng have been isolated and identified to be saponin (ginsenosides), polysaccharides, antioxidants, peptides, fatty acids, alcohols, vitamins, and phenolic compounds *(6,10)*. In recent years, ginseng polysaccharides have been regarded as useful compounds with important pharmacologic effects such as immune stimulation, as well as antitumor, antihepatitis, mitogenic, and hypoglycemic activities *(11)*. As cell-wall components (primary metabolite), the total cellular content of ginseng is fairly stable and a high productivity is more easily obtained compared with other components.

In the present study, we investigated the effect of liquid-to-flask volume ratio on the growth of *P. ginseng* hairy roots in flask cultures and compared culture characteristics and metabolite production in shake-flask and various bioreactors.

Materials and Methods

Plant Materials and Maintenance

Hairy roots of *P. ginseng* C.A. Meyer, induced and established by the root-disk method *(12)*, were used. They were maintained on hormone-free 1/2 MS (1/2-macro MS) *(13)* liquid and solid medium containing 30 g/L of sucrose at 23°C under dark conditions, and subcultured once every 3 wk.

Medium and Culture of Hairy Roots

In all experiments, hairy roots were cultivated in liquid hormone-free 1/2 MS medium containing 30 g/L of sucrose. The pH of the medium was adjusted to 5.8 with 2 *N* NaOH, and the medium was autoclaved at 121°C for 15 min prior to use. Cultivations were carried out at 23°C in dark or light conditions, using the bioreactors described next.

Shake-Flask Cultures

For shake-flask cultures, a 500-mL Erlenmeyer flask with 200 mL of MS medium was shaken at 70 rpm in dark conditions on a rotary shaking incubator (Vision Scientific). About 1 g fresh wt of hairy roots was inoculated into the flask. All data were obtained from three samples.

Solid Culture of Hairy Roots

Solid culture experiments were performed in 250-mL flasks containing 50 mL of 1/2 MS solid medium containing 30 g/L of sucrose and 0–8 g/L of agar incubated in dark, static conditions at 23°C.

Stirred Bioreactor

A 1-L bioreactor (800-mL working volume) was used for agitated cultivation. The agitator was a magnetic bar (5 mm id, 25 mm length). About 1 g hairy roots was inoculated in the bioreactor, and filtered air was supplied at a rate of 0.1 vvm at the bottom.

Bubble Bioreactors

A 3-L column (2.5 L [w/v]), 5-L bioreactor (4 L [w/v]), and 19-L bioreactor (17 L [w/v]) were used as bioreactors. These bioreactors have height/diameter aspect ratios of 7.14, 1.41, and 1.48, respectively. The bubble bioreactors are free of internal mechanical agitation parts. Each bioreactor was inoculated with 4, 4, and 36 g fresh wt of hairy roots, respectively. The supplied aeration rate was 0.1 vvm.

Experiment Using Different Liquid-to-Flask Volume Ratios

Erlenmeyer flasks (250 or 500 mL) contained 50, 100, 150, and 200 mL of liquid medium were covered with aluminum foil or a cotton stopper. About 1 g fresh wt of hairy roots was inoculated into every flask and cultured in a rotary shaking incubator (70 rpm) at 23°C under dark conditions for 26 d.

Analytical Methods

To determine cell mass, hairy roots were harvested, rinsed with distilled water, and the extra water was eliminated. Treated hairy roots were measured as fresh wt and dry wt. The dry wt was measured gravimetically after drying the roots at 60°C for 24 h. In the medium, reducing sugar was measured colorimetrically by the dinitrosalicylic acid method *(14)* using a spectrophotometer (DR/4800; HACH) and glucose was used as the standard. Total sugar was measured by the phenol–sulfuric acid method *(15)*, and standard curves were made by sucrose. The conductivity of culture medium was carried out at 20°C using a Model CM-20E conductivity meter, (cell constant k = 1.013; TOA Electronics, Japan).

Extraction and Analysis of Intracellular Polysaccharide

To determine intracellular polysaccharide, 100 mg of powdered dry hairy roots was suspended in 10 mL of distilled water, sonicated for 10 min, and centrifuged twice at 5030*g* for 10 min. The collected supernatant was used to determine intracellular polysaccharide by the phenol–sulfuric acid method *(12)*.

Calculation of Growth Rate and Metabolite Productivity Yields

Cell growth was evaluated in terms of average cell growth rate (GR). Average GR was defined as follows:

$$\text{GR} = (\text{final cell mass} - \text{initial cell mass})/\text{initial cell mass}/\text{time}$$

The yield coefficients (carbon) of cell in the hairy root cultures were calculated as follows:

$$Y_{X/S} = \text{mass of cell increased}/\text{substrate consumed}$$

Results and Discussion

Effect of Liquid-to-Flask Volume Ratio

In flask cultures, liquid-to-flask volume ratio had a significant effect on the biomass growth, requiring nutrients and oxygen. Figure 1 shows the results of hairy root growth according to liquid-to-flask volume ratio in 250- and 500-mL flask after 26 d. The 250-mL flask containing 100 mL of medium showed higher growth than the others. The final cell mass in flasks containing 50 and 150 mL of medium was lower than that in flasks containing 100 mL of medium. In the 250-mL flasks containing 150 mL of medium, the flasks covered with a cotton stopper showed about 1.4-fold higher growth than those with aluminum foil. In the case of 500-mL flask cultures, flasks containing 100 and 200 mL of medium were comparable with 250-mL flasks containing 100 mL medium. These results indicated that initial liquid volume ratio was optimum at 1.5 of mL air/mL of medium in the flask culture of hairy roots. With *Atropa belladonna* hairy

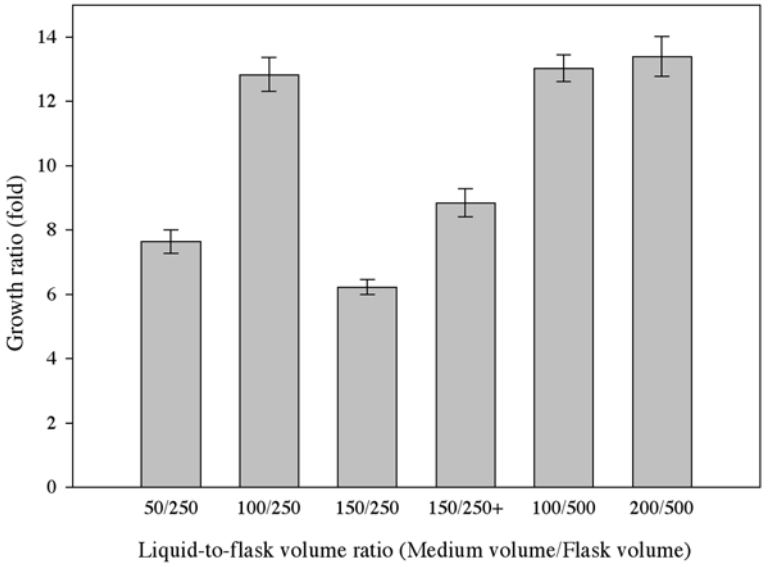

Fig. 1. Effect of medium volume size on growth of hairy roots cultured for 26 d in 250 and 500-mL flasks. All flasks were covered with aluminum foil except for 100/250+ (covered with cotton stopper).

root cultures, initial specific growth rates were higher at a 50-mL medium volume than at 100 mL *(16)*. In addition, Auro et al. *(8)* and van Suijdam et al.*(17)* reported that reducing the liquid volume in flask culture allows an increase in oxygen transfer rate. These reports suggested that air/liquid volume ratio is a limiting factor affecting the plant cell growth, and this parameter influences the effectiveness at mass transfer of gas-liquid and liquid-solid in flask cultures.

Characteristics of Hairy Roots in Flask Cultures

Figure 2 shows the growth characteristics, such as biomass growth on semilogarithmic coordinates, sugar consumption, changes in pH, and conductivity of the medium, of *P. ginseng* hairy roots in 500-mL Erlenmeyer flask culture. Two phases of lag and exponential growth were observed from a semilogarithmic representation of the data. Diauxic growth in plant cell and tissue cultures may occur when the cell is offered a catabolizable energy source in the presence of a more readily catabolizable energy source *(18)*. After a short lag period of about 4 d, the first exponential growth phase occurred between d 4 and 12 of the culture periods, and hairy roots had a specific growth rate (μ) of 0.057 d^{-1}. The second growth phase was between d 12 and 42 of the culture periods ($\mu = 0.019\ d^{-1}$). Generally, exponential growth has been reported to occur only during the first few d of hairy root culture periods by several researchers and for a range of plant

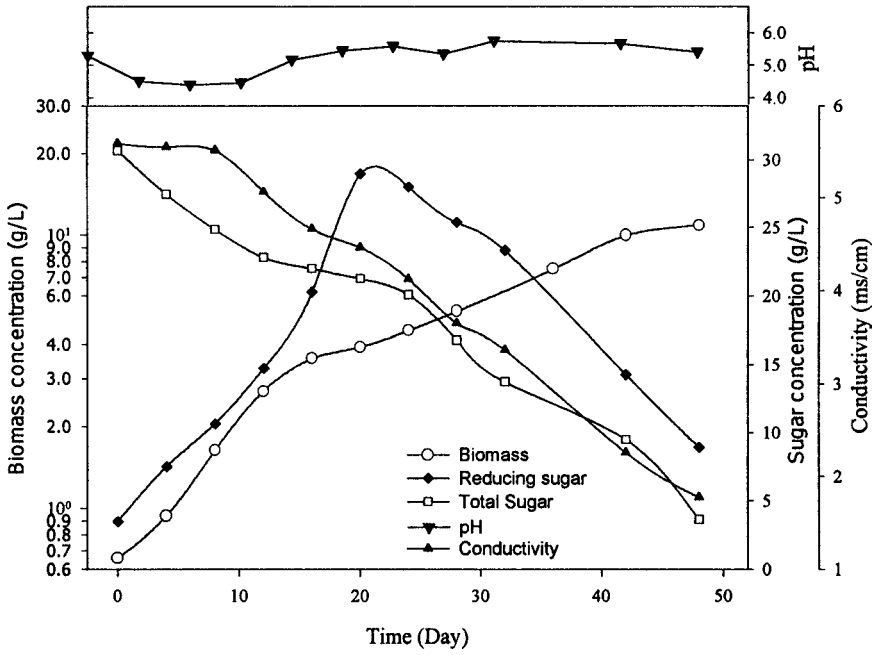

Fig. 2. Growth properties of *P. ginseng* hairy roots in 500-mL flask culture.

species *(16)*. After 42 d, the cultures entered stationary phase by substrate depletion and insufficiency of culture space. Finally, at 48 d, hairy roots grew about 16.5-fold of inoculum. The pH of the medium dropped from 5.3 to 4.5 after 12 d, but it gradually increased to 5.6 towards the end of the growth. Total sugar and medium conductivity decreased inversely to the increase in biomass. This decrease in medium conductivity appeared to reflect the amount of nutrients and electrolytes consumed by the cell *(16)*. Reducing sugar increased during the first 20 d of the culture and decreased afterward. The average specific growth rate of hairy roots was about 0.34 g dry wt/(g inoculum·d) for total culture periods.

Figure 3 shows the growth of hairy roots on 1/2 MS solid medium containing 0–8 g/L of agar in a solid culture environment. Hairy roots grew about 5.8-fold on medium containing 8 g/L of agar after 28 d. Solid medium conditions containing <3 g/L of agar showed lower growth. These results occurred by mass-transfer resistance, which was caused by hairy roots immersed in liquid or semisolid medium in static culture conditions.

Comparison of Performance of Various Bioreactors

Stirred Bioreactor

In the stirred bioreactor, agitation is performed by magnetic bar and the filtered air is introduced from the bottom of the bioreactor. During 27

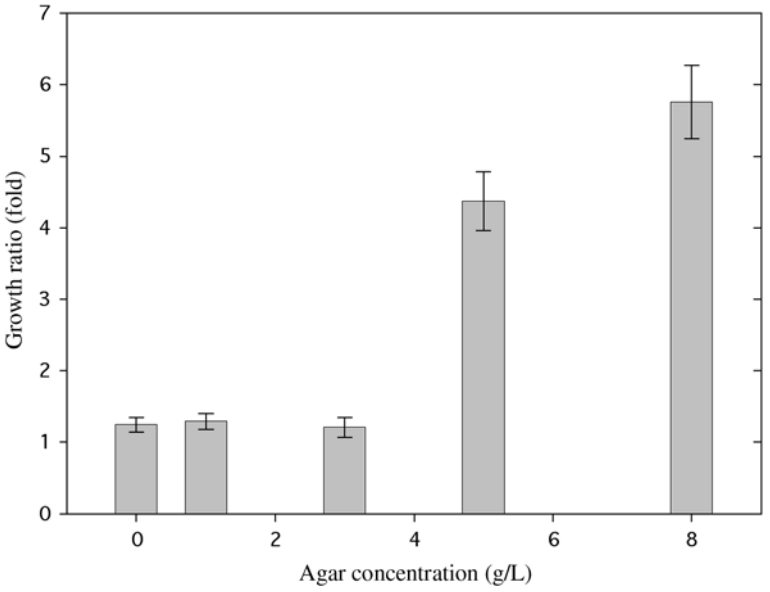

Fig. 3. Growth of hairy roots cultured for 27 d in 1/2 MS solid medium containing agar as solidifier.

d of cultivation, hairy roots grew about 24-fold of inoculum. The growth rate of hairy roots, 0.85 g dry wt/(g inoculum·d), was about 2.65-fold higher than in the 250-mL flask culture.

Bubble Bioreactor

In the bubble bioreactor, aeration and mixing are achieved by air sparging. Like an airlift bioreactor, the bubbles create less shear stress in a bubble column, so that the bubble reactor is useful for hairy root culture. Bubble column bioreactors are structurally very simple and therefore require only a low initial investment and maintenance capital, and have a low possibility of contamination *(4)*. In a 3-L bioreactor with a 7.14 height/diameter aspect ratio, the growth rate of hairy roots, 0.44 g dry wt/(g inoculum·d), showed low growth. This result explained that the culture space was limited by the floating of hairy roots on the upper side of the bioreactor by a high aspect ratio and air bubbles. Air bubbles were entrapped in the hairy root mat and resulted in floating of the root mat and inefficient contact with the liquid phase during the culture period.

Figure 4 shows the growth characteristics of *P. ginseng* hairy roots in a 5-L bioreactor for 39 d. Hairy roots increased about 55-fold of inoculum and the growth rate was 1.38 d^{-1}. The intracellular polysaccharide content, 0.11 g/g on a dry wt basis, was lower than in flasks. For extended culture periods, hairy roots caused several problems in the bubble column reactor.

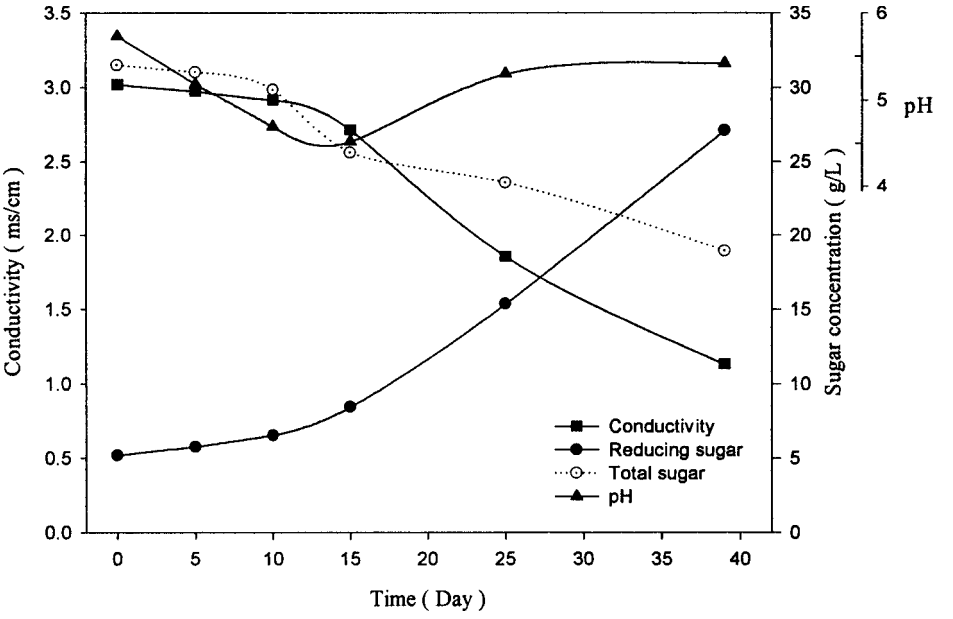

Fig. 4. Growth properties of hairy roots cultured for 39 d in 5-L bioreactor.

First, hairy roots grew on the upper partition of the bioreactor, causing a more limited use of culture space than suspension cultures. Second, at liquid culture, the meristem-dependent growth pattern of hairy roots constructed a root ball or mat, which consists of old tissue at the core, and grew young lateral roots around it. This root mat inhibited the mass transfer of nutrients and oxygen to the root mat core. Hairy roots in large-scale culture constructed greater root mats than those in small-scale culture at latter periods of cultivation.

Figure 5 shows the time course of the growth and nutrition consumption of *P. ginseng* hairy roots in a 19-L bioreactor. After 40 d, hairy roots increased about 38-fold of inoculum and showed a growth rate of 0.96 d^{-1}. This result was about 2.6 times higher than that observed in the 250-mL flask. Kim *(19)* reported that hairy roots grew about 37 times (2.7–101 g dry wt) in a 20-L airlift bioreactor using *Phytolacca esculeuta* hairy root culture. These results showed the possibility that the mass cultivation of *P. ginseng* hairy roots could be accomplished in a bioreactor. Sucrose was first hydrolyzed and continuously consumed afterward from 32.1 to 16.8 g/L during the culture periods. The pH of the medium was consistently maintained at 4.87 after the first 20 d and slowly increased to 5.2 afterward. Medium conductivity decreased from 2.82 to 0.82 ms/cm during the culture period. The intracellular polysaccharide content was 0.13 g/g on a dry wt basis. This value was lower than that of natural roots *(12)*.

Table 1 summarizes the results of hairy root growth characteristics and metabolite production in bioreactors. The growth rates obtained from

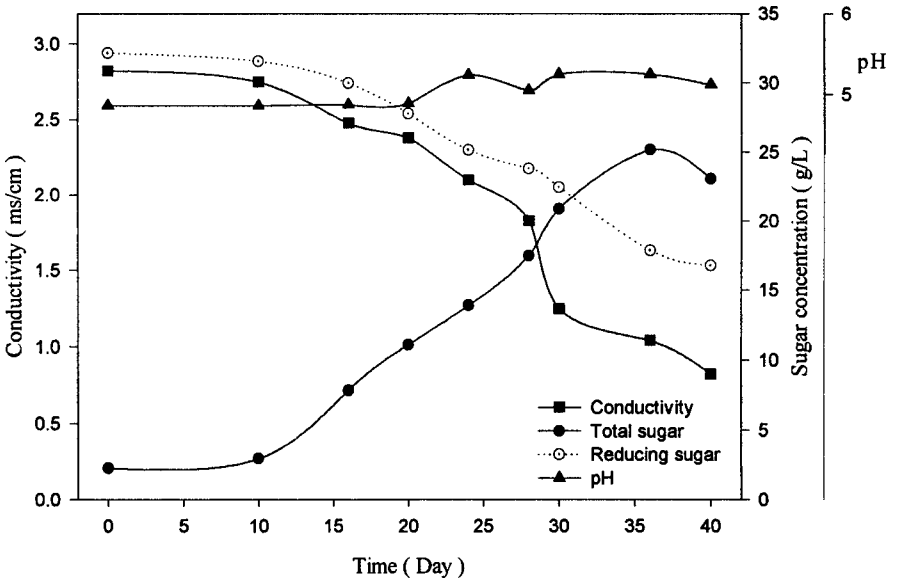

Fig. 5. Growth properties of hairy roots cultured for 40 d in 19-L bioreactor.

bioreactors were higher than from flask cultures. Intracellular polysaccharide content of hairy roots on bioreactors was in a range of 0.11–0.13 g/g on a dry wt basis. These results were lower than that of natural *P. ginseng* roots, 0.45–0.79 g/g on a dry wt basis. Natural *P. ginseng* root generally contained a lot of ginseng starch at the main root part *(12)*. Carbon yield was greatest in the 19-L bioreactor, but it did not show any linear relationship to the growth rate of hairy roots in bioreactors. The relationship between growth of carbon yield and the ionic effect needs to be studied in more detail.

Conclusion

In flask and bioreactor cultures, oxygen transfer to hairy roots submerged in medium have three external mass-transfer resistances. Thus, the most important parameters affecting flask culture properties are flask size, shaking speed, closure type, and medium volume. The liquid-to-flask volume ratio was optimum at 1.5 mL of air/mL of medium in flask cultures. In 500-mL flask culture, hairy roots showed two growth phases. After the first exponential growth phase, specific growth rate was decreased. Hairy roots showed a growth rate of about 1.38 and 0.96 d^{-1} in 5 and 19-L bioreactors, respectively. The yields of sugar and conductivity were higher in the 19-L bioreactor than in the others, but there was no linear relationship to the growth rate of hairy roots in bioreactors. Intracellular polysaccharide content of hairy roots on various bioreactors was in the range of 0.11–0.13 g/g on a dry wt basis. It was lower than those of natural ginseng roots, 0.45–0.79 g/g on a dry wt basis.

Table 1
Comparison of Growth Kinetics and Metabolite Production of *P. ginseng* Hairy Roots in Bioreactors[a]

Type of bioreactor	Culture time (d)	Aspect ratio (height/diameter)	Working volume (L)	Growth ratio (fold)	Growth rate (d^{-1})	Intracellular polysaccharide (g/g)	Carbon yield (g dry wt/g sugar)
Static culture	28	—	0.05	5.8	0.21	NT	NT
Shake-flask culture	48	—	0.20	16.5	0.34	0.19	0.19
Stirred type	27	1.43	0.80	24.0	0.85	NT	0.19
Bubble column type	25	7.14	2.60	11.0	0.44	0.11	0.13
Bubble column type	39	1.41	4.00	55.0	1.38	0.11	0.22
Bubble column type	40	1.48	17.00	38.0	0.96	0.13	0.18

[a]NT, not tested.

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