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Studies on Mass Production of Transformed *Panax ginseng* Hairy Roots in Bioreactor

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Abstract

The growth properties of *Panax ginseng* hairy roots transformed by *Agrobacterium rhizogenes* were compared between flask and aerated column or stirred bioreactor. In flask cultures, sucrose, initially 30 g/L, was nearly exhausted after 45 d of culture. The pH of the medium dropped from 5.5 to 4.96 after 10 d, but afterward it gradually increased to 6.4. After 45 d, hairy roots grew about 16-folds. The growth rate of hairy roots in air-bubble column or stirred bioreactor cultures was 1.13 (1.11) to 1.23 (1.20) g fresh wt (dry wt)/(g of cells·d), respectively. For both bioreactors, growth was about three times as high as in the flask cultivation.

Index Entries: *Panax ginseng*; transformed hairy roots; ginseng crude saponin; polysaccharide; *Agrobacterium rhizogenes*.

Introduction

The large-scale production of economically valuable biochemicals such as alkaloids, flavones, perfumes, pigments, terrenes, terpenoids, peptides, and polysaccharides by field-grown plants has been limited mainly by low growth rate, restricted cultivation area, dependency on climate, and labor shortages *(1,2)*. The development of plant cell and tissue culture technology has extended to the production of important

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phytochemicals. These techniques of plant cell and tissue culture are expected to minimize these disadvantages *(3)*.

Transformed root cultures provide a promising alternative for the biotechnologic exploitation, which is a method for the constant and standardized production of useful metabolites from plant cells. For a long time, researchers have had a great interest in plant hairy roots because of their fast growth, low doubling time, ease of maintenance on phytohormone-free medium *(4)*, and ability to synthesize a number of chemical compounds of root-derived secondary metabolites at a level similar to that found in the original plants *(5,6)*.

Seversal bioreactor designs for large-scale production of hairy roots have been demonstrated *(3,7,8)*. Bioreactors used in hairy root culture are complex owing to continuous growth of hairy roots. They must have a unique configuration to compensate for the heterogeneous, structured, and entangled nature of fibrous roots *(7)*.

Hairy roots cultured in either shake flasks or bioreactors tend to distribute unevenly throughout the vessel and form dense and highly tangled root clumps, which may resist flow, by branching the growth pattern of hairy roots. Conditions in the interior of the clump may be very different from the bulk liquid owing to mass-transfer effects. The nutrient limitation caused by the formation of dense root clumps leads to cell death and necrosis at the core of the root clumps *(9)*.

Scale-up of hairy root culture becomes difficult because of the need to simultaneously provide nutrients from both liquid and gas phases. The design of bioreactors for hairy root culture should consider factors such as the growth characteristics, the nutrient requirements and utilization rates, mass transfer, the mechanical properties, the requirement of a support matrix, and the possibility of flow restriction by the root mass in certain parts of the bioreactor *(9,10)*. Moreover, to obtain optimal biomass yield, the roots should be evenly distributed in the bioreactor *(11,12)*.

In this article, we describe the growth characteristics and secondary metabolite production of *Panax ginseng* hairy roots in flask and bioreactor cultures.

Materials and Methods

Plant Materials and Maintenance

The hairy roots of *P. ginseng* C. A. Meyer were used. These tissues were induced and established by the root-disc method *(13)*. The hairy roots were maintained on hormone-free 1/2 MS *(14)* liquid and solid medium containing 30 g/L of sucrose at 23°C under dark conditions, and subcultured once every 3 wk.

Medium and Cultures of Hairy Roots

The hairy roots were cultivated in liquid hormone-free 1/2 MS medium with 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. The cultivations were carried out at 23°C in dark or light conditions, using the bioreactors described next.

Rotary Shaking Incubator with Erlenmeyer Flask

For flask cultures, a 250-mL flask with 100 mL of medium was shaken at 70 rpm in dark conditions on a rotary shaking incubator (Vision Scientific).

Air Bubble Column Type of Bioreactor

A glass column (35 mm Ø × 500 mm, working volume of 1600 mL) was used as a bioreactor. This vessel had four compartment stages. Each was separated by stainless steel mesh. About 1 g fresh weight of hairy roots was inoculated into each compartment stage. The aeration rate was 0.5 vvm.

Modified Stirred Type of Bioreactor

A 7-L bioreactor (working volume of 5000 mL; Bioflo New Brunswick, NJ) was used for agitated cultivation. The reactor was installed with a cylindrical frame (65 mm Ø × 150 mm) which was made of stainless steel mesh (void size of 1 × 1 mm), and the frame was fixed in the reactor. The impeller with six flat blades was agitated at 70 rpm. Hairy roots (3 g fresh weight) were inoculated in the frame of the bioreactor, and air was supplied at a rate of 0.5 vvm at the bottom of the frame.

Analytical Methods

To determine cell mass, the hairy roots were harvested, rinsed with distilled water, and the extra water was eliminated. Treated hairy roots were measured as fresh weight and dry weight. The dry weight was measured gravimetrically after drying the roots at 60°C for 24 h. In the medium, reducing sugar concentration was measured colorimetrically by the dinitrosalicylic acid method *(15)*, and total sugar concentration was measured by the phenol–sulfuric acid method *(16)* using a spectrophotometer (DR/4800, HACH). Standard curves were made by glucose and sucrose, respectively.

The conductivity measurement of culture medium was carried out at 20°C using a conductivity meter, Model CM-20E (cell constant k = 1.013; Japan TOA Electronics).

Extraction and Analysis of Crude Saponin

To determine crude saponin, 100 mg of powdered dry hairy roots was soaked in 5 mL of *n*-BuOH saturated with water, stored at 4°C for 24 h, sonicated for 30 min, and centrifuged twice at 5,030*g* for 10 min. The collected supernatant solution was evaporated to dryness below 60°C. Crude saponin, the evaporated supernatant, was measured by gravimetric methods *(13)*.

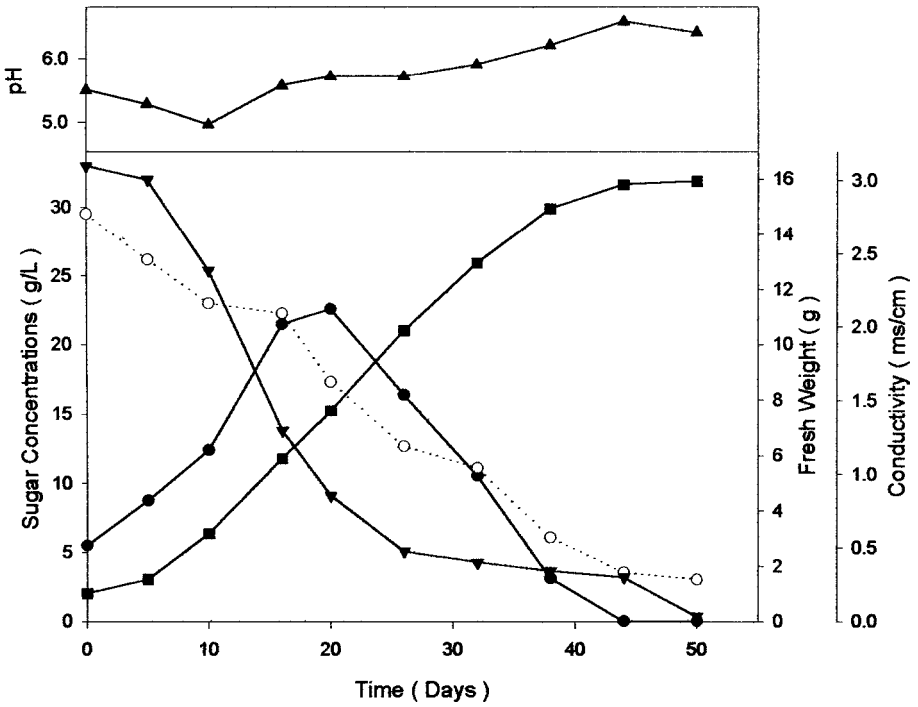

Fig. 1. Growth properties of *P. ginseng* hairy roots in flask culture. (—■—), biomass; (—●—), reducing sugar; (—○—), total sugar; (—▼—), conductivity; (—▲—), pH.

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 5,030*g* for 10 min. The collected supernatant was used to determine intracellular polysaccharide by the phenol–sulfuric acid method *(13)*.

Results and Discussion

Characteristics of Hairy Roots in Flask Cultures

Figure 1 shows the cultivation results, such as biomass growth, sugar consumption, changes in pH of the medium, and conductivity, of *P. ginseng* hairy roots in the shake flask. Hairy roots entered an exponential growth phase after a short lag period of about 5 d, and exponential growth occurred between 5 and 36 d. During the exponential phase, hairy root cultures had a growth rate of 0.368 g dry wt/(g of cells·d) (0.393 fresh wt/[g of cells·d]). After 40 d, substrate was exhausted, and after 45 d, total mass of hairy roots showed about a 16-fold increase over the inoculated amount. The ph of the medium dropped from an initial 5.5 to 4.96 after 10 d, but it gradually increased to 6.4 toward the end of the growth. Total sugar concentration

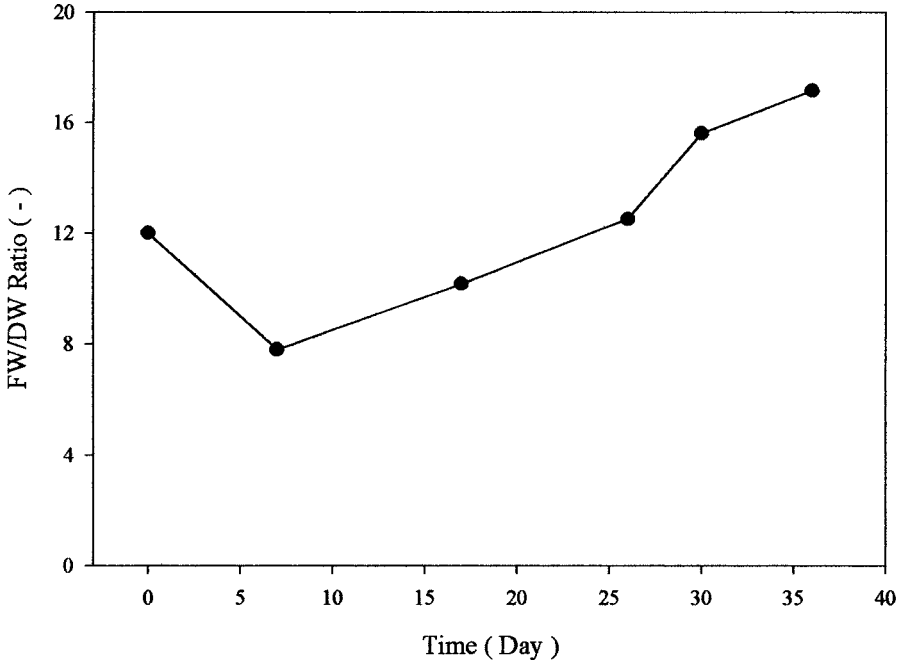

Fig. 2. Change in fresh wt/dry wt (FW/DW) ration of hairy roots during cultivation.

decreased inversely to the increase in biomass. Reducing sugar concentration increased in the first 20 d of the culture and decreased afterward. Initial sucrose, 30 g/L, was nearly exhausted after about 45 d. Medium conductivity decreased inversely to the increase in biomass. The decrease in medium conductivity appeared to reflect the amount of electrolytic or inorganic nutrients consumed by the cell *(6,17)*.

Figure 2 shows the change in the fresh wt/dry wt ratio of hairy roots during the culture period. The water content decreased in the first 7 d, and linearly increased afterward. The fresh wt/dry wt ratio of hairy roots increased with the decrease in osmotic pressure, which was caused by nutrient uptake *(18)*.

Figure 3 shows changes in crude saponin and polysaccharide content during the culture period. Crude saponin and polysaccharide content in the hairy roots fluctuated only slightly over the whole culture period, and its productivity had no correlation with the growth of hairy roots.

Estimation of Weight of Hairy Roots by Medium Conductivity

Conductivity measurement of culture medium is a convenient method for cell mass estimation in plant cell and tissue cultures. The effects of environmental changes on medium conductivity are almost negligible in plant cell culture *(7,19)*. In the present study, the estimation of hairy root growth was performed on the basis of the conductivity

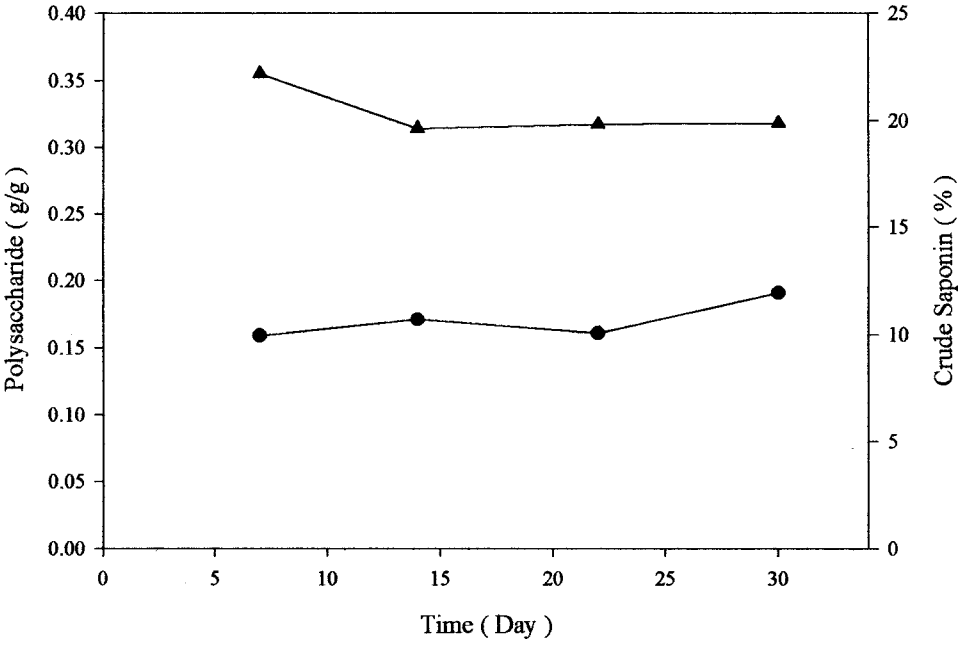

Fig. 3. Change in secondary metabolites of hairy roots during cultivation in flask cultures (—●—), polysaccharide; (—▲—), crude saponin.

change in the culture medium. Conductivity of the medium was correlated with the fresh weight of the hairy roots, which were harvested at different growth stages. Figure 4 shows the relationship between increase in fresh wt and decrease in conductivity of the medium in flask culture. The conductivity correlation obtained from the calibration curve is indicated in Eq. 1:

$$X = 0.085 + 2.666 \times C \ (R^2 = 0.973) \quad (1)$$

in which X is the fresh wt of hairy roots (g), and C is the medium conductivity (ms/cm).

Figure 5 shows the results of estimation of cell growth in various bioreactor cultures based on conductivity change. The dashed lines indicate the cell mass calculated using Eq. 1. In all bioreactor cultures, the calculated values did not match up well with experimental values. These results were caused by different culture conditions in flasks and bioreactors such as aeration, agitation, mass transfer, and culture space. Another cause is overestimation of biomass. As the mass of hairy roots increased, the actual medium volume decreased because hairy roots contain a great deal of intracellular water. Thus, if the reduction in actual medium volume were ignored, the biomass estimation based on conductivity change would not be matched.

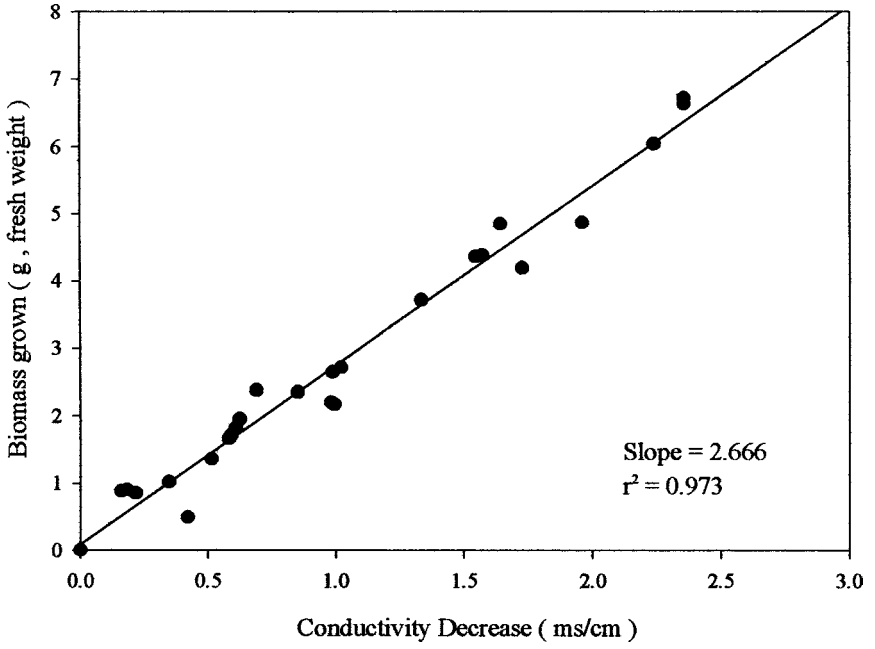

Fig. 4. Relationship between growth of hairy roots and decrease in conductivity during flask cultures of *P. ginseng*.

Growth Kinetics

Figure 6 represents the growth kinetics of *P. ginseng* hairy root cultures in the flask. The growth of hairy roots can be described according to a logistic model *(20)* as follows:

$$X = X_m/1 + \exp(b - kt) \quad (2)$$

in which X is the fresh wt of hairy roots (g); X_m is the maximal fresh wt of hairy roots (here 16.5 g); t is the time (d); and b and k are model constants, in which model constant b is 2.72, and k is 0.13 the doubling time, calculated at the linear phase, was 6.5 d with a specific growth rate of 0.15 d^{-1}.

Production of Crude Saponin and Intracellular Polysaccharide by Hairy Roots in Flask Cultures

The production of crude saponin and intracellular polysaccharide by hairy root culture was examined. The crude saponin and polysaccharide content of the hairy roots and ordinary field-grown, 5-yr-old ginseng root are shown in Table 1. Hairy roots produced almost the high or similar amounts of metabolites in the flask cultures. The crude saponin content of hairy roots, 19.9% on a dry wt basis, was 1.2 times as high as that of the main root part of native plant roots, 16.1%. However, it was lower than in lateral

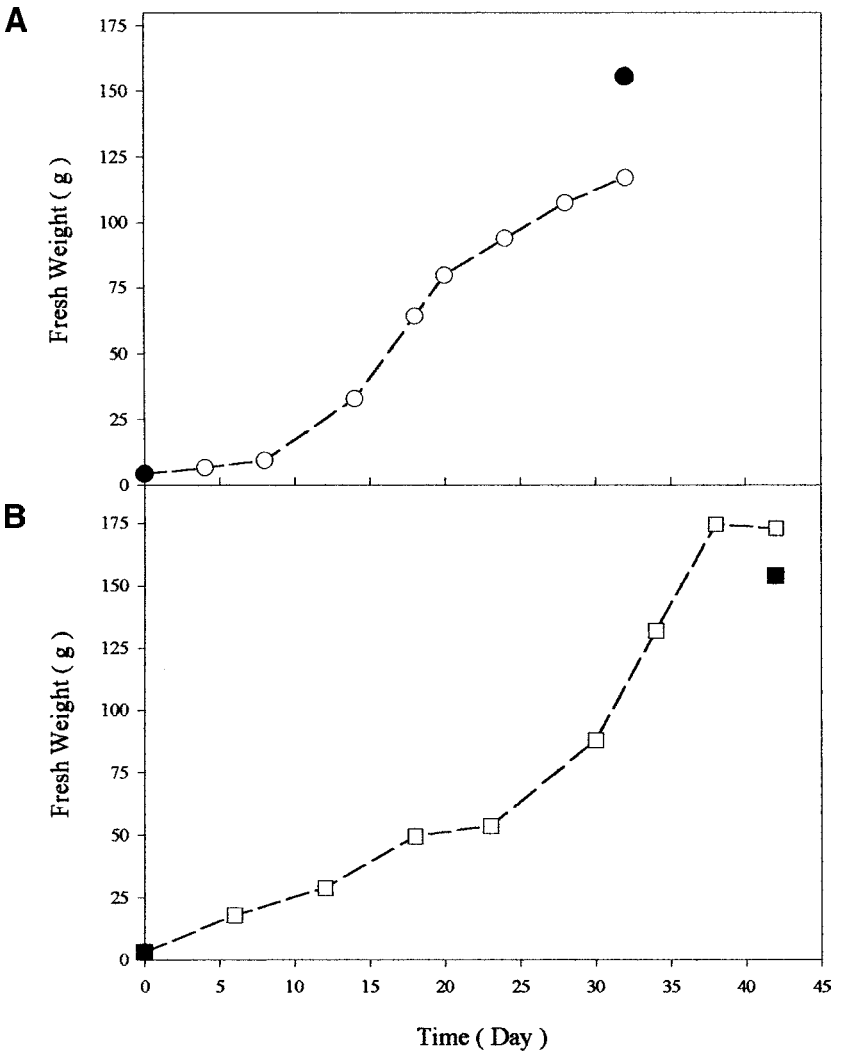

Fig 5. Estimation of cell growth based on conductivity in various bioreactors. **(A)** Air bubble column bioreactor: ●, experimental value; (—○—), estimated value; **(B)** modified stirred bioreactor: ■, experimental value; (—□—), estimated value.

root or root hair parts. Root hair and lateral root parts of natural ginseng roots showed higher crude saponin content than main root parts. In the natural ginseng roots, saponin content is higher in the periderm and cortex than in the xylem *(21)*. Intracellular polysaccharide content of hairy roots cultured in flasks, 0.19 g/g on a dry wt basis, was lower than that of natural ginseng roots, 0.45–0.79 g/g on a dry wt basis. The main part on natural roots has a higher polysaccharide content than the lateral root or root hairs. Okuda *(22)* reported that in natural ginseng roots, polysaccharide has a high content in the internal tissue of roots in contrast to saponin distribution.

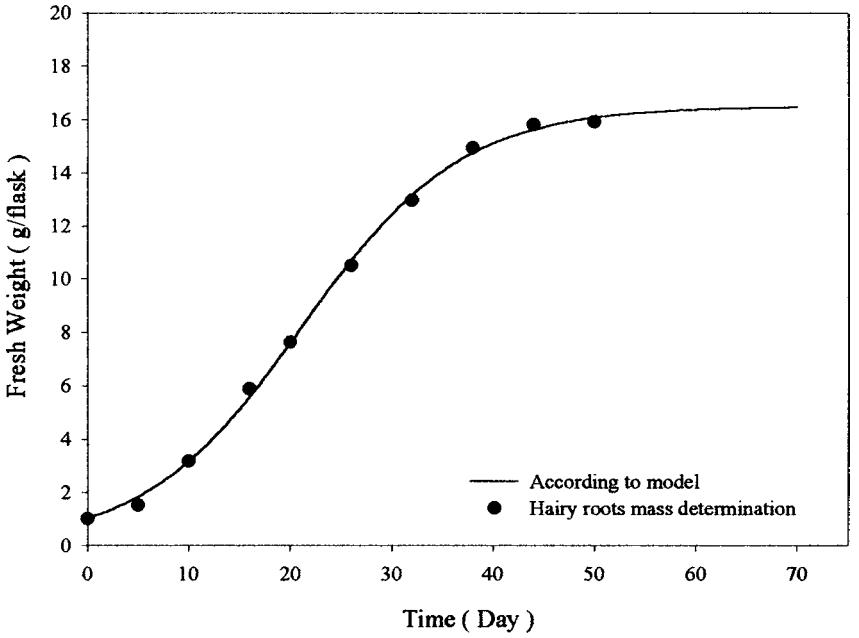

Fig. 6. Growth kinetics of *P. ginseng* hairy roots in flask cultures.

Table 1
Comparisons of Content of Polysaccharide and Crude Saponin on Natural *P. ginseng*

Natural roots	Polysaccharide (g/g)	Crude saponin (%)	Fresh wt/dry wt ratio
Main root (Ø 15–25 mm)	0.789	16.06	3.004
Lateral root (Ø 2–6 mm)	0.641	27.34	3.449
Root hair (below Ø 2 mm)	0.449	30.48	4.115
Hairy roots	0.191	19.89	7.8–17.2

Table 2
Comparison of Growth and Secondary Metabolite Content on Various Bioreactors

Type of Bioreactor	Culture time (d)	Growth ratio (times)	Growth rate fresh wt/dry wt (g/[g·d])	Polysaccaride (g/g)	Crude saponin (%)
250-mL flask	38	14.93	0.393 (0.368)	0.191	19.89
Air bubble column	42	51.680	1.230 (1.112)	0.106	16.8
Modified stirred	32	36.290	1.134 (1.198)	0.096	20.03

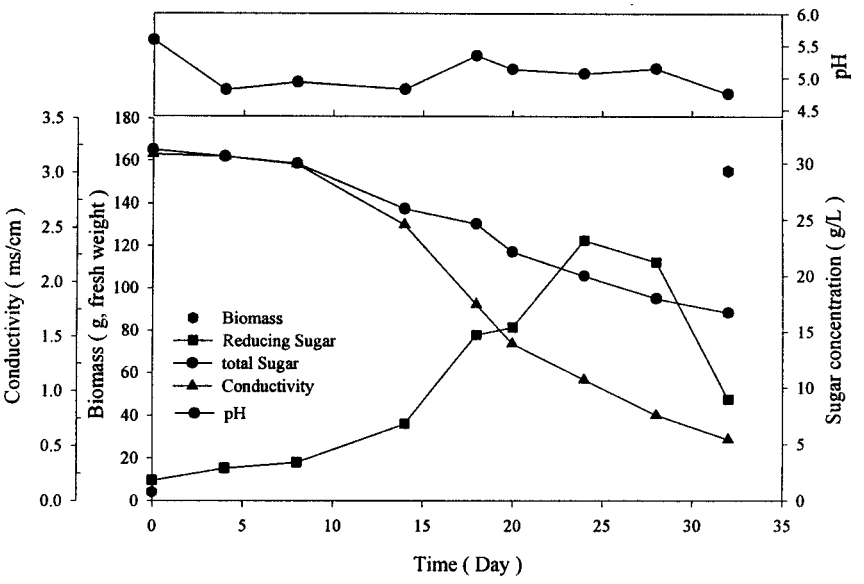

Fig. 7. Growth properties of hairy roots cultured in air bubble column bioreactor.

Hairy Root Cultures in Bioreactor

Two bioreactor systems were investigated for large-scale hairy root cultures of *P. ginseng*. As shown in Table 2, the cultivations with an air bubble column bioreactor and a modified stirred bioreactor were successful in both systems.

Air Bubble Four-Stage Column Reactor

Like an airlift bioreactor, in a bubble column, the bubbles create less shear stress, so that it is useful for organized structures such as hairy roots *(12)*. Figure 7 shows the time course of the growth and nutrient consumption of *P. ginseng* hairy roots in the air bubble column bioreactor. After 32 d, hairy roots increased 36.3 times (4.3 [0.27] to 155 [9.65] g fresh wt [dry wt]) or 1.13 (1.11) g fresh wt (dry wt)/(g of cells·d) in this type of bioreactor was about 2.9 (3.0) times higher than that observed in the flask cultures. This result showed that floating of hairy roots on the upper medium by air bubbles during culture periods was prevented by the stainless steel mesh at each stage. Therefore, the tissues have a sufficient culture space. Sucrose was first hydrolyzed and continuously consumed afterward from 31.2 to 16.8 g/L during the culture period. Reducing sugar increased to 23.2 g/L until 24 d, then decreased to 8.96 g/L. The pH of the medium dropped initially from 5.59 to 4.83 after the first 14 d, but it was stable after that time. Medium conductivity inversely decreased from 3.17 to 0.56 ms/cm during the culture period. As also shown in Fig. 8A, the hairy roots showed good growth, and each stage of the column was closely packed with hairy roots after 32 d. The crude saponin content of hairy

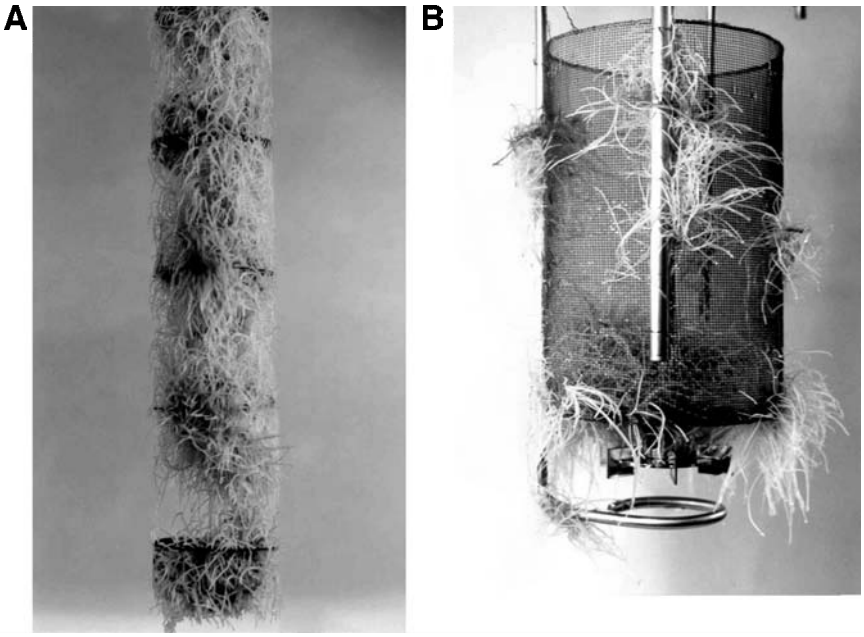

Fig. 8. *P. ginseng* hairy roots cultured in bioreactors: **(A)** air bubble column bioreactor; **(B)** modified stirred bioreactor.

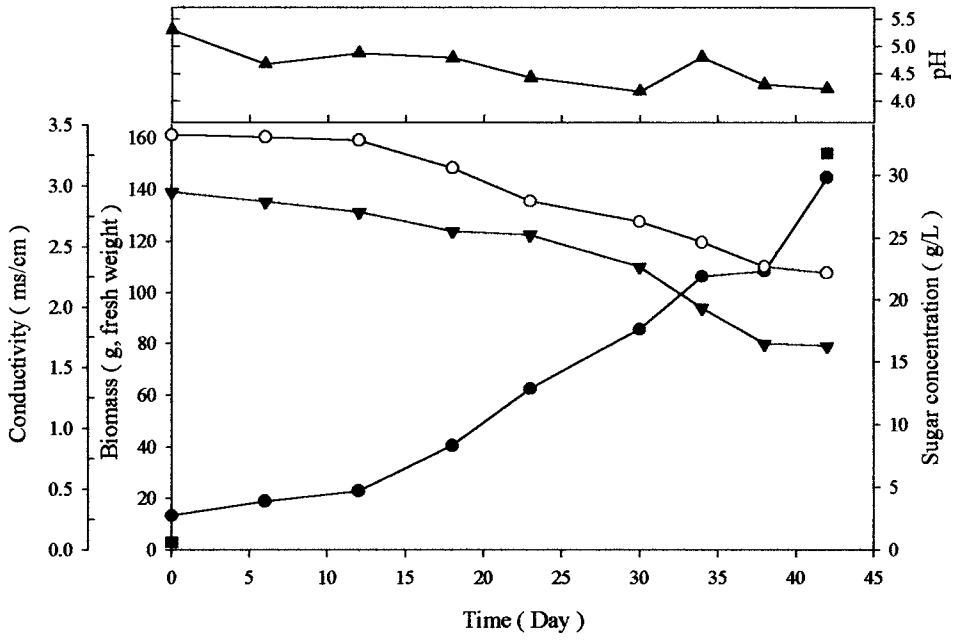

Fig. 9. Growth properties of hairy roots cultured in modified stirred bioreactor. ■, biomass; (—●—), reducing sugar; (—○—), total sugar; (—▼—), conductivity; (—▲—), pH.

roots in this type of bioreactor was 20.0% on a dry wt basis. This was similar to flask cultures, but intracellular polysaccharide content, 0.096 g/g on a dry wt basis, was lower than in flasks (Table 2).

Modified Stirred Bioreactor (Frame-Fixing Type of Bioreactor)

In the modified stirred bioreactor, cultivation space is separated from agitation space by stainless steel mesh frame, so that hairy roots do not contact the impeller and air is introduced from the bottom of the bioreactor. Figure 9 shows the time course of the growth and nutrient consumptions of *P. ginseng* hairy roots in the modified stirred bioreactor. After 42 d, hairy roots increased 52-fold from the initial inoculum 3 (0.19) to 154 (9.5) g fresh wt (dry wt). The growth rate of hairy roots, 1.23 (1.2) g fresh wt (dry wt)/(g of cells·d), in this bioreactor was about 3.1 (3.3) times higher than that of the flask cultures. The pH of the medium was generally maintained low. Figure 8B shows the hairy roots cultivated in the bioreactor for 42 d. The crude saponin and polysaccharide content of hairy roots in this type of bioreactor (17%, 0.11 g/g on a dry wt basis, respectively) were lower than those of the flask cultures (Table 2). In this bioreactor system, problems were mass-transfer inhibition by formation of root clumps and damage to frame-outgrowing hairy roots by the impeller during the latter culture period.

Conclusion

In flask cultures, hairy roots grew about 16-fold after 45 d. A linear correlation was obtained between the increase in fresh cell weight and decrease in medium conductivity in *P. ginseng* hairy root culture. The crude saponin and intracellular polysaccharide content of hairy roots in the bioreactor were similar or lower than those in the flask culture.

In the cases of air bubble column or stirred bioreactor cultures, the growth rate of hairy roots was 1.13 (1.11) to 1.23 (1.20) g fresh wt (dry wt)/(g of cells·d), respectively. For both, growth was about three times as high as in the flask cultivation.

Acknowledgment

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References

1. Fransworth, R. N. and Morris, P. M. (1976), *Am. J. Pharm.* **148,** 46–52.
2. Park, S. H., Park, D. H., Kim, J. H., Hur, B. K., and Jo, Y. I. (1990), *Korean J. Biotechnol. Bioeng.* **5(3),** 207–214.
3. Giri, A., Narasu, M. L. (2000), *Biotechnol. Adv.* **18,** 1–22.
4. Mukundan, U. and Hjortso, M. A. (1991), *Plant Cell Rep.* **9,** 627–630.
5. Shanks, J. V. and Morgan, J. (1999), *Curr. Opin. Biotechnol.* **10,** 151–155.
6. Nobuyuki, V. and Takesh, K. (1994), in *Advances in Plant Biotechnology*, Ryu, D. D. Y. and Furusaki, S., eds., Elsevier, pp. 307–338.

7. Taya, M., Yoyama, A., Kondo, O., and Matsui, C. (1989), *J. Chem. Eng. Japan* **22(1),** 84–89.
8. Rodriguez-Mendiola, M. A., Stafford, A., Cresswell, R. and Arias-Castro, C. (1991), *Enzyme Microb. Technol.* **13,** 697–702.
9. Yu, S. and Doran, P. M. (1994), *Biotechnol. Bioeng.* **44,** 880–887.
11. Kondo, O., Honda, H., Taya, M., and Kobayashi, T. (1989), *Appl. Microbiol. Biotechnol.* **32,** 291–294.
12. Sajc, L., Grubisic, D., and Vunjak-Novakovic, G. (2000), *Biochem. Eng. J.* **4,** 89–99.
13. Jeong, G. T. (2001), MS thesis, Chonnam National University, Gwangju, Korea.
14. Murashige, T. and Skoog, F. (1962), *Physiol. Plant* **15,** 473–497.
15. Miller, G. L. (1959), *Anal. Chem.* **31(3),** 426–428.
16. Chapline, M. F. and Kenedy, J. (1986), *Carbohydrate Analysis: A Practical Approch,* IRL, Oxford.
17. Ryu, D. D. Y., Lee, S. O., and Romani, R. J. (1990), *Biotechnol. Bioeng.* **35,** 305–311.
18. Ramakrishnan, D., Luyk, D., and Curtis, W. R. (1999), *Biotechnol. Bioeng.* **62(6),** 711–721.
19. Taya, M., Hegglin, M., Prenosil, J. E., and Bourne, J. R. (1989), *Enzyme Microb. Technol.* **11,** 170–176.
20. Edwards, V. H. and Willce, C. R. (1968), *Biotechnol. Bioeng.* **10,** 205–232.
21. Kim, M. W., Ko, S. R., Choi, K. J., and Kim, S. C. (1987), *Korean J. Ginseng Sci.* **11(1),** 10–16.
22. Okuda, H. (1992), *Ginseng Rev.* **15,** 34–37.