Biotechnology and Bioprocess Engineering **2006**, 11: 43-47

Characteristics of Transformed *Panax ginseng* C.A. Meyer Hairy Roots: Growth and Nutrient Profile

Gwi-Taek Jeong[1,2] and Don-Hee Park[2,3,4,5]*

[1] Engineering Research Institute, Chonnam National University, Gwangju 500-757, Korea
[2] School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Korea
[3] Biotechnology Research Institute, Chonnam National University, Gwangju 500-757, Korea
[4] Research Institute for Catalysis, Chonnam National University, Gwangju 500-757, Korea
[5] Institute of Bioindustrial Technology, Chonnam National University, Gwangju 500-757, Korea

Abstract Ginseng (*Panax ginseng* C.A. Meyer) hairy root cultures, which are established via the infection of ginseng root discs with *Rhizobium rhizogenes*, have been used to construct profiles of both biomass growth and nutrient consumption in flask cultures. In a 250 mL shake flask culture, the maximum biomass was observed on the 59th day of the culture period, at 216.8 g (fresh wt) per liter or 11.4 g (dry wt) per liter. The hairy roots were determined to have a growth rate of 0.355 g-DW/g cells/day during the exponential growth phase and a maximum specific growth rate on day 7. Total ginseng saponin and phenolic compound contents were noted to have increased within the latter portion of the culture period. Linear correlations between increases in biomass weight and nutrient uptake were used to imply the conductivity yield 2.60 g-DW/(L·mS) and carbon yield 0.45 g-DW/(g sugar) in the 250 mL flask cultures. The biomass yield when two different nitrogen sources were used (ammonia and nitrate) was shown to remain approximately constant, at 0.47 g-DW/(L·mM NH_4) and 0.33 g-DW/(L·mM NO_3); it remained at these levels for 16 days with the ammonia, and for 24 days with the nitrate. The biomass yield when a phosphate source was used was also shown to remain approximately constant for 9 days, at 3.17 g-DW/(L·mM PO_4), with an R^2 of 0.99.

Keywords: *Panax ginseng*, transformed hairy roots, growth profile, nutrient, secondary metabolites

INTRODUCTION

Both plants and plant-derived natural products have been, and will continue to be, important chemical resources. Approximately 100,000 compounds are currently known to be directly obtainable from plants. Approximately 4,000 new compounds are discovered each year. These compounds are connected not only with important traits of the plant itself, including color, fragrance, taste, and resistance to pests and diseases, but have also proven useful in the production of a host of valuable components, including drugs, antioxidants, flavorings, fragrances, pigments, insecticides, and other important industrial and medicinal raw materials [1-3]. Plant cell and tissue cultures constitute an attractive alternative to whole plants in terms of the generation of high-value secondary metabolites. Plant cells are known to be biosynthetically totipotent, in that each cell retains the complete genetic information of the plant, and thus is capable of generating the entire range of chemicals which would be found in the parent plant [4]. The relationship between the plant cell/tissue and its culture condition is very complex. The relationships inherent to cell growth and operating parameters should be determined from the results of laboratory experiments conducted in scaled-down versions of a production bioreactor [5].

When induced by *Rhizobium rhizogenes*, transformed hairy root cultures constitute a promising alternative technique for use in the biotechnological exploitation of plants. These hairy root cultures synthesize amounts of material comparable to, and sometimes in excess of, the amounts generated by the original plants [6]. Transformed hairy root lines appear to be a promising source for constant standardized production of useful plant metabolites [7]. Hairy roots are characterized by a high growth rate, high secondary metabolite productivity, and inherent genetic stability. The transformed hairy roots of a multitude of plant species have been studied; the aim of these studies was *in vitro* secondary metabolite generation [8,9].

A great deal of chemical, biochemical, and pharmacological research has been conducted using the ginseng plant. The primary compounds relevant to pharmaceutical interaction in the ginseng plant have been previously

***Corresponding author**
Tel: +82-62-530-1841 Fax: +82-62-530-1909
e-mail: dhpark@chonnam.ac.kr

isolated, and these include ginseng saponin (ginsenosides), phenolic compounds, polysaccharides, antioxidants, peptides, fatty acids, alcohols, and vitamins [10]. The pharmacological characteristics and effects of the ginsenoside Rb_1 and Rg_1 are distinct, and are sometimes antagonistic. Ginsenoside Rb_1 manifests sedative, anticonvulsive, analgesic, antipyretic, anti-inflammatory, and antipsychotic properties, and also has been shown to improve gastrointestinal motility. Ginsenoside Rg_1 exerts stimulant and antifatigue effects, and also appears to enhance motor ability. In recent years, the non-saponin components of ginseng have attracted a great deal attention for their antioxidant, anticancer, antidiabetic, and immunomodulatory effects. These non-saponin components include compounds such as phenolic compounds, polyacetylene, ginsenoyne, sesquiterpenes, methoxypyrazine, alkypyrazine derivatives, sesquiterpene alcohol, panasinsanols, β-caboline, and a host of neutral or acidic polysaccharides. A variety of vitamins, inorganic substances, free monosaccharides, and organic acids are also included in this list [11,12]. Ginseng polysaccharides, in particular, exert rather striking pharmacological effects, including immune stimulation, antitumor functions, and anti-hepatitis functions, all of which have received increased attention in recent years. As components of the cell wall (primary metabolites), their total cellular content has been found to be fairly stable, and it is easier to obtain a high production titer with these compounds than with ginsenosides [10]. Therefore, the objective of this work is to characterize the growth profiles and metabolite biosynthesis capabilities of *Panax ginseng* C.A. Meyer hairy roots in flask cultures. We also performed an indirect evaluation of biomass growth for these cultures.

MATERIALS AND METHODS

Hairy Root Cultures

The hairy roots of *P. ginseng* C.A. Meyer were initiated and maintained as previously described [13]. In order to determine the growth characteristics and nutrient consumption properties of *P. ginseng* hairy roots in the flask cultures, we inoculated 1 g fresh weight of the hairy roots into 250 mL Erlynmeyer flasks that contained 100 mL phytohormone-free 1/2 MS liquid medium, which had been supplemented with 3% sucrose. The hairy roots were then cultured at 23 ± 1°C on a rotary shaking incubator (80 rpm) in darkness. In order to determine both the biomass and the composition of the medium, the hairy roots and medium were harvested at regular intervals.

Analytical Methods

In order to determine the weight of the biomass, the hairy roots were harvested and rinsed in distilled water. All excess water was then eliminated. The treated hairy roots were measured in terms of both fresh weight and dry weight. Dry weights were measured gravimetrically after the roots had been dried at 60°C for 24 h. In the medium, the concentrations of reducing sugar were determined colorimetrically with a spectrophotometer (DR/4800, HACH, USA) via the 3,5-dinitrosalicylic acid (DNS) method. The standard curve was constructed with glucose. The total sugar concentration was measured via the phenol-sulfuric acid method, using sucrose as the standard. The conductivity (expressed in milli Siemens; mS) in the culture medium was measured with a conductivity meter, a Model CM-20E (TOA Electronics Ltd., Japan; cell constant k = 1.013), at a constant temperature of 25°C. The concentrations of the ammonium ion (NH_4^+), nitrate ion (NO_3^-), and inorganic phosphate (PO_4^{3-}) were measured colorimetrically *via* the phenate method, the ultraviolet spectrometric screening method, and the stannous chloride method, respectively [13].

In order to determine the total contents of ginseng saponin in the cultures, we soaked 100 mg of powdered dry hairy roots in 5 mL of *n*-BuOH saturated with distilled water. This was then stored for 24 h at 4°C, sonicated for 60 min in an ultrasonic cleaning bath, and centrifuged twice at 10,000 rpm for 10 min each time. The supernatants were collected, and were then used for total ginseng saponin analysis. Total ginseng saponin levels were determined via the Vanillin-H_2SO_4 colori-metric method. In order to determine the concentrations of phenolic compounds, 100 mg of powdered dry hairy roots were soaked in 5 mL of 70% MeOH, sonicated for 30 min, and then centrifuged twice at 10,000 rpm for 10 min each time. The supernatants were collected and used for phenolic compound analysis, which was conducted according to the modified Folin-Denis method [13].

RESULTS AND DISCUSSION

Growth Characteristics and Nutrient Consumption

Quantitative information regarding growth kinetics, nutrient utilization, respiratory requirements, and biomass and product yields are all prerequisites for the proper evaluation of the potential of hairy root cultures. The growth of many organisms is amenable to macroscopic mass balance analyses, in which the system exchanges a certain number of components with the environmental conditions, despite the biochemical complexity of the method [14]. Hairy root cultures are known to require a different set of inorganic nutrients and carbon sources for proper growth and propagation. Fig. 1A depicts the growth profiles of the *P. ginseng* hairy roots in the shake flask cultures. In our 250 mL shake flask cultures, we observed the maximum amount of biomass on the 59th day of the culture period; this was recorded as 216.8 g (fresh wt) per liter, or 11.4 g (dry wt) per liter. The hairy roots were shown to have entered an exponential growth phase after a short lag period of approximately 4 days, and the exponential growth proceeded from the 4th day to the 45th day of the culture period. During the exponential phase, the hairy root cultures exhibited a growth rate of 0.355 g-DW per g cells per day (0.433 g-FW per g cells per day). After 59 days, the total mass of the hairy roots, on the basis of dry weight (21.7-

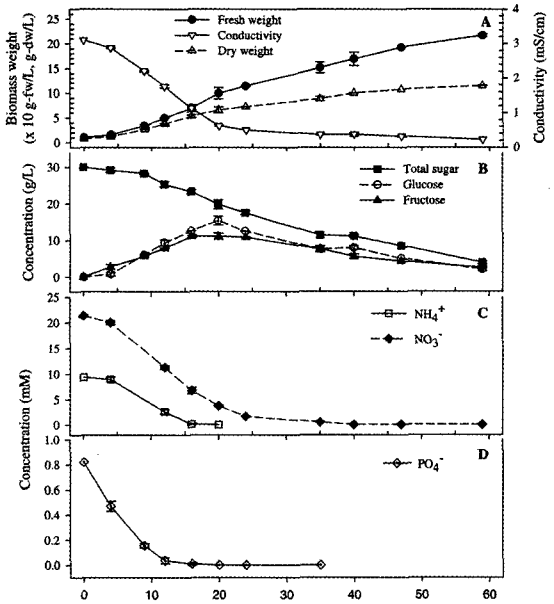

Fig. 1. Time course of biomass growth profiles and consumption of major medium components of *P. ginseng* hairy roots in 250 mL flask cultures. (A) Biomass growth and conductivity, (B) Sugars, (C) Ammonium and nitrate, (D) Phosphate.

fold on a fresh weight basis), had increased by a factor of about 16.1-fold the amount originally inoculated into the culture. After 9 days, the pH value of the medium dropped, from an initial pH of 5.4, to 4.98, but the pH continued to fluctuate, and a pH of 6 was recorded toward the end of growth (data not shown). This fluctuation in pH was attributed to the uptake of ammonium ions in exchange for the hydrogen ion within the medium [15]. Hence, the uptake of these ionic materials induces a reduction in the conductivity of the medium, as shown in Fig. 1A. The conductivity of the medium was shown to decrease inversely to the increase in the biomass. The conductivity dropped rapidly, from an initial 3.1 mS to a value of 0.62 mS on the 20th day. After the 20th day, the conductivity was found to decrease slightly, toward the end of the growth. The attenuation in the conductivity of the medium appeared to reflect the degree to which the electrolytic or inorganic nutrients were being consumed by the cells [16].

Sugar uptake was also monitored throughout the entirety of the culture period. The typical growth curve of a batch plant cell culture exhibits a phase of decline in biomass growth subsequent to the exhaustion of the carbon source [17]. We noticed a reduction in total sugar concentration which occurred inversely with increases in the biomass, as shown in Fig. 1B. Sucrose was initially hydrolyzed as glucose and fructose, and was then continuously consumed from at concentrations of 30 to 3.89 g/L during the culture period. After about 25 days, the initial 30 g/L of sucrose was almost completely exhausted. The concentrations of both glucose and fructose were determined to have increased over the initial 20 days of the culture period, and subsequently decreased.

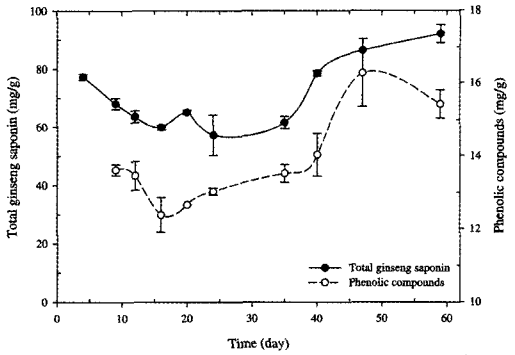

Fig. 2. Biosynthesis of secondary metabolites of hairy roots during cultivation in flask cultures.

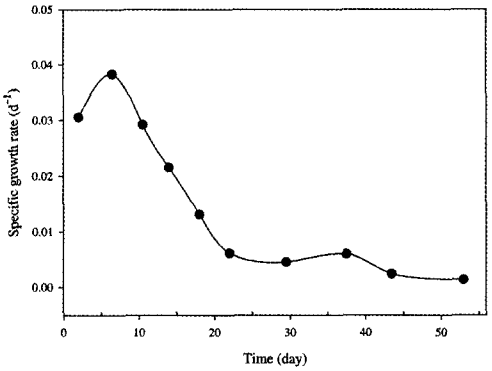

Fig. 3. Variations in the specific growth rate of *P. ginseng* hairy roots in the 250 mL flask cultures.

In general, the growth medium contains more nitrate than ammonium. However, the ammonium is taken up preferentially by the plant cells. This uptake of ammonium induces a drop in pH during the initial stage of the batch growth cycle, after which pH increases with the subsequent uptake of nitrate. Furthermore, the specific ammonium and nitrate uptake rates tend to vary with the specific growth rate, resulting in a variation in the biomass yields depending on the concentrations of ammonium and nitrate [5]. Nitrate exhaustion uniformly precedes carbohydrate exhaustion, and tends to occur earlier as the initial nitrate concentration is reduced [17]. As shown in Fig. 1C, the ammonium was continuously consumed afterwards, from 9.4 to 0 mM within the first 20 days of the culture period. Nitrate was also determined to have been exhausted within the first 40 days of the culture period. Ammonium was taken up more rapidly than was nitrate. The observed increase in biomass was often preceded by the exhaustion of the nitrogen and phosphate sources. The phosphate concentration in the medium exhibited an initial drop, from 0.82 to 0.009 mM within the first 16 days.

Fig. 2 shows the changes in the total ginseng saponin and phenolic compound contents of the hairy roots throughout the culture period. The total ginseng saponin and phenolic compound contents of the harvested hairy roots were observed to increase during the latter portion

of the culture period. The maximal total ginseng saponin and phenolic contents, 92.09 and 16.29 mg/g, were observed on the 59 and 47th days of the culture period, respectively. The observed reduction in total ginseng saponin and phenolic compound contents during the growth phase implies that metabolite generation is a non-growth-associated process. These biosynthetic patterns are well-known for the formation of secondary metabolites in plant cell/tissue cultures.

Instantaneous specific growth rates, as evaluated by the graphical differentiation of concentration data, are presented in Fig. 3. The specific growth rates of the *P. ginseng* hairy roots reached maximal levels in the 250 mL flask cultures with 1% (w/v) inoculum at day 7. After day 7, the specific growth rate was shown to decline with culture time, from a value of 0.038 per day. The pattern of specific growth rate over time was determined to be similar to those reported previously for the hairy roots of *Atropa belladonna* [14].

Correlationship of Hairy Root Weight and Medium Components

During the scale-up of the plant cell and tissue cultures in bioreactors, particularly in the case of hairy root cultures, biomass cannot be obtained for the purposes of analysis for a period. Generally, the progress of a hairy root culture appears to be accompanied by a reduction in the conductivity of the medium, in addition to a host of associated nutrimental and environmental changes, including glucose/sucrose concentrations, biomass concentration, and pH values. The effects of environmental changes on the conductivity of the medium appear to be all but negligible in plant cell cultures. Indirect methods of growth measurement, therefore, might prove to be useful. Measurements of conductivity in culture media have been employed as convenient tools for the indirect estimation of biomass in experiments involving the continuous on-line monitoring of plant cell and tissue cultures [17,18]. Ballica *et al.* provided a reliable estimation method for superior correlation, involving the $y = mx + c$ formula [19].

In the present study, we estimated hairy root growth characteristics based on changes in the conductivity and components of the culture medium. The conductivity of the medium was correlated with the weight of the biomass of the hairy roots, which had been harvested at different stages of growth. Fig. 4A depicts the relationship between increases in the dry weight and reductions in the conductivity of the medium in the 250 mL flask cultures. The conductivity correlation obtained from this calibration curve is indicated as: $X = -0.063 + 0.260 \times C$ ($R^2 = 0.982$), where X is the dry weight of the hairy roots (× 10 g-DW/L), and C is the conductivity of the medium (mS/cm). This method develops several limitations when fit is taken into account. Poor fit occurs due to differences in the change of conductivity and growth rate during the latter portion of the culture period.

In our estimation of the biomass yield coefficients, biomass growth was correlated with the uptake of carbon,

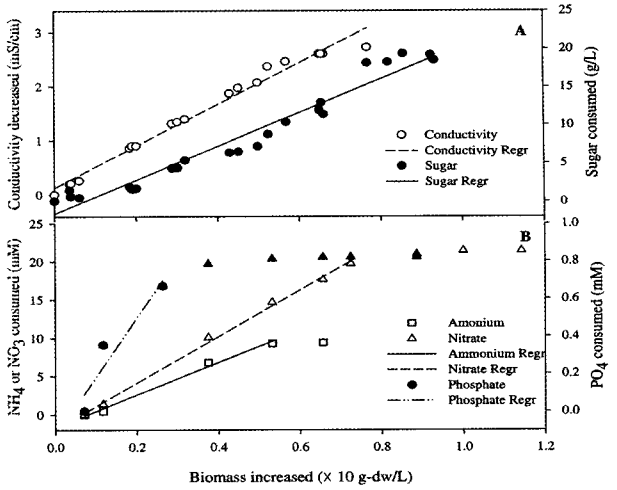

Fig. 4. Linear relationship between biomass growth and nutrient consumption by the *P. ginseng* hairy roots in the 250 mL flask cultures. (A) Conductivity and sugar, (B) Ammonium, nitrate, and phosphate. (Conductivity: $R^2 = 0.982$; Sugar: $R^2 = 0.956$; Ammonium: $R^2 = 0.992$; Nitrate: $R^2 = 0.997$; Phosphate: $R^2 = 0.898$).

nitrogen, and phosphate sources. These relationships are shown in Fig. 4A and B. The apparent biomass yields from the substrates were evaluated from a plot of increases in biomass versus the degree to which the substrates had been consumed (sugar, ammonium, nitrate, and phosphate). In this study, we estimated the growth of the hairy roots on the basis of the total sugar consumption in the culture medium. The total sugar concentrations of the medium were correlated with the weight of the biomass of the hairy roots, which had been harvested at various stages of growth.

Fig. 4A depicts the relationship between increases in dry weight and the total sugar consumption of the medium in the 250 mL flask cultures. The total sugar correlation acquired from the calibration curve was determined as: $X = 0.076 + 0.045 \times C$ ($R^2 = 0.956$), where X is the dry weight of the hairy roots (× 10 g-DW/L) and C is the total sugar concentration consumed in the medium (g/L).

The 1/2 MS medium harbors two distinct nitrogen sources, ammonia and nitrate, at a molar ratio of 1:1.91. The relationships between the consumption of ammonia and nitrate ions and the increase in the biomass of the hairy roots are shown in Fig. 4B. The biomass yield from the two distinct nitrogen sources (ammonia and nitrate) remained at approximately zero when assessed at 16 and 24 days, respectively; for each g of dry weight per liter increase in the biomass of the hairy roots, 2.12 mM NH_4 and 3.04 mM NO_3 were consumed. The ratio of NO_3 (mM) to NH_4 (mM) consumed was determined to be 1.43:1. The biomass yield from phosphate remained approximately constant for 9 days; for each g of dry weight per liter of increase in the biomass of the hairy roots, 0.32 mM PO_4 was consumed. As was the case with conductivity, the $y = mx + c$ formula proved to be a superior

method for the estimation of both biomass and nutrients (ammonia, nitrate, and phosphate) during the culturing of *P. ginseng* hairy roots (Fig. 4B). These correlations with the medium components can also be successfully applied to indirect on-line estimations of the growth of hairy root cultures conducted in bioreactors.

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[Received July 26, 2005; accepted November 15, 2005]