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Optimum Conditions for Transformed *Panax ginseng* Hairy Roots in Flask Culture

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Abstract

Panax ginseng hairy roots were transformed by *Agrobacterium rhizogenes* KTCT 2744. They showed an active branching pattern and fast growth in hormone-free medium, and good growth at 23°C, pH 5.8, 1/2 MS medium, and 3% sucrose. Sucrose provided the highest growth among seven carbon sources tested. Six complex media were also tested. In the combined sugar study, hairy roots grew better on sucrose without glucose or fructose than with glucose or fructose. In the 1/2 MS basal medium, 30 m*M* in nitrogen and 0.62 m*M* phosphate salt concentration was the optimum. The growth ratio was maximal at an inoculum size of 0.4 % (w/v). Crude saponin and polysaccharide levels were also measured.

Index Entries: *Panax ginseng*; transformed hairy roots; optimal condition; ginseng crude saponin; polysaccharide.

Introduction

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 Korea *(1)*. 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 *(1,2)*. The major compounds of pharmaceutical interaction in *P. ginseng* have been isolated and identified to be

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saponins (ginsenosides), polysaccharides, antioxidants, peptides, and fatty acids. In recent years, ginseng polysaccharides have been regarded as useful compounds of ginseng plants with antitumor and immune-stimulating activities *(1,3–5)*.

Hairy roots are induced by the genetic transformation of plant cells by the pathogenic soil bacterium *Agrobacterium rhizogenes*, which has a plasmid vector that seems to be feasible for the improvement of plant properties and for the production of transgenic plants *(6)*. Transformed hairy roots are characterized by a high growth rate; high secondary metabolite production; and inherent genetic stability, reflected in stable growth and production. Although hairy root cultures follow definite growth patterns, metabolite production may not be related to hairy root growth *(7,8)*.

In plant cell and tissue cultures, cell growth and metabolite production are influenced by nutritional and environmental conditions such as cultivation temperature, pH of the medium, carbon source, nature of the nitrogen and phosphate sources and their relative amounts, presence of chemicals, aeration rate, and mass transfer rate *(9–11)*. To obtain a high growth rate of plant cell tissue cultures, the culture conditions should be maintained at the optimum level. This article focuses on a systematic investigation of the effect of culture conditions on the growth and formation of metabolites in transformed *P. ginseng* hairy root cultures.

Materials and Methods

Plant Materials and Bacterial Strains

Five-year-old, field-grown ginseng (*P. ginseng* C. A. Meyer) root was purchased from Kwangju, Korea. For the hairy root induction, *A. rhizogenes* KCTC 2744 was used for transformation. This bacterial strain was cultured at 27°C on potato dextrose agar solid medium under dark conditions. All chemicals used were reagent grade.

Induction and Culture of Hairy Roots

Ginseng roots, in which lateral roots were eliminated, were washed in 70% EtOH for 5 min, sterilized in 6% (v/v) NaOCl with a small amount of Tween-80 for 15 min, and rinsed three times with sterile distilled water. Surface-treated ginseng roots were sliced into 1- to 1.5-cm-long sections. Bacteria were inoculated onto the excised upper surface of sliced ginseng roots using a sterile cotton stick or loop. The inoculated roots were cultured on 1/2 MS (1/2-macro MS *[12]*) solid medium containing 3% sucrose, 0.8% agar, and without plant growth regulators at 23°C under dark conditions. Transformed hairy roots were maintained by 3-wk subculturing on hormone-free 1/2 MS liquid and solid media, and were used for this study.

Batch Experiment Procedure

MS, 1/2 MS, N6 *(13)*, White *(14)*, R2 *(15)*, and B5 *(16)* media were used to investigate the growth of hairy roots on each medium. In addition, the

growth of hairy roots on 1/2 MS medium under various conditions was compared. The investigated conditions were cultivation temperature, various carbon sources, initial pH, sugar concentration, nitrogen and phosphate concentration, and size of the inoculum.

To study the kinetics of the growth and secondary metabolite production in a shake flask, 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. About 1 g fresh weight of hairy roots was inoculated into a 250-mL flask containing 100 mL of hormone-free 1/2 MS liquid medium with 30 g/L of sucrose and cultured in a shaking incubator (70 rpm) at 23°C under dark conditions for 26 d.

Analytical Methods

To determine cell mass, the hairy roots were harvested, rinsed with distilled water, and the water was thoroughly drained. Treated hairy roots were gravimetrically measured for fresh weight. In the medium, reducing sugar concentration was measured colorimetrically by the dinitrosalicylic acid method *(17)*, and total sugar concentration was measured by phenol–sulfuric acid methods *(18)* using a spectrophotometer (DR/4800, HACH). Each standard curve was made by glucose and sucrose, respectively.

Extraction and Analysis of Crude Saponin

To determine crude saponin, 100 mg of powdered dry hairy roots was soaked in 5 mL of *n*-bButanol 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 was evaporated to dryness below 60°C. Crude saponin (as the evaporated supernatant) was measured by gravimetric methods *(19)* as a percentage of the original 100 mg.

Extraction and Analysis of Intracellular Polysaccharide

To determine intracellular polysaccharide, 100 mg of powdered dry hairy roots was suspended with 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 of hairy roots by the phenol–sulfuric acid method *(19)*.

Results and Discussion

Induction and Culture of Hairy Roots

After 7 wk of infection with *A. rhizogenes* KCTC 2744, hairy roots induced from the surface of the ginseng root sections were cultured on hormone-free 1/2 MS solid medium. Induced hairy roots were excised from the parent root section and transferred to fresh 1/2 MS medium with 300 mg/L of cefatoxime to eliminate bacteria. Bacteria-eliminated hairy roots were subcultured for 3 wk at 23°C under dark conditions in hor-

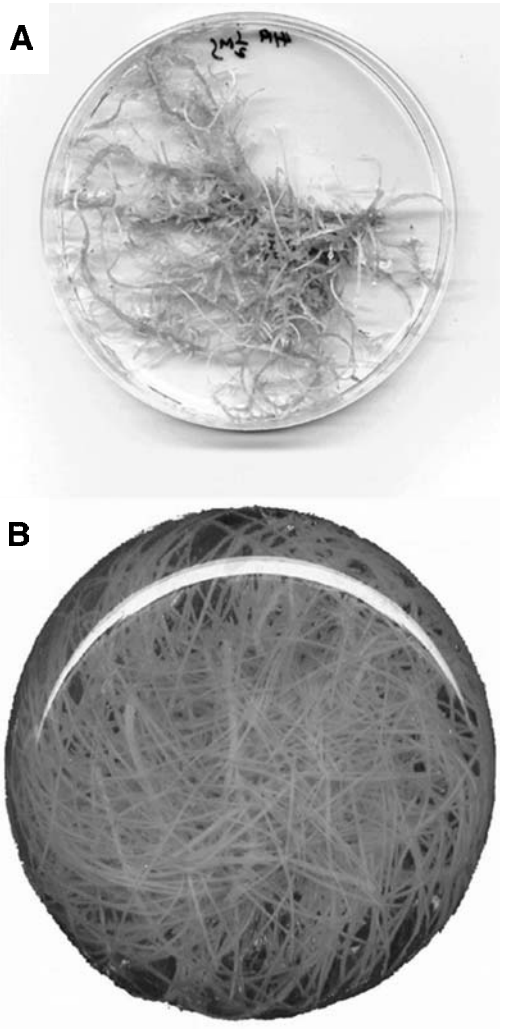

Fig. 1. Transformed *P. ginseng* hairy roots: **(A)** Petri dish; **(B)** 250-mL flask.

mone-free 1/2 MS solid and liquid media. Figure 1 shows *P. ginseng* hairy roots that grew in hormone-free 1/2 MS solid (Fig. 1A) and liquid (Fig. 1B) media. Transformed *P. ginseng* hairy roots showed an active branching pattern and fast growth in hormone-free medium. This is characteristic of transformed plant tissue by *Ri*-plasmid of *A. rhizogenes*.

Effects of Medium Composition and Initial pH

The medium composition, which is necessary for plant cell culture, varies in plant species with optimum medium salt types and concentration. MS, 1/2 MS, White, B5, R2, and N6 liquid media were used to investigate hairy root growth. The results showed that 1/2 MS and B5 media appeared better than MS, White, R2 and N6 media (Fig. 2). Hairy roots grew up to 12.8

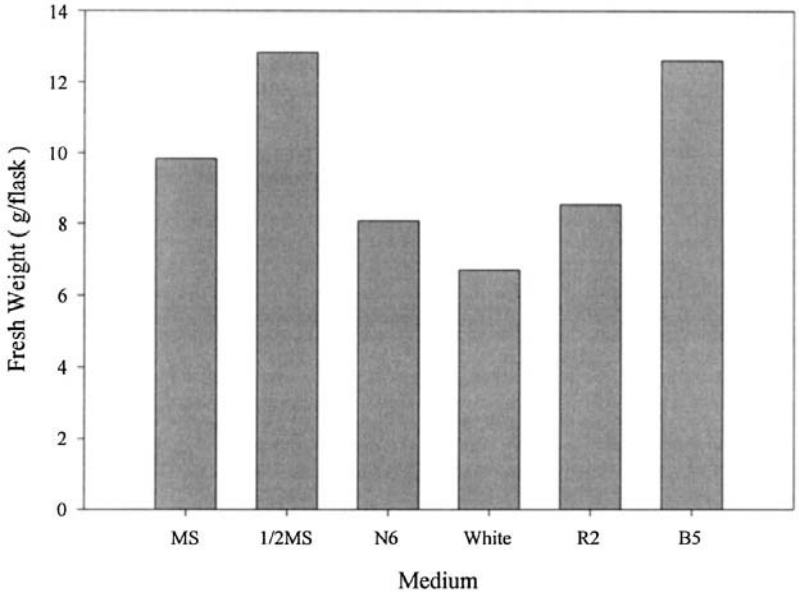

Fig. 2. Effect of type of medium on growth of hairy roots cultured for 26 d in 250-mL flask.

and 12.5 g fresh weight per flask in 1/2 MS and B5 media, respectively. In the case of N6 and R2 media, hairy roots showed lower growth and thick roots. The White medium showed thick roots with long lateral roots. Washida et al. *(20)* reported that *P. ginseng* hairy roots showed two times higher growth in B5 medium than in 1/2 MS medium.

The effect of the growth of hairy roots at different initial pH of the medium is shown in Fig. 3. Better growth of hairy roots was obtained in cultures with an initial pH of 4.5 followed by pH 7.0. At a pH of <4.0 and >pH 8.0, growth was lower. For pH 5.8–8.5, the pH of the medium initially decreased for the first 10 d and then increased toward the end of the growth (data not shown). This may be explained by the fact that when two types of nitrogen sources, ammonium salts and nitrate salts, were added to the medium, initially the ammonium salts were absorbed by the cells, so the pH decreased to 4.2–4.7. Then, nitrate salts absorption was facilitated by the low pH of the medium, resulting in an increase in the pH *(21)*.

Crude saponin content of hairy roots at various initial pH conditions of the midium was similar, and polysaccharide content was highest at pH 7.0.

Effects of Sugars

To optimize the carbon source, *P. ginseng* hairy roots were cultivated in the hormone-free 1/2 MS medium with various sugars as carbon sources (30 g/L). As shown in Fig. 4, hairy roots exhibited the highest growth (12.8 g/flask) in the sucrose-added medium, and showed high

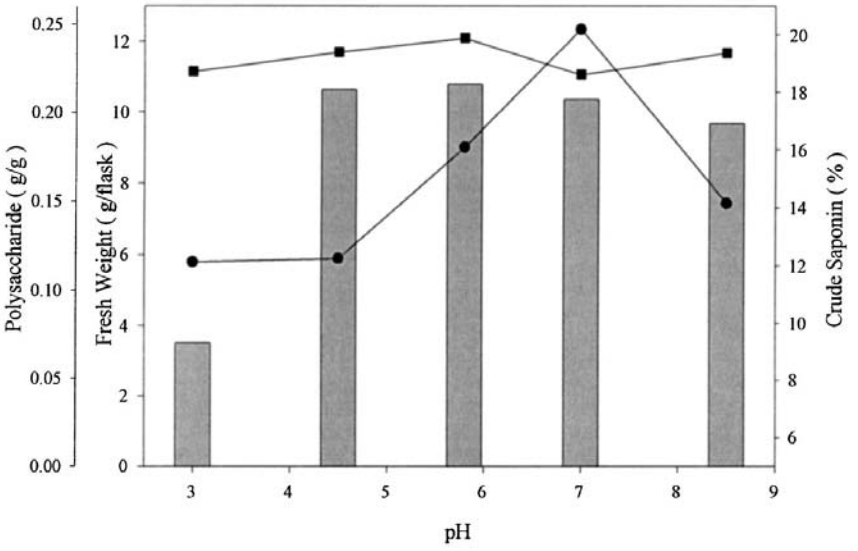

Fig. 3. Effect of initial pH on growth and secondary metabolite production of hairy roots cultured in 1/2 MS for 26 d in 250-mL flask (■), biomass; (—●—), polysaccharide; (—■—), cude saponin.

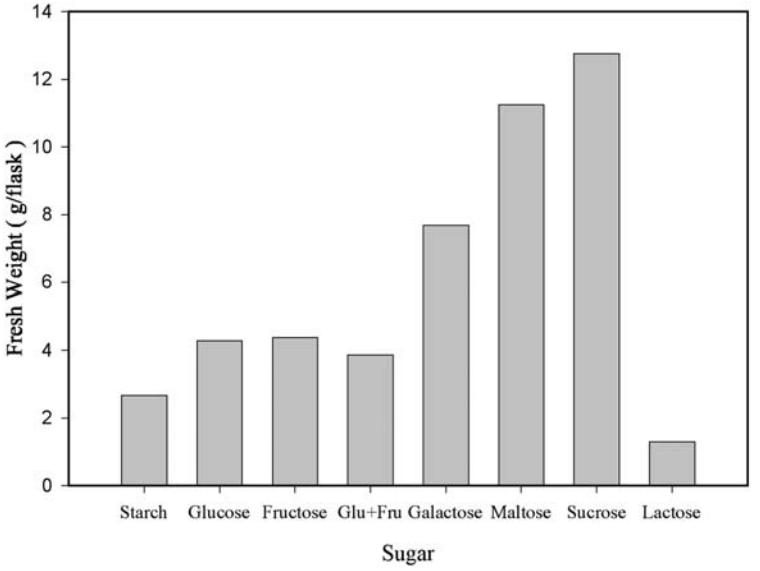

Fig. 4. Effect of type of sugar on growth of hairy roots cultured for 26 d in 250-mL flask.

growth (11.2 g/flask) in the maltose-added medium. Although hairy roots could utilize glucose (4.3 g/flask) and fructose (4.4 g/flask) as the alternative carbon source, the growth was lower than on sucrose. In addition, in the combined medium with glucose and fructose as carbon source,

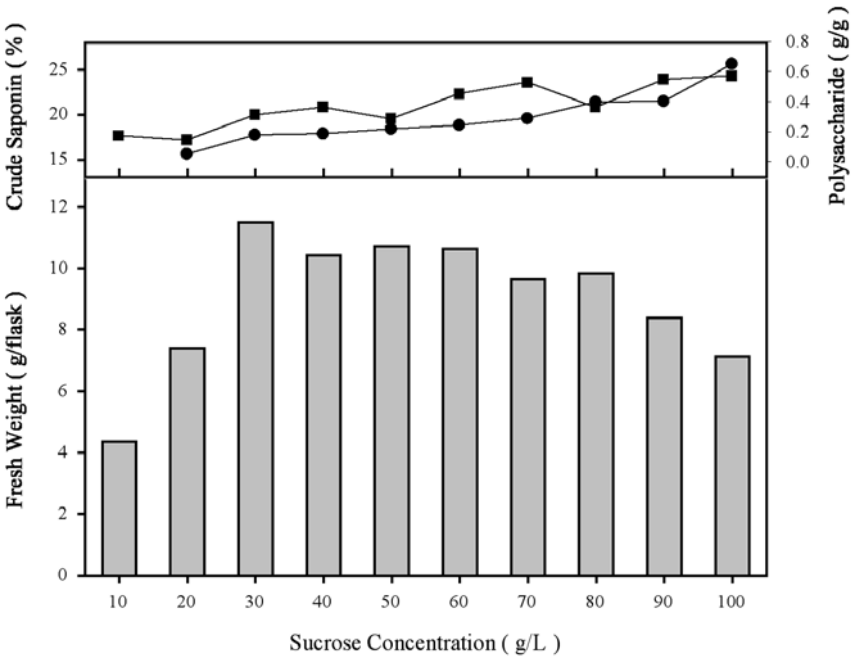

Fig. 5. Effect of sucrose concentration on growth and secondary metabolite production of hairy roots cultured for 25 d in 250-mL flask. (■), biomass; (—●—), polysaccharide; (—■—), crude saponin.

which are component monosaccharides of sucrose, hairy roots showed even lower growth (3.9 g/flask). Lactose and starch were not suitable as carbon sources. In the medium with sucrose, hairy roots formed mainly long branches of hairy roots, but with maltose, they formed short branches. Wu and Ho *(22)* reported that suspension cultures of *P. ginseng* showed good growth in the combined medium with sucrose and glucose or fructose as carbon source. Thus, sugar utilization is different for hairy root cultures and suspension cultures.

Effects of Initial Sucrose Concentration

Sucrose is a widely used carbon source in plant tissue culture. The effect of initial sucrose concentration on the growth and production of hairy roots was investigated. The results for its growth after 25 d of culture are shown in Fig. 5. Hairy root growth was the highest in the medium with 3% sucrose. With low initial sucrose concentration (1 to 2%), sugar was exhausted early in the culture period. Hairy root growth with high sucrose concentration was inhibited by substrate and high osmotic pressure in the medium. The osmotic pressure of culture medium is directly proportional to sucrose concentration *(23)*. Crude saponin content was generally higher at high sucrose concentrations, and polysaccharide content was proportional to increased sucrose.

Effects of Combined Sugar

Sucrose, which is an optimum carbon source, may be hydrolyzed to glucose and fructose during high-temperature sterilization (data not shown). Thus, the effect of the hydrolyzed glucose and fructose concentration on the growth was investigated at a total sugar concentration of 30 g/L by changing the ratio of sucrose to glucose to fructose (g/L:g/L:g/L) to 30:0:0, 20:10:0, 20:0:10, 20:5:5, 25:5:0, 25:0:5, and 25:2.5:2.5, respectively. Hairy root growth was highest in the medium with sucrose as the only carbon source compared with medium with any combination of sugars. Hairy roots grew better on added sucrose with glucose or with fructose than on the single glucose or fructose. Glucose provided somewhat better growth than fructose for sucrose-added medium. When three sugars were combined, as more glucose and fructose was added, stronger inhibition was observed. Wu and Ho *(22)* also reported that in the suspension culture of *P. ginseng*, cell growth rate was higher in the medium with sucrose as the only carbon source than in the medium with combined sugars.

Effects of Concentration of Nitrogen and Phosphate Source

In plant tissue culture, ammonium and nitrate salts are used as general nitrogen sources. The effect of the initial concentration of nitrogen source in the medium for the cell growth was studied in the *P. ginseng* hairy roots cultures with an NO_3^-/NH_4^+ ratio of 2 to 1. The initial total nitrogen salts level was adjusted to 15, 30, 60, and 120 m*M*, respectively. As shown in Table 1, the optimal nitrogen level for the growth of hairy roots was 30 m*M* in 1/2 MS basal medium, and it was obvious that the growth was inhibited by a high initial nitrogen concentration. Zhong and Wong *(24)* reported that the maximum cell growth was 15 g/L with a total initial nitrogen level of 40 m*M* in the suspension cultures of *P. ginseng*.

In the experiments investigating optimal phosphate salt level, the initial phosphate concentration was adjusted as in Table 1. The hairy root growth optimum was 0.62 m*M* initial phosphate. Zhong and Zhu *(25)* reported an optimum phosphate level of 1.25 m*M* in suspension culture of *P. notoginseng*.

Effects of Inoculum Size

To investigate the effects of inoculum size for hairy roots growth, hairy roots were inoculated at 0.2, 0.4, 0.7, 1.0, 2.0, and 3.0% (w/v). Figure 6 shows the dynamic profile of *P. ginseng* hairy roots at various inoculum sizes. Hairy roots had the highest growth rate at 0.4% (w/v) inoculum ratio and maintained active growth after 20 d at a 0.2% inoculum size. The growth ratio of hairy roots was decreased with an increase in inoculum size from 0.7 to 3.0% (Table 2). The ratio of inoculum and the final cell weight was not proportional to the increase in inoculum size. It was caused by limited substrate, dissolved oxygen, and limited space at the flask culture. Carvalho and Curtis *(26)* reported that on *Hyoscyamus muticus* root culture, growth

Table 1
Nigrogen and Phosphate Effects in 1/2 MS Basal Medium

Nitrogen salts (m*M*)	15.0	30.0	60.0	125.0
Fresh weight (g/flask)	9.4	9.8	8.1	4.7
Phosphate salts (m*M*)	0.31	0.82	1.25	2.50
Fresh weight (g/flask)	7.8	9.8	7.4	7.6

Table 2
Properties of Hairy Root Growth and Secondary Metabolite Production on Various Inoculum Ratios

Inoculum ratio (%)	Growth ration (times)	Growth rate (d^{-1})	Polysaccharide (g/g)	Crude saponin (%)
2	23.51	0.717	0.181	17.50
0.4	25.47	0.849	0.268	19.06
0.7	17.22	0.574	0.169	19.91
1.0	14.09	0.470	0.180	19.84
2.0	9.07	0.302	0.193	19.48
3.0	5.90	0.236	0.172	20.06

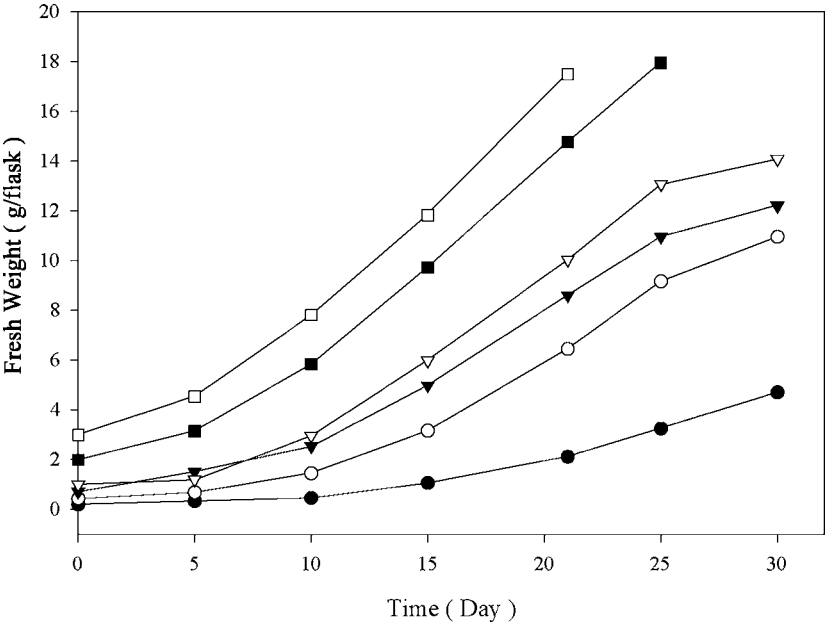

Fig. 6. Effect of inoculum size on growth of hairy roots cultured for 30 d in 250-mL flask. (—●—), 0.2%; (—○—), 0.4%; (—▼—), 0.7%; (—▽—), 1.0%; (—■—), 2.0%; (—□—), 3.0%.

rate decreased from 0.43 to 0.24 d^{-1} by increasing the inoculum size in the range of 0.1–4 g fresh weight/L.

According to inoculum size, crude saponin content showed a slight difference, and polysaccharide content was only slightly fluctuated over the whole inoculum size except for 0.4% (w/v) (Table 2).

Conclusion

P. ginseng hairy roots, transformed by *A. rhizogenes*, were incubated in various conditions in flask cultures. Transformed *P. ginseng* hairy roots showed an active branching pattern and a fast growth in hormone-free medium. The optimal culture conditions were 23°C, pH 5.8, 1/2 MS medium, and 3% sucrose. In the 1/2 MS basal medium, hairy roots maintained a high growth at 30 m*M*, 0.62 m*M* in nitrogen and phosphate salt concentration, respectively. In addition, the optimal inoculum size was 0.4% (w/v), and the ratio of inoculum size and the final cell weight was not proportional to the increase in inoculum size.

Crude saponin generally had much content at high sucrose concentration, and polysaccharide content was also higher at high sucrose concentration, pH 7.0, and 0.4% (w/v) inoculum.

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