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Assessment of Various Carbon Sources and Nutrient Feeding Strategies for *Panax ginseng* Cell Culture

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Abstract

Ginseng (root of *Panax ginseng* C. A. Meyer) cells were cultivated on medium supplemented with various carbohydrates including sucrose, glucose, and fructose, at initial concentrations ranging from 10 to 110 g/L. Sucrose was shown to be the superior carbon source to the monosaccharides for ginseng cell growth and the optimal concentration was between 30 and 50 g/L. An increase in the initial concentration within this range increased the maximum cell density and growth index significantly, whereas much higher concentrations inhibited cell growth. Feeding of sucrose and some other medium components during the growth (fed-batch mode) was more effective in enhancing the cell growth and biomass productivity, increasing the growth index by more than 60–70% and biomass productivity by more than 50%.

Index Entries: Biomass productivity; carbon source; nutrient feeding; *Panax ginseng*; plant cell culture; sugar concentration.

Introduction

Ginseng, the dried root of *Panax ginseng* C. A. Meyer of the Araliacae family, is a well-known herbal tonic, which has been used in China and some other oriental countries for thousands of years. In recent years, various ginseng health products have also been widely distributed in many Western countries. The current supply of ginseng mainly relies on field cultivation, which is a slow and laborious process *(1)*. Ginseng is highly priced on the market because of its unique medicinal functions and the

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costly production process. Since the 1960s, the use of plant tissue culture methods for the production of ginseng and its active ingredients has attracted considerable academic and industrial interest *(2)*. Ginseng tissue and cell culture have actually found commercial application in Japan at Nitto Denko Corporation *(3)* and China at Ganon Biochemistry Ltd. (China Pharmaceutical University, Nanjing, China). However, ginseng tissue and cell culture are still not widely used in industry, and the effort to optimize culture conditions and improve biomass and secondary metabolite productivity is far from complete.

For rational optimization of ginseng production in plant tissue and cell culture, there is a need for a more complete characterization and understanding of the key factors controlling the biomass growth and metabolite productivity. One of the most important factors is the nutrient supply to the culture. Among the various nutrient components in a plant cell medium, carbohydrates are the most prominent, serving as the major carbon and energy source in cell metabolism. In a batch culture, the time when cell growth starts to level off usually corresponds to depletion of the major carbon source and some other nutrients *(4)*. To prolong growth, we may either raise the initial concentrations of these components in the medium or add to the culture when they become growth limiting. Indeed, some previous studies have shown the effects of such measures on the growth and metabolism of ginseng cells *(5–7)*.

There have been only a few studies on the growth of ginseng cells at different sucrose concentrations and with alternative carbon sources. The objective of the present study was to conduct a comprehensive assessment of the effects of different carbon sources, sugar concentrations, and feeding schemes on ginseng cell growth and biomass productivity in suspension culture and to determine the physiological responses of the cells to high sugar concentrations. The culture media for most plant cells including ginseng are supplemented with 30 g/L of sucrose as the carbon source. In our study, we tested a wide range of sucrose concentrations, from 10 to 110 g/L, and alternative carbon sources, glucose, fructose, and their combinations with sucrose. By manipulating the sugar concentrations and applying nutrient feeding schemes, we were able to increase the ginseng biomass density and productivity significantly.

Materials and Methods

Plant Cells, Medium, and Culture Conditions

The ginseng cell strain used in this study was induced from the root of a 4-yr-old plant *P. ginseng* C. A. Meyer, which was harvested in northern China. The culture medium was formulated with Murashige–Skoog (MS) *(8)* basal salts and supplemented with 1 mg/L of 2,4-dichlorophenoxyacetic acid as the growth regulator and 30 g/L of sucrose as the (routine) carbon source, and 500 mg/L of casein hydrolysate. The MS basal salts were purchased from Gibco/Life Technologies (Grand Island, NY; cat. no. 10632)

and the supplements from Sigma (St. Louis, MO). The medium was prepared by dissolving all the components in deionized water, adjusting the pH to about 5.7, and then autoclaving at 121°C for 20 min. The cells were maintained in suspension culture in shake flasks, with 120-mL Erlenmeyer flasks each containing 25 mL or 250-mL flasks each containing 50 mL of medium, on an orbital shaker shaking at 110–120 rpm. The culture was placed in the dark at 25°C and subcultured every 14–16 d.

The experiments with various carbon sources, sugar concentrations, and nutrient feeding schemes were all carried out in the shake-flask culture. Glucose and fructose were tested as alternative carbon sources to sucrose, and initial concentration in the fresh medium was varied from 10 to 110 g/L. In the nutrient feeding studies, a 10× concentrated nutrient solution was added to the culture flasks at suitable volumes to reach a desired final concentration. Because it was difficult to withdraw uniform samples from the flasks, whole flasks were usually taken each time for various measurements. For each sample point, there were three to five replicate flasks. Therefore, the total number of flasks at the beginning of a test for each condition was equal to the sample frequency times the number of replicates.

Ginseng Cell Culture in Bioreactors

A 2-L KFL 2000 Laboratory Fermenter (Bioengineering AG, Wald, Switzerland) was used for studying batch and fed-batch culture of ginseng cells in stirred-tank bioreactors. The fermenter was equipped with four wall baffles (~12 mm wide), agitated with a four-blade hydrofoil impeller (ϕ48) (suitable for cell culture), and aerated through a ring sparger below the impeller. The bioreactor was inoculated with cells from 14- to 16-d-old shake-flask culture as we previously described, and the total culture liquid in the bioreactor was 1.70–1.72 L. During the experiment, the impeller speed was fixed at 250 rpm, and the gassing rate was adjusted between 0.1 and 0.3 vvm (volume of air/volume of liquid-min) to maintain dissolved oxygen (DO) at 20–50% air saturation. Sample (~5 mL) was taken from the culture every 2–5 d for various assays. The sample was forced out of the bioreactor by the input air pressure when the exhaust line was closed. Deionized water was added in due time to make up water losses owing to evaporation and sampling and to maintain a fairly constant liquid level in the tank. In the sugar-feeding experiments, a concentrated sucrose solution was added to the bioreactor in several aliquots (multiple feeding), starting from a day when the growth nearly reached stationary phase.

Quantification of Growth

The following growth characteristics were used for evaluation of the effects of various factors on the ginseng cell culture:

Growth index:

$$GI = X_{max}/X_o$$

Biomass productivity or the average growth rate (mg dry wt/L·d):

$$P_r = 1000(X_{max} - X_o)/t$$

in which X_o = the initial biomass density (g dry wt/L); X_{max} = maximum biomass density (g dry wt/L); and t = the day on which the maximum density was measured.

Determination of Cell and Sugar Concentrations

Fresh weight (fresh wt) of cells in the suspension was determined by centrifugation. The sample suspension was centrifuged at ≈1865*g* for 15 min. The pellet in the centrifuge tube was rinsed with deionized water at least twice and then centrifuged again. The water was then removed with a pipet, and the residual pellet was collected and weighed as the fresh weight. Dry weight was obtained by drying the fresh biomass for about 2 d to constant weight at 80°C in an oven.

The concentration of individual sugars was determined using a refractometer based on a linear correlation between sugar concentration and the refractive index. However, this method does not differentiate the types of sugar, so it was used only when one was added to the fresh medium. If other sugars were produced from cell metabolism during the culture, the measurement might represent the total sugar concentration since the calibration curves for the three different sugars were nearly identical.

Results and Discussion

Effects of Initial Sucrose Concentration

The growth curves and sugar consumption of the ginseng cell culture in shake flasks were measured at 30 and 40 g/L sucrose concentrations to determine the typical time courses of the culture (Fig. 1). The growth usually reached stationary phase between 20 and 26 d when medium sugar became nearly depleted. The specific growth rate in the exponential growth period [=the slope of $\ln(X/X_o) \sim t$ plot] based on the dry cell weight was 0.10 ± 0.02 d^{-1}. In the following experiments on different sugars and sugar concentrations, only the cell density between 20 and 30 d was measured from which the maximum cell density and growth index were obtained. In most cases of our experiments (with various sugar types and concentrations), maximum cell density was achieved within this time period.

Figure 2 presents the biomass density (fresh wt and dry wt) and sucrose concentration of the ginseng cell suspension culture at different initial sucrose concentrations. The sugar levels measured on d 0 in the culture medium were usually lower than the initial sugar concentrations in the fresh medium (as labeled Fig. 2) because of dilution by the inoculum. The biomass growth rate was the highest with 30 g/L of initial sucrose in the first 20 d, and with 50 g/L of sucrose, the growth was slower initially but showed a rapid increase on d 20–25. A further increase in the initial sucrose concentration, i.e., to 70 g/L and higher, resulted in serious growth

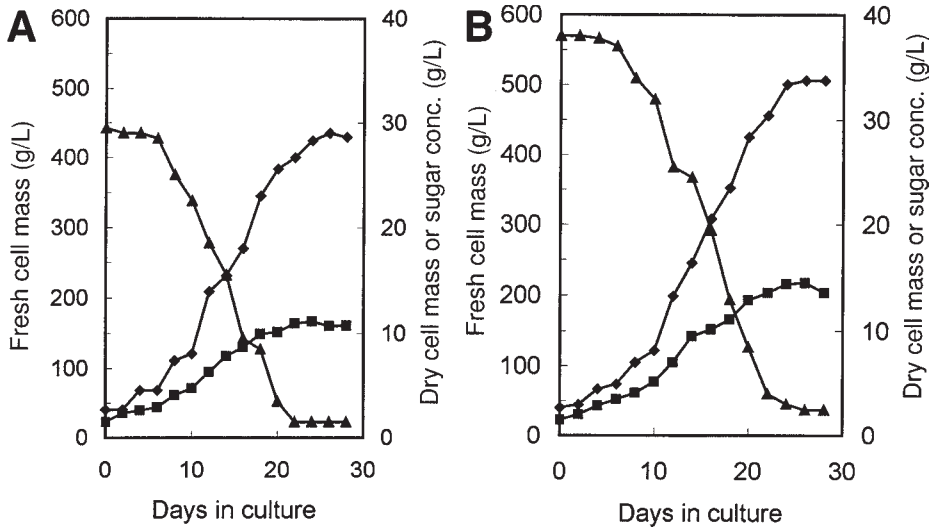

Fig. 1. Time courses of biomass and sucrose concentrations of ginseng cell culture in shake flasks. **(A)** 30 g/L sucrose; **(B)** 40 g/L sucrose. (—◆—), fresh wt; (—■—), dry wt; (—▲—), sucrose.

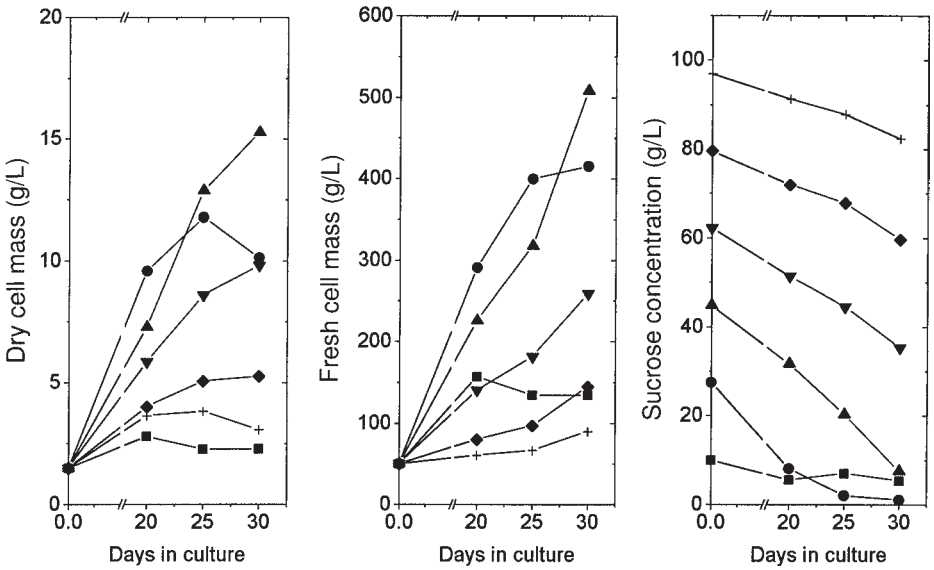

Fig. 2. Cell growth and sugar consumption of ginseng cell culture at different initial sucrose concentrations: (—■—), 10 g/L; (—●—), 30 g/L; (—▲—), 50 g/L; (—▼—), 70 g/L; (—◆—), 90 g/L; (—+—), 110 g/L.

retardation and significantly lower biomass concentrations. When the sucrose concentration was too low, e.g., 10 g/L, the growth was also slow and the maximum biomass density was low as a result of insufficient carbon nutrition. The optimal sucrose concentration for ginseng cell growth, there-

fore, was in the range of 30–50 g/L, the same as for the cell growth of a different type of ginseng, *Panax notoginseng*, reported by Zhang and Zhong *(7)*.

From the time courses of biomass and sucrose concentration (Figs. 1 and 2), we can also see that the biomass growth was closely correlated to sugar consumption and that active cell growth usually corresponded to rapid sugar consumption. The average biomass yield on sucrose was estimated as 0.40 ± 0.05 g dry wt/g of sucrose, which was in the same range as for the *P. notoginseng* cells in suspension culture (0.35–0.37 g of cells/g of sucrose) obtained by Zhang and Zhong *(7)*, and for various suspension cultures of tobacco cells [0.36–0.53 g of cells/g of sucrose cited by Dougall *(9)*].

The results also show a gradual decrease in the fresh wt to dry wt ratio with an increase in the sucrose concentration. For example, the fresh wt to dry wt ratio (Fig. 2) on d 25 was 34.0 at 30 g/L, 24.8 at 50 g/L, and 21.0 at 70 g/L, respectively. This decrease in the cell volume with sugar concentration was likely caused by a hypertonic effect of the higher osmotic pressures at higher sucrose concentrations. A cell in a hypertonic medium tends to lose water and shrink owing to osmosis. A smaller cell volume is favorable for maximization of the biomass concentration and productivity in suspension culture bioreactors, which are often limited by the packed volume of the cells *(10)*.

The trend of ginseng cell growth vs sucrose concentration is indicative of the Monod-type kinetics with substrate inhibition at high concentrations *(11)*. It is suspected that the growth depression at higher concentrations of sucrose may be partially caused by the high osmotic pressures. The osmotic pressure of the culture medium is directly proportional to sugar concentrations (data not shown). However, this postulation on osmotic effects needs to be further verified through experiments.

Growth on Different Carbon Sources

Table 1 presents the growth characteristics of the ginseng cells on medium supplemented with different carbohydrates, sucrose, glucose, and fructose. In general, sucrose was better than the monosaccharides as the carbon source for ginseng cell growth, and the replacement of sucrose with glucose and fructose partially or completely resulted in lower growth indexes and lower productivities. The highest productivity in the batch culture was obtained at 40 g/L of sucrose concentration, estimated as 500 mg/L·d (Table 1). With glucose, the growth indexes were only slightly lower than with sucrose; with fructose, the growth experienced a significant depression, especially at higher concentrations, e.g., 50 g/L and higher. Although the growth index with 30 g/L of fructose was not significantly lower than with 30 g/L of sucrose or glucose, the time required to reach the maximum was much longer (30 vs 25 d), giving rise to a lower productivity. The growth depression experienced by ginseng cells on the fructose medium was not unusual since the phenomenon has been observed for a number of other plant tissues and cells *(12–14)*. Usually the growth inhibition was appreciable only when the medium containing fructose had been autoclaved

Table 1
Ginseng Cell Growth and Biomass Productivity
on Different Types and Concentrations of Carbohydrate[a]

Sugar type (g/L)	Maximum biomass density (g dry wt/L)	Growth index	Productivity (mg/L·d)
Suc (30)	11.9 (25)[b]	7.2	408
Suc (40)	14.2 (25)	8.6	500
Suc (50)	15.3 (30)	9.3	454
Glc (30)	11.1 (25)	6.7	378
Glc (40)	12.1 (25)	7.3	418
Glc (50)	13.6 (30)	8.2	397
Fru (30)	11.2 (30)	6.8	318
Fru (50)	9.6 (30)	5.8	266
Suc (25) + Glu (5)	11.5 (25)	7.0	393
Suc (25) + Fru (5)	11.0 (25)	6.7	374
Glu (20) + Fru (20)	8.5 (25)	5.2	274

[a]Suc, sucrose; Glc, glucose; Fru, fructose; X_o = 1.65 g dry wt/L. Each data point is the average of three to five replicates, and the standard deviation (SD) among the data is within 7.5% of the mean (the same is true for the data in Table 2).
[b]The day on which the maximum was measured.

or steam-sterilized but not with the filter-sterilized fructose medium, as a result of thermal decomposition to growth-inhibiting compounds.

Feeding of Sucrose and Other Nutrients for Cell Growth

We have shown that raising the initial sucrose concentration within a certain range can increase the maximum biomass concentration in a batch culture. However, the cell growth was often temporarily retarded by the higher sugar concentrations and the productivity could be lower. To avoid both carbon nutrient limitation and high sugar inhibition, we could add smaller amounts of sucrose during the culture period (sucrose feeding), at time(s) when the sucrose is near depletion.

In a batch culture without nutrient feeding, the cell growth approached maximum cell density on d 21–25 and then started to drop (with initial 30 g/L of sucrose). With sucrose feeding, the cell density continued to rise after d 30 and the growth index increased by more than 60% (Table 2). The biomass productivity was in the range of 557 to 585 mg/L·d with feeding, which is 35–42% higher than that achieved in the control without feeding, about 410 mg/L·d. The feeding schedule did not affect the results significantly, nor did the use of a multiple feeding scheme, i.e., intermittent addition of smaller amounts. A possible explanation for these results may be that with the feeding on d 15, the sucrose concentration was in the range of 15–20 g/L (Fig. 2), and the addition of 20 g/L would make a total of no more than 40 g/L, which is below the inhibitory level; with feeding on d 23, the cells were still in the exponential growth phase and were not subjected to significant sugar limitation.

Table 2
Enhancement of Ginseng Cell Growth and Biomass Productivity Through the Feeding of Sucrose and Medium Components[a]

Feed component (g/L)	Feeding schedule (d)	Maximum biomass density (g dry wt/L)	Growth index	Productivity (mg/L·d)
None	—	11.8 (25)	7.8	410
Suc (20)	15	19.1 (30)	12.7	585
Suc (20)	19	18.6 (30)	12.4	570
Suc (20)	23	18.2 (30)	12.1	557
Suc (20)	15–23, five times	18.8 (30)	12.5	577
Suc (20) + Org[b]	17	20.3 (30)	13.6	628
Suc (20) + Inorg[c]	17	17.1 (30)	11.4	518
Fresh medium[d]	17	18.7 (30)	12.5	573

[a]Initial Suc (sucrose) = 30 g/L; X_o = 1.5 g dry wt/L.
[b]MS vitamins plus glycine, at concentrations the same as in the original MS medium.
[c]MS inorganic salts, at concentrations the same as in the original MS medium.
[d]Complete MS medium with 20 g/L of sucrose.

Previous studies on plant cell culture indicate that some other major nutrients, such as phosphorus and nitrogen, in addition to the carbon source may also become exhausted as the cell growth is approaching the stationary phase *(4)*. Therefore, feeding of these components may be fruitful to enhance the growth near the stationary phase. We therefore further tested several other feeding formulations: complete medium, sucrose + organic nutrients (vitamins and glycine), sucrose + MS inorganic salts. However, only with the addition of the organic nutrients was there a slight increase in the biomass growth indexes and productivity, and feeding of the MS salts even adversely affected growth. Similarly, in a study by Zhang and Zhong *(7)*, feeding sucrose together with MS basal medium ± phytohormones did not further improve the growth of *P. notoginseng* cells in suspension culture with respect to sucrose feeding. The negative effect caused by adding the MS salts could be owing to a high osmotic pressure at the high salt concentration. In another test, we compared the growth of ginseng cells on medium containing diluted and concentrated MS salts (Table 3). It was found that the growth was significantly retarded with 1.5× concentrated MS salts, as shown by a lower growth index (on d 21); the growth index on medium with 3/4 MS salts was comparable to that on the full MS salt medium. This implies that the MS medium, which is commonly regarded as "high-salt," is sufficiently enriched for the ginseng cell culture.

Effect of Sucrose Feeding on Bioreactor Culture

Figure 3 shows the growth and sucrose concentration curves of batch and fed-batch culture of ginseng cells in the 2-L stirred-tank bioreactor. Without sucrose feeding (batch mode, Fig. 3A), the growth characteristics

Table 3
Growth of Ginseng Cells in Medium with Different Proportions of MS Salts[a]

Maximum biomass[b]	Fraction of MS salts				
	1/4	1/2	3/4	1.0	1.5
Dry wt ± *s*[c]	8.46 ± 0.6	12.1 ± 0.9	14.9 ± 0.8	14.6 ± 0.3	10.6 ± 0.4
Fresh wt ± *s*	280.4 ± 26.8	345.9 ± 18.9	433.8 ± 32.2	435.1 ± 15.9	206.2 ± 10.2

[a]Initial sucrose = 30 g/L, X_o = 2.0 g dry wt/L and 68.31 g fresh wt/L, respectively.
[b]Obtained on d 21.
[c]Mean of five flasks ± *s* (standard deviation).

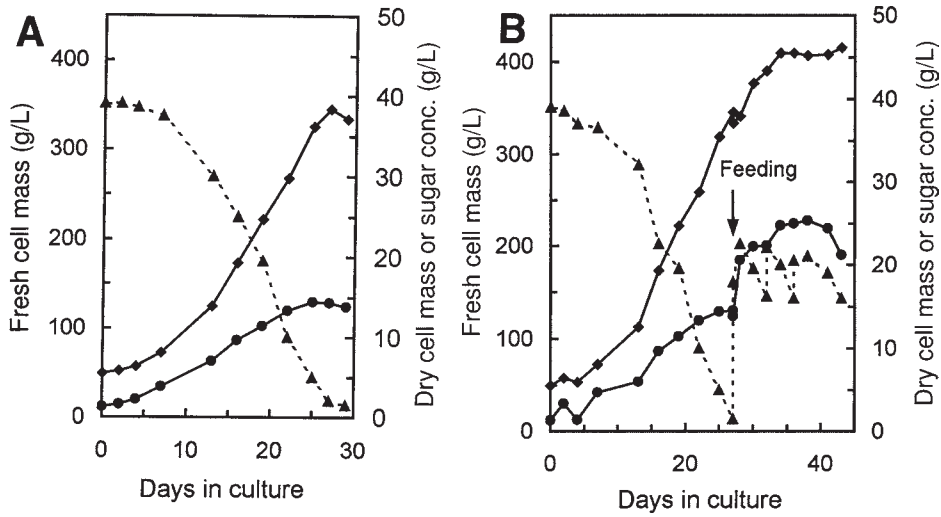

Fig. 3. Batch and fed-batch culture of ginseng cells in a 2-L stirred-tank bioreactor: **(A)** batch growth; **(B)** fed-batch with sucrose feeding (day of feeding: 27, 28, 32, 36, and 38). (—◆—), fresh wt; (—●—), dry wt; (– –▲– –), sucrose.

including the biomass growth rate, biomass productivity, and biomass yield on sucrose were similar to those found in the shake flasks at the same initial sucrose (specific growth rate ≈0.10 d^{-1}, biomass productivity ≈523 mg dry wt/L·d, and yield ≈0.41 g dry wt/g). With sucrose feeding (fed-batch mode, Fig. 3B), the growth period was much longer and the maximum biomass concentration higher than those achieved in the batch run (25.3 vs 15.4 g/L). The maximum biomass productivity in the fed-batch culture was 658 mg/L·d, about 25% higher than that in the batch culture of 523 mg/L·d.

Conclusion

Although ginseng cells could utilize glucose and fructose as the alternative carbon source to sucrose, the growth rates were generally lower than on sucrose. The carbon source appeared to be the main growth-limiting nutrient for ginseng cell growth, and the biomass growth of ginseng cells

was directly correlated to the sugar consumption. An increase in the initial sucrose concentration in the medium within a certain range and feeding sucrose to the culture before the stationary phase could both prolong the growth phase, whereas the latter was more effective at increasing the biomass productivity. Therefore, the fed-batch process is an effective process strategy for ginseng production in plant cell culture.

Acknowledgments

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