Chapter 6
Ginseng Cell Culture for Production of Ginsenosides

Nguyen Trung Thanh, Hosakatte Niranjana Murthy, and Kee-Yoeup Paek

Abstract *Panax ginseng* C. A. Meyer (Araliaceae) is one of the most valuable oriental herbs and has been used as a healing drug and a health tonic in Korea, Japan and China since ancient times. Cultivation of ginseng in fields takes a long time, generally 4–6 years, and needs extensive efforts for quality control since plant growth is susceptible to many environmental factors including soil, shade, climate, pathogens and pests. On the other hand, the culturing of plant cells has been considered as a potential alternative for the efficient production of ginseng biomass and its active ingredients, such as ginseng saponins. In this chapter, the research work on cell suspension cultures of Korean ginseng (*P. ginseng*) using bioreactor technology, the various culture factors and the process variables such as growth regulators, sucrose concentration, types of bioreactors, inoculum density, aeration volume, gaseous composition such as oxygen, carbon dioxide and ethylene and elicitation on suspension cultures have been presented.

Keywords Bioreactor cultures • Ginseng • Ginsenosides • Panax ginseng • Suspension cultures

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Abbreviations

2 4-D	2, 4-Dichlorophenoxy acetic acid
DW	Dry weight
FW	Fresh weight
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MJ	Methyl jasmonate
MS medium	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
vvm	Air volume per medium volume per minute

6.1 Introduction

Panax ginseng C. A. Meyer (Araliaceae) has been widely used as a tonic and medicine since ancient times, particularly in oriental countries including Korea, Japan and China. It is effective for gastro-enteric disorders, diabetes, blood circulation, and has been used as an adjuvant to prevent various disorders. Thus, ginseng has been recognized as a miraculous medicine in preserving the health and attaining the longevity. The principal bioactive constituents of *P. ginseng* are the ginsenosides, a group of triterpene glycosides also known as saponins [1]. Currently, 30 ginsenosides have been recognized and they have been grouped under protopanaxadiol saponins (Rb group) and protopanaxatriol saponins (Rg group). Antiplatelet, hypocholesterolemic, antitumor, immunomodulatory functions, and their activity of improving the central nervous system have been attributed to the pharmacological importance of various ginsenosides [2, 3].

Plant cell suspension cultures are more advantageous for the for large scale production of secondary metabolites such as ginsenosides using bioreactor technologies [4]. The tissue culture of ginseng was first documented in 1964, after that numerous studies on ginseng were reported in the succeeding years [1]. Various successful efforts on *in vitro* culture of ginseng cells or tissues for the production of ginsenosides have been reported [5–8]. Murashige and Skoog (MS) [9] medium is commonly used for establishing cell and tissue cultures and other various physiological and physical factors have been investigated for the production of biomass and ginsenosides [1]. The addition of growth regulators is essential for cell growth, biomass accumulation and product formation. Choi [10] has investigated the *in vitro* culture of *P. ginseng* extensively and indicated that plant growth regulators such as 2, 4-D and kinetin in the medium affected the levels of saponins in callus and cell suspension cultures. For example, 3.62 % of total saponins were detected in the callus cultivated in MS medium containing 5 mg L^{-1} 2, 4-D and 1 mg L^{-1} kinetin, while 8.78 % was produced in 10 mg L^{-1} 2, 4-D and 1 mg L^{-1} kinetin medium. Zhong [11] found that a higher concentration of 7 mg L^{-1} kinetin inhibited the cell growth. The highest saponin content (13.9 %) was achieved in a medium containing 2.0 mg L^{-1} IAA and 0.07 mg L^{-1}

kinetin. Sucrose is a common carbon source used in ginseng cell cultures and the rate of biomass growth is usually directly correlated with the sugar consumption. Many researchers have worked out different types and concentration of sugars depending upon the cell lines used for cultures. Choi et al. [12, 13] found that the optimal concentration of sucrose for cell growth was between 30–50 g L^{-1}, and 70 g L^{-1} sucrose inhibited cell growth, while the saponin content showed a steady increase with the increase in sucrose concentration up to 60 g L^{-1}. In suspension cultures of *Panax notoginseng* cells, Zhang and Zhong [14] found that the constant or intermittent feeding of sucrose or other sugars was more effective than increasing the initial concentration to enhance the biomass and yield of secondary metabolites. Effects of nitrogen sources on the production of ginsenosides by cell cultures were investigated by Zhang et al. [15] and Ushiayama [16] and they have reported that a lower NH_4^+ to NO_3^- ratio is more favorable for saponin production. Zhang and Zhong [14] found that an increase in initial phosphate from 1.25 to 3.75 mM enhanced both cell growth and saponin yield in cell suspension cultures of *P. notoginseng*. The effect of K^+ and Cu^{2+} ions have also been investigated on cell growth and metabolite production in ginseng [17, 18].

Bioreactor cultures (stirred tank and airlift bioreactors) were established for large scale production of ginseng cell biomass and saponin production [19–21]. Impact of fed-batch cultures [14, 22], condition of the medium [23] and high-density cultures [14, 24–26] were experimented and the increased biomass and productivity of saponins and polysaccharides was achieved.

Recent reports show that saponins account for about 3–4 % in Korean ginseng, and more than 30 kinds of ginsenosides have been found in it, double the number of ginsenosides occurring in the ginsengs of other countries [2]. Considering that each of these ginsenosides has different pharmacological activities, it becomes apparent that Korean ginseng has a pharmacological effectiveness superior to those of other ginseng species [2]. Therefore, we were interested in establishing cell cultures of Korean ginseng and carried out a series of experiments for the production of ginsenosides in bioreactor cultures, and here we have summarized various aspects of ginseng cell cultures for the production of useful metabolites in a large scale.

6.2 Cell Suspension Cultures of Ginseng in Shake Flasks

6.2.1 Induction of Callus

Calli were induced from Korean ginseng (*P. ginseng* C.A. Meyer) root on MS semi-solid medium supplemented with 1.0 mg L^{-1} 2, 4-D and 3 % sucrose in the dark at 25 °C [27]. The callus proliferation was achieved on MS semi-solid medium supplemented with 2.0 mg L^{-1} NAA, 0.1 mg L^{-1} kinetin, and 30 g L^{-1} sucrose. Suspension cultures were established in 300 mL conical flasks containing 100 mL MS medium by adding 6 g callus and were maintained on rotary shaker at 105 rpm, in the dark at 25 °C. Cells were maintained by subculturing on to a fresh medium once every 15 days.

6.2.2 *Effects of Auxins on Cell Growth and Ginsenosides Production in Cell Suspension Culture of P. ginseng*

To understand the growth characteristics of cell suspension cultures of ginseng in shake flasks, the effects of plant growth regulators (2, 4-D, IBA, and NAA) on cell growth and saponin production, and nutrient utilization by the cultured cells were studied. The maximum biomass yield was obtained in medium containing 2, 4-D as compared to IBA or NAA. It was observed that a relatively lower concentration of IBA and NAA was unfavorable for cell growth and production of ginseng saponins (Table 6.1). With an increase in IBA or NAA concentration from 1 to 9 mg L^{-1} in the medium, the dry weight of cells increased and this phenomenon was reported from other cultures as well, in which high auxin levels were often good for cell growth [28]. In our experiments, the highest dry weight of cells was obtained with IBA (10.5 g L^{-1}) and NAA (9.7 g L^{-1}) at a concentration of 9 mg L^{-1}.

The effects of auxin (2, 4-D, IBA, and NAA) concentrations on saponin accumulation by *P. ginseng* cells were also studied. Total saponin production was significantly enhanced as the initial auxin level was raised from 1 to 7 mg L^{-1} of IBA and from 1 to 3 mg L^{-1} of NAA. However, a further increase in auxin concentration (up to 9 mg L^{-1}) led to a decrease in saponin accumulation (Table 6.1). The maximum saponin production of 7.29 ± 0.2 mg g^{-1} DW and 8.76 ± 0.1 mg g^{-1} DW was achieved at IBA concentration of 7 mg L^{-1} and NAA concentration of 3 mg L^{-1} respectively. These results are considered to be useful for exploration of the biosynthesis mechanism and in a large-scale bio-processing of the ginseng cell cultures.

Plant growth regulators are one of the key factors to influence the biomass accumulation and secondary metabolite production. In safflower cell cultures, high concentration of auxin was suitable for the cell growth, while high concentration of cytokinin was favorable for red and yellow pigment production in *Carthamus tinctorius* [29]. Son et al. [30, 31] found that 4 mg L^{-1} IBA was suitable for the mountain ginseng adventitious roots growth. So, the influence of growth regulators during *in vitro* culture is species specific. In this study, IBA (7 mg L^{-1}) was found to be

Table 6.1 Effect of auxins on cell growth and ginsenosides production in cell suspension culture

Auxin	Concentration (mg L^{-1})	Fresh wt. (g L^{-1})	Dry wt. (g L^{-1})	Ginsenosides (mg g^{-1} dry wt.)		
				Rg	Rb	Total
2,4 D	1	328 a[a]	11.9 a	1.81 ± 0.1	2.35 ± 0.2	4.16 ± 0.3
IBA	1	144 d	7.5 d	2.16 ± 0.3	3.05 ± 0.1	5.21 ± 0.3
	3	170 c	8.8 cd	2.09 ± 0.6	3.49 ± 0.2	5.58 ± 0.3
	5	178 c	9.1 c	2.43 ± 0.2	3.31 ± 0.4	5.74 ± 0.2
	7	216 b	10.1 b	2.6 ± 0.3	4.69 ± 0.2	7.29 ± 0.2
	9	226 b	10.5 b	1.42 ± 0.6	4.22 ± 0.2	5.64 ± 0.3
NAA	1	132 d	7.1 e	2.85 ± 0.4	5.33 ± 0.2	7.18 ± 0.2
	3	134 d	7.3 de	3.28 ± 0.3	5.48 ± 0.1	8.76 ± 0.1
	5	152 cd	7.8 d	2.61 ± 0.1	4.83 ± 0.2	7.44 ± 0.1
	7	164 c	7.6 cd	2.16 ± 0.1	4.08 ± 0.3	6.24 ± 0.3
	9	188 c	9.7 b	2.16 ± 0.5	2.45 ± 0.2	4.61 ± 0.3

[a]Mean separation by Duncan's multiple range test at $P \leq 0.05$

favorable auxin for the increase in cell mass as well as increase in total saponin yield (10.1 g L^{-1} DW and 7.29 ± 0.2 mg g^{-1} DW, respectively), while NAA at 3 mg L^{-1} was favorable for saponin accumulation but not effective for increasing cell biomass (7.3 g L^{-1} DW and total saponin 8.76 ± 0.1 mg g^{-1} DW). For this reason IBA (7 mg L^{-1}) was used during the cell suspension cultures of *P. ginseng*.

6.2.3 *Effect of IBA and Cytokinin Combinations on Cell Growth and Ginsenosides Production in Cell Suspension Culture of P. ginseng*

To determine the effect of types and concentrations of cytokinins on increase in cell biomass and ginsenoside production, kinetin and BA at 0.1, 0.5 and 1 mg L^{-1} were combined with 7 mg L^{-1} IBA. The results (Table 6.2) clearly showed that the addition of cytokinins (BA and kinetin) did not affect the proliferation of cells in culture.

The saponin productivity (particularly Rb group) was increased when the medium was supplemented with 0.1–0.5 mg L^{-1} BA or kinetin (Table 6.2). The highest saponin content was (7.08 ± 0.1 and 7.34 ± 0.2 mg g^{-1} DW) obtained with a combination of IBA at 0.5 mg L^{-1} of BA or kinetin. Further increase in cytokinin concentrations led to the decrease in ginsenoside content. A relatively high cytokinin level was not favorable to secondary metabolite synthesis. A similar phenomenon was also reported in other plant cell cultures. In *P. notoginseng* suspension cell cultures, Zhong [11] found that both the saponin content and cell biomass production were decreased with an increase in kinetin concentration.

Flow Cytometric Analysis of *Panax ginseng* Cells

To verify the genetic stability of regenerated cells, 2C DNA values of suspension cultures were analyzed by flow cytometry and compared with donor plant. The histograms obtained with *P. ginseng* cells of different inoculum densities cultured for 25 days in MS medium with 7 mg L^{-1} IBA and donor plant are shown in Fig. 6.2a.

Table 6.2 Effect of IBA and cytokinin combinations on cell growth and ginsenoside production in cell suspension culture of *P. ginseng*

Cytokinin	Concentration (mg L^{-1})	Fresh wt. (g L^{-1})	Dry wt. (g L^{-1})	Ginsenoside (mg g^{-1} dry wt.)		
				Rg	Rb	Total
	0	221 a[a]	11.0 ab	2.17 ± 0.1	4.36 ± 0.2	6.43 ± 0.2
BA	0.1	230 a	11.0 ab	1.81 ± 0.1	2.75 ± 0.2	4.56 ± 0.3
	0.5	252 a	11.5 a	1.75 ± 0.4	5.33 ± 0.1	7.08 ± 0.1
	1	242 a	11.0 a	1.79 ± 0.1	3.53 ± 0.2	5.33 ± 0.2
Kinetin	0.1	224 ab	11.1 ab	1.16 ± 0.1	4.61 ± 0.3	5.76 ± 0.3
	0.5	240 a	11.7 a	1.49 ± 0.3	5.85 ± 0.2	7.34 ± 0.2
	1	242 a	11.4 a	1.56 ± 0.2	3.51 ± 0.3	5.07 ± 0.1

[a]Mean separation by Duncan's multiple range test at $P \leq 0.05$

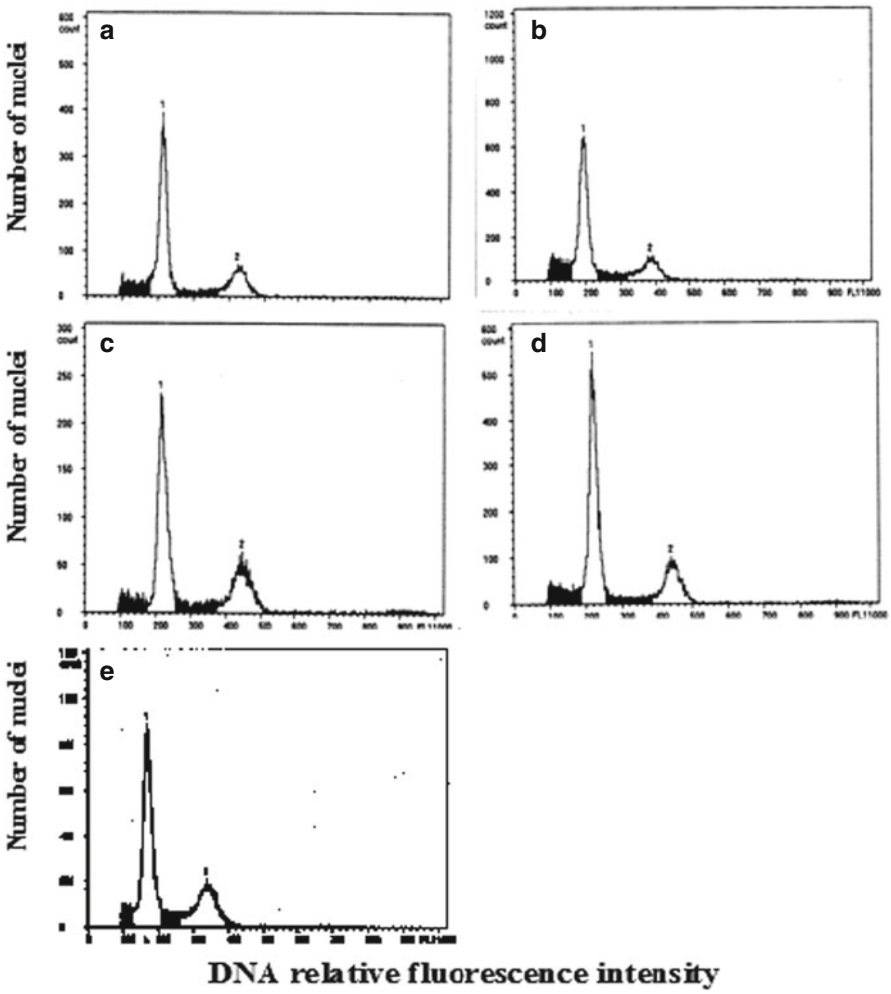

Fig. 6.1 Flow cytometric analyses of *P. ginseng* at different density cell. (**a**) 4 g, (**b**) 6 g, (**c**) 8 g, (**d**) 10 g/100 mL medium and (**e**) mother plant

In suspension culture, most of the cells were diploid (Fig. 6.1a–d), revealing a peak at nearly the same position as the standard diploid donor plant (Fig. 6.1e). However, in other plant species high chromosomal variability during *in vitro* cultures has been reported [32]. Nevertheless, *P. ginseng* callus line has mostly retained its ploidy level even after 4 years of culture on the callus induction medium containing relatively high levels of 2,4-D.

To examine more closely the cell division activity within a culture passage of 25 days, the percentage of cells in the three phases of the cell cycle, G1, S and G2 + M, were evaluated by flow cytometery in both donor plant and suspension culture cells with different inoculum densities (4, 6, 8, 10 g FW/100 mL MS medium). The

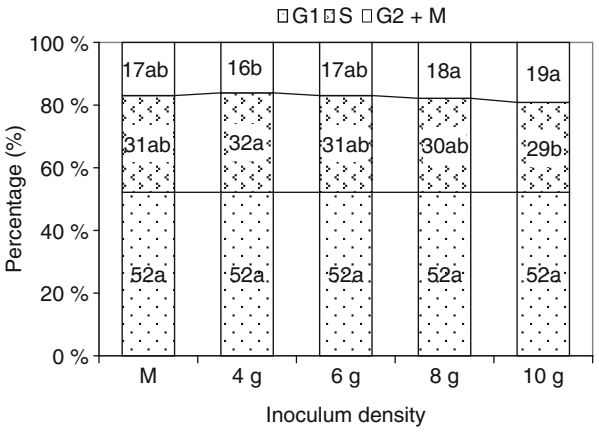

Fig. 6.2 Cell cycle analyses of *P. ginseng* cells

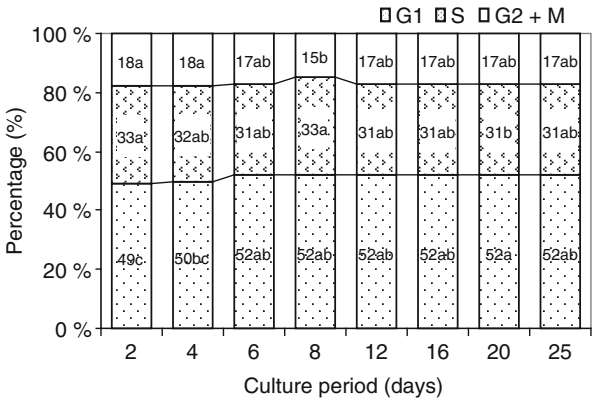

Fig. 6.3 Cell cycle analyses of *P. ginseng* cell cultured for 25 days

results are summarized in Figs. 6.2 and 6.3. The ratio of cells in S phase clearly show a parallel development at G1 (52 %), other phase variants show as S (29–33 %); G2 + M (15–18 %; Fig. 6.2).

The results of cell cycle analysis of cells at different phases of cell cycle over a period of 25 days also revealed the stability of cells without much variation (Fig. 6.3).

6.2.4 Effect of Sucrose Concentrations on Cell Growth and Ginsenoside Production in Cell Suspension Culture of P. ginseng

The effect of sucrose concentration at a range of 10–70 g L^{-1} were studied to find out the optimal sucrose concentration for cell growth and saponin production and the results are presented in Table 6.3. Dry cell weight increased with an increase in sucrose concentration from 10 to 30 g L^{-1}. Further, increase in sucrose

Table 6.3 Effect of sucrose concentrations on cell growth and ginsenoside production in cell suspension culture of *P. ginseng*

Sucrose concentration (g L^{-1})	Fresh wt. (g L^{-1})	Dry wt. (g L^{-1})	Ginsenoside (mg g^{-1} dry wt.)		
			Rg	Rb	Total
10	26.6 d[a]	2.9 d	0.32 ± 0.1	0.62 ± 0.2	0.92 ± 0.1
30	180.6 a	10.8 a	3.17 ± 0.1	4.19 ± 0.1	7.36 ± 0.2
50	98.2 b	8.4 b	1.06 ± 0.2	2.23 ± 0.1	3.29 ± 0.1
70	52.1 c	5.7 c	0.08 ± 0.1	1.56 ± 0.2	1.64 ± 0.2

[a]Mean separation by Duncan's multiple range test at $P \leq 0.05$

concentration of up to 50 or 70 g L^{-1} decreased the cell biomass. The highest biomass yield was obtained (180.6 g L^{-1} FW and 10.8 g L^{-1} DW) at 30 g L^{-1} of sucrose concentration.

In case of *Vitis vinifera* cell culture, both a lag phase and reduced cell concentration were observed under a relatively high sucrose concentration of 50 g L^{-1} [33]. For cell culture of *Coleus blumei*, a high initial sucrose concentration of 60 g L^{-1} led to a higher biomass accumulation without an obvious lag phase [34]. With suspension cultures of *Perilla frutescens*, the growth rate increased with an increase in initial sucrose level of up to 60 g L^{-1} in the medium [26]. Choi et al. [12, 13] found that the optimal concentration of sucrose for cell growth was between 30 and 50 g L^{-1} and 70 g L^{-1} sucrose inhibited cell growth, while the saponin content showed a steady increase with sucrose concentration of up to 60 g L^{-1} in *Panax ginseng*. It is clear that initial sucrose concentration is important for the proliferation of plant cells and its effect depends on a specific cell lines. Similarly, the saponin content of the cells was also dependent on initial sucrose concentration in the medium as that of cell mass (Table 6.3). Initial sucrose concentration of 30 g L^{-1}, significantly increased the saponin accumulation in the cells (7.36 ± 0.2 mg g^{-1} DW) and total saponin production decreased at a higher sucrose concentration of up to 70 g L^{-1}. In cell suspension cultures of *P. notoginseng* also, manipulation of medium sucrose could effectively enhance the saponin production [35].

6.3 Ginseng Cell Culture in Bioreactors

6.3.1 Effect of Bioreactor Types on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng

The effect of various types of airlift bioreactors such as cylinder, cone, balloon and bulb type bioreactors of 5 L capacity containing 4 L of optimized medium (MS medium with 7 mg L^{-1} of IBA, 0.5 mg L^{-1} kinetin and 30 g L^{-1} sucrose) was tested for biomass accumulation and metabolite production and results are presented in Table 6.4. Balloon type bioreactor was found suitable for biomass accumulation and

Table 6.4 Effect of bioreactor types on k_La coefficient and cell growth during cell culture of *P. ginseng*

Bioreactor type	Initial k_La (h^{-1})	Biomass			Growth rate[a]
		Fresh wt. (g L^{-1})	Dry wt. (g L^{-1})	% dry wt.	
Cylinder	5.25	240 b[b]	9.1 b	3.8	4.14
Balloon	6.98	255 a	10.6 a	4.0	4.82
Bulb	6.95	251 a	10.1 a	4.0	4.59
Cone	5.69	245 ab	9.8 ab	3.9	4.45

[a]Growth rate is the quotient of the dry weight after culture and the dry weight of the inoculum size
[b]Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

Table 6.5 Effect of bioreactor types on ginsenoside production

Bioreactor type	Ginsenoside (mg g^{-1} wt)			
	Rg	Rb	Total[a]	Rb: Rg[b]
Cylinder	1.45 ± 0.2	2.37 ± 0.3	3.82 ± 0.4	1.67 ± 0.4
Balloon	0.79 ± 0.1	3.38 ± 0.6	4.17 ± 0.6	4.27 ± 0.7
Bulb	1.09 ± 0.3	3.07 ± 0.5	4.16 ± 0.5	2.98 ± 1.2
Cone	1.35 ± 0.3	2.59 ± 0.1	3.95 ± 0.2	1.98 ± 05

[a]Total content = Rb + Rg
[b]Rb: Rg = (Rb1 + Rc + Rb2 + Rd)/(Rg1 + Re + Rf)

255.4 g L^{-1} FW, 10.6 g L^{-1} DW were recorded. The highest ginsenoside amount of 4.17 mg g^{-1} DW was also documented with balloon type bioreactors and this might be due to high initial k_La values (Table 6.5). Kim et al. [36] have also reported that balloon type bioreactors are suitable among the various configurations of bioreactors tested for ginseng adventitious root cultures.

6.3.2 *Effect of Aeration Volume on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng*

The cell multiplication, growth and accumulation of secondary metabolites in bioreactors were strongly influenced by aeration volume [37] and it is essential to investigated suitable aeration volume to achieve biomass and metabolite productivity. Constant aeration of 0.05, 0.1, 0.2 and 0.3 vvm as well as variable aeration volume of 0.5/0.1/0.2/0.3 (i.e., aeration volume was changed for every 6 days) were tested and the results of effect of aeration volume on cell growth and yield of ginsenosides are presented in Tables 6.6 and 6.7. Increment of aeration volume with the increasing time duration was found suitable for both biomass and ginsenoside productivity.

Table 6.6 Effect of aeration volumes on k_La and cell growth in bioreactor culture

Aeration volume (vvm)	Initial k_La (h^{-1})	Biomass			Growth rate[a]
		Fresh wt. ($g\ L^{-1}$)	Dry wt. ($g\ L^{-1}$)	% dry wt.	
0.05	4.95	165 d[b]	5.7 d	3.44	2.58
0.1	7.84	244 b	10.3 a	4.22	4.68
0.2	11.42	225 c	9.1 b	4.02	4.08
0.3	16.81	211 c	7.9 c	3.76	3.61
0.05/0.1/0.2/0.3[c]	5–16.58	263 a	10.6 a	4.04	4.82

[a]Growth rate is the quotient of the dry weight after culture and the dry weight of the inoculum size
[b]Mean separation within columns by Duncan's multiple range test at $P \leq 0.05$
[c]Aeration volume increased at 6-day intervals

Table 6.7 Effect of aeration volumes on ginsenoside production in bioreactor culture

Aeration volume (vvm)	Ginsenoside (mg g^{-1} wt)			
	Rg	Rb	Total[a]	Rb: Rg[b]
0.05	0.72 ± 0.1	1.82 ± 0.2	2.55 ± 0.3	2.52 ± 0.1
0.1	1.47 ± 0.3	2.54 ± 0.1	4.01 ± 0.4	1.76 ± 0.3
0.2	1.06 ± 0.6	2.65 ± 0.2	3.71 ± 0.3	3.58 ± 0.3
0.3	1.29 ± 0.2	2.32 ± 0.4	3.61 ± 0.2	1.86 ± 0.6
0.05/0.1/0.2/0.3[c]	0.98 ± 0.3	3.31 ± 0.2	4.28 ± 0.3	3.57 ± 0.8

[a]Rb: Rg = (Rb1 + Rc + Rb2 + Rd)/(Rg_1 + Re + Rf)
[b]Total content = Rb + Rg
[c]Aeration volume was increase at 6-day intervals

6.3.3 *Effect of Inoculum Density on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng*

The effect of inoculum density of the cultured cells on biomass and metabolite accumulation is well established by various studies [38–40]. For example, Jeong et al. [40] investigated the production of ginsenosides from adventitious root suspension cultures at an inoculum density of 2.5, 5.0, 7.5 and 10.0 g L^{-1} and reported 10 % increment in ginsenosides with an inoculum density of 5.0 g L^{-1}. The results of the effect of inoculum density on biomass and secondary metabolites accumulation in the present study are depicted in Fig. 6.4. Of the varied inoculums tested (40, 60, 80 and 100 g) 100 g L^{-1} fresh weight was good for fresh and dry biomass accumulation (Fig. 6.4a, b). However, metabolite accumulation was optimum with inoculum density of 60 g L^{-1} (Fig. 6.4c). The maximum saponin production of 4.4 mg g^{-1} DW was achieved and therefore, 60 g L^{-1} inoculum density was used for further experiments.

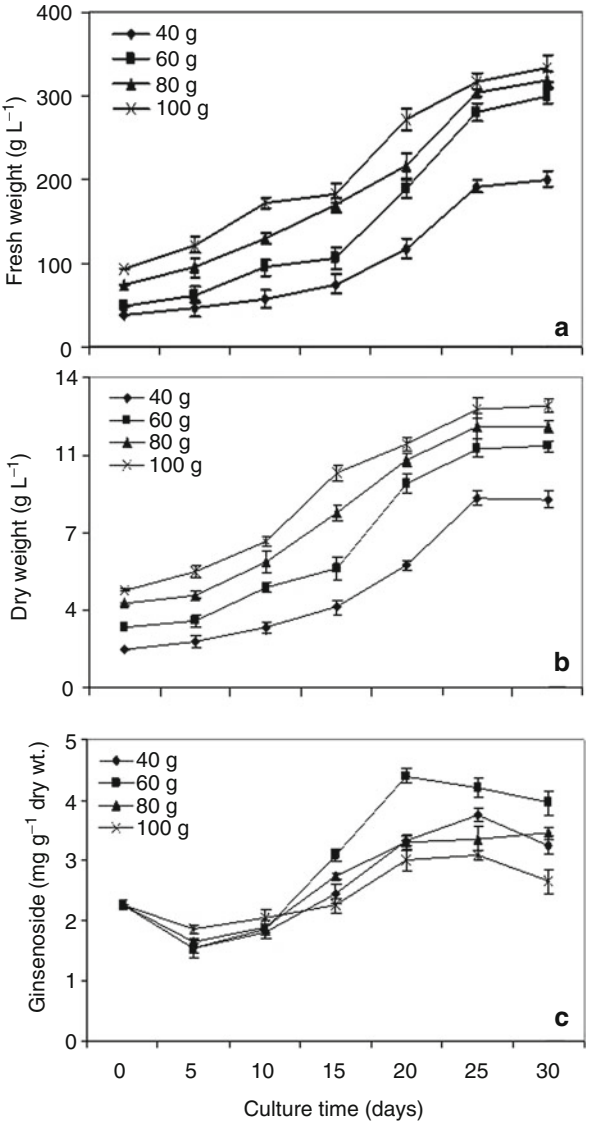

Fig. 6.4 Effect of inoculum density on biomass and ginsenosides accumulation of *P. ginseng* cells cultured in large-scale suspension cultures. Fresh weight (**a**), dry weight (**b**) and ginsenosides content (**c**)

6.3.4 Effect of Oxygen Supply on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng

Ginseng cells were cultured in 5 L capacity airlift bioreactors and the effect of oxygen levels was tested on accumulation of biomass and ginsenosides. The growth kinetics of *P. ginseng* cells cultivated in balloon type-bubble bioreactors at four

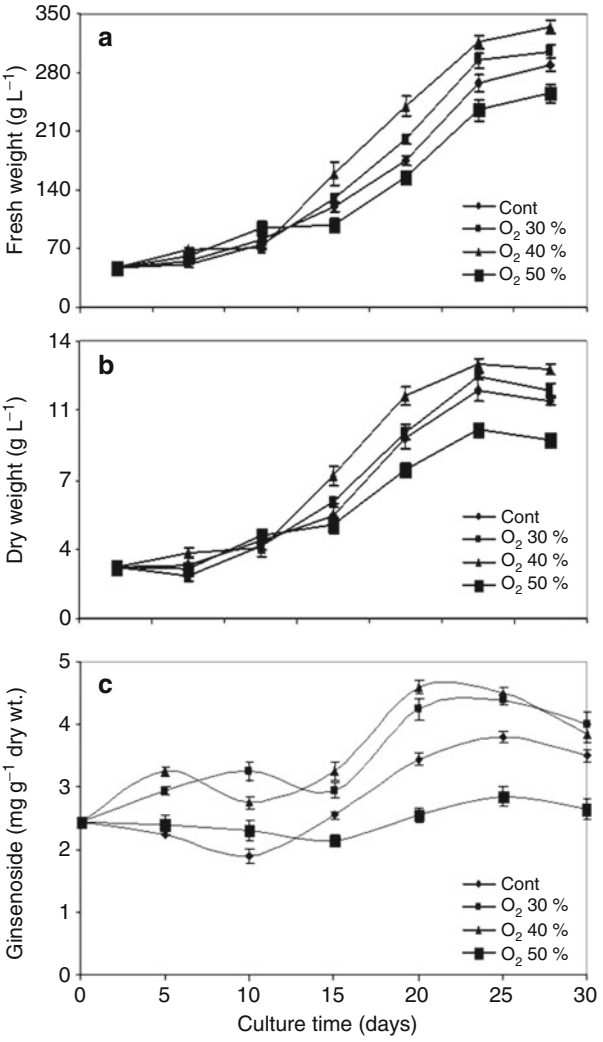

Fig. 6.5 Time profiles for (**a**) fresh cell weight, (**b**) dry cell weight and (**c**) saponin production in *P. ginseng* suspensions cultivated in 5 L balloon-type bubble reactors

different levels oxygen supply [20.8 % (control), 30 %, 40 %, and 50 %] are presented in Fig. 6.5 [41]. The cell growth and biomass accumulation increased gradually with the time duration and maximum biomass level was reached after 25 days. Similar growth patterns have been previously reported for *P. notoginseng* cell cultures [24]. The maximum FW (316 g L^{-1}) was achieved at 40 % oxygen; the corresponding DW was 12.8 g L^{-1} (Fig. 6.5a, b). Increase of 15 % in fresh cell biomass and 10 % in dry cell biomass were evident, compared to the control (20.8 % O_2 supply) cultures. Similar positive effects of oxygen supply on cell growth have been reported for tobacco suspension cultures [42].

Profiles of total saponin (ginsenoside) production are shown in Fig. 6.5c. Highest saponin accumulation was recorded on 20–25 days and declined thereafter. The maximum total saponin concentrations were 3.8, 4.4, 4.5 and 2.85 mg g^{-1} DW at 20.8, 30, 40, and 50 % O_2 supply respectively (Fig. 6.5c). Highest saponin levels

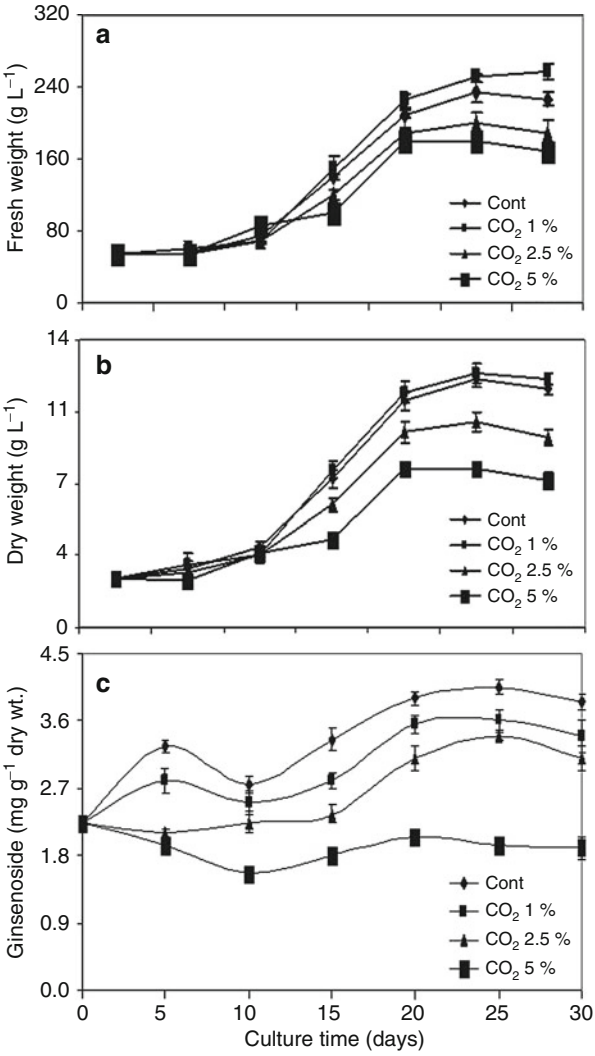

Fig. 6.6 Effect of CO_2 concentration on accumulation of cell fresh mass (**a**), dry mass (**b**), and production of saponins (**c**) of *Panax ginseng* cells cultivated in balloon type bubble bioreactors (rhomb – control, square – 1 % CO_2, triangle – 2.5 % CO_2, cross – 5 % CO_2)

were achieved at 40 % O_2 supply; the lowest levels were achieved at 50 % O_2 supply. The results indicate that oxygen supplementation to bioreactor-based ginseng cultures was beneficial for biomass accumulation and saponin production.

6.3.5 *Effect of Carbon Dioxide on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng*

Ginseng cell cultures were supplemented with various concentrations of carbon dioxide (1, 2.5 and 5 %) and their effect was tested on biomass accumulation and productivity of ginsenosides [43]. The fresh mass of cells with 0.03 % CO_2 (control) was 227 g L^{-1} and corresponding dry mass was 11.6 g L^{-1} (Fig. 6.6a, b). It was

found that optimum accumulation of fresh (258 g L^{-1}) and dry mass (12.1 g L^{-1}) was with the supply of 1 % CO_2 in the bioreactors. Thus 13.7 and 4.3 % increase in fresh and dry cell mass was evident with the supply of 1 % CO_2. This finding is in agreement with the earlier reported results that carbon dioxide is required for the growth of plant suspension cultures [44, 45]. The biomass accumulation declined with the further increase in CO_2 concentration. Fresh and dry cell mass was decreased by 30.1 and 38.3 %, respectively with the supply of 5 % CO_2.

The ginsenoside production increased with the lapse of time and significantly higher saponin content was observed in control condition (Fig. 6.6c). After 30 day of culture increased CO_2 supply 1, 2.5 and 5 % led to decrease in saponin accumulation up to 11.6, 19.5 and 50.6 %, respectively. However, the enhancement in secondary metabolites with an increase in CO_2 concentrations has been reported in the cell cultures of *Thalictrum minus* [46], *T. rugosum* [47], *Stizolobium hassjoo* [48] and *Catharanthus roseus* [49].

6.3.6 *Effect of Ethylene on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng*

The influence of ethylene supplementation at 5, 10 and 20 ppm levels were tested with ginseng cell cultures and results are depicted in Fig. 6.7a, b. Growth kinetics revealed similar trends as in case of O_2 and CO_2 supplementation. Increment of 16 and 8 % in dry biomass was recorded with supplementation of 5 and 10 ppm of ethylene respectively, whereas supplementation of 20 ppm decreased the biomass accumulation significantly when compared to control. This result suggests that ethylene had stimulatory or inhibitory effect on cell growth in bioreactor culture system. Similar results have been reported in cell culture of different *Taxus* species [50].

Figure 6.7c shows the profile of saponin content under different concentrations of ethylene in *P. ginseng*. The yield of ginsenoside production was decreased significantly in all the ethylene concentrations compared to control. Recently, Zhang and Wu [51] reported that ethylene inhibitors induce or stimulate the secondary metabolite production by inhibiting ethylene production endogenously or supplied concentration in the medium. Ethylene effects on growth and differentiation is highly variable and it is not yet clear why ethylene promotes growth, differentiation and secondary metabolite production in some case and inhibits them in others [52].

6.4 Elicitation

6.4.1 *The Effect of MJ on Cell Growth and Ginsenosides Production*

The growth and secondary metabolite accumulation in *Panax ginseng* cell culture are represented in Tables 6.8 and 6.9 [53]. Cell growth was significantly affected by the application of MJ. The fresh weight, dry weight and growth ratio of the cells, decreased with increasing MJ concentration, resulting in a cell growth ratio of 3.48 at 400 μM MJ.

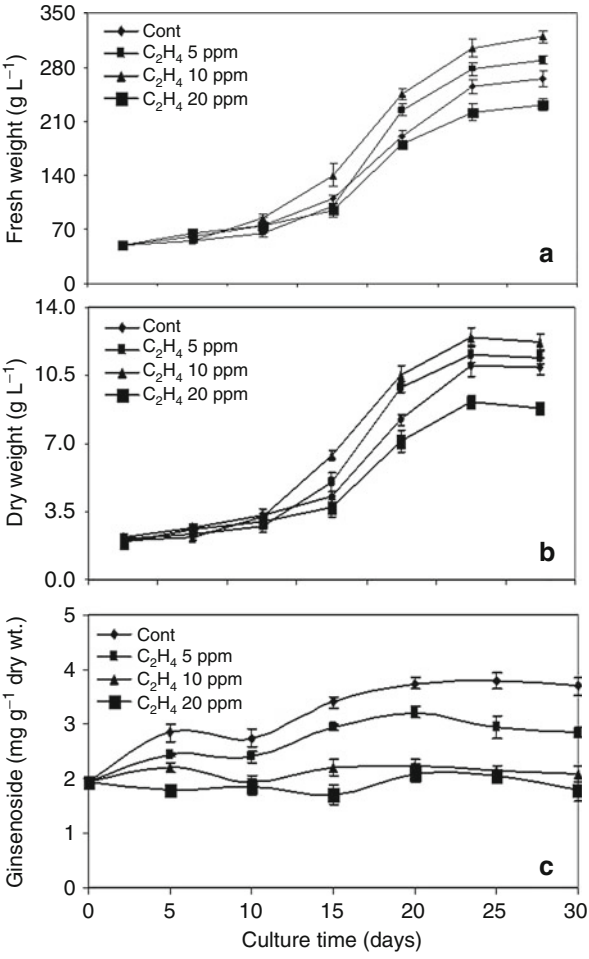

Fig. 6.7 Growth kinetics of cell fresh weight (**a**), dry weight (**b**) and ginsenoside accumulation (**c**) of *P. ginseng* in bioreactor cultures under different C_2H_4 concentrations

Table 6.8 The effect of methyl jasmonate (MJ) on ginseng cell growth after 25 days of bioreactor culture

MJ concentration (μM)	Biomass		Growth rate[a]
	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	
0	311.9 a[b]	12.6 a	4.62
50	304.2 ab	11.3 b	4.14
100	294.9 b	11.4 b	4.28
200	293.1 b	11.3 b	4.26
400	240.2 c	9.4 c	3.48

[a]Growth ratio is the quotient of the dry weight after cultivation and the dry weight of the inoculum
[b]Mean separation within column by Duncan's multiple range test at $P<0.05$

Table 6.9 Effect of MJ on biosynthesis of ginsenosides after 25 days of bioreactor culture

MJ concentration (μM)	Ginsenosides [mg g^{-1} DW]			
	Rg	Rb	Total[a]	(Rb: Rg)[b]
0	1.87 ± 0.1	2.09 ± 0.2	3.96 ± 0.3	1.12 ± 0.1
50	1.84 ± 0.5	2.80 ± 0.2	4.63 ± 0.4	1.59 ± 0.4
100	2.35 ± 0.1	4.84 ± 0.2	7.19 ± 0.3	2.06 ± 0.1
200	2.65 ± 0.1	6.17 ± 0.3	8.82 ± 0.3	2.32 ± 0.1
400	2.32 ± 0.3	4.62 ± 0.2	6.94 ± 0.3	1.97 ± 0.2

Values represent mean with standard error
[a]Total saponin content = Rb + Rg
[b]Rb: Rg = (Rb1 + Rc + Rb2 + Rd)/(Rg1 + Re + Rf)

On the other hand, ginsenosides content was significantly enhanced by the addition of MJ. Total ginsenosides content increased with increasing MJ concentration, and reached a maximum of 8.82 mg g^{-1} DW at 200 μM MJ, representing a 2.2-fold increase over the control (3.96 mg g^{-1} DW). Both Rb group and Rg group ginsenosides reached a maximum at 200 μM MJ but the content of Rb group ginsenosides increased more significantly than that of Rg group ginsenosides. There was 1.3-fold increment in Rg group ginsenosides, whereas threefold increment of Rb group ginsenosides was evident compared to the control. The Rb/Rg group ratio was 2.32 with an application of 200 μM MJ. Figure 6.8a shows the dynamic changes in the content of the Rb/Rg ratio with 200 μM MJ treatment over the control.

6.4.2 Accumulation of Ginsenosides After MJ Treatment in a Two-Stage Bioreactor Operation

Based on the results of the first experiment, bioreactor cell cultures were established and ginseng cells were cultured for 15 days without MJ treatment. Two hundred micrometer MJ was added to the cultures after 15 days for elicitation and accumulation of ginsenosides. Figure 6.8b–d shows ginsenosides accumulation in ginseng cell culture during 10 days of 200 μM MJ addition. The content of ginsenosides and Rb group ginsenosides gradually increased, reaching maximum values 8 days after treatment and showing a little change thereafter. Contents of total ginsenosides, Rb, and Rg group ginsenosides increased 2.9, 3.7, and 1.6 times, respectively. Among the Rb group ginsenosides, Rb1 content increased significantly by four times but the contents of Rb2, Rc and Rd increased only slightly. Among Rg group ginsenosides, Rg1 and Re showed 2.3-fold and 3.0-fold increments, whereas there was only a slight increment in Rf group ginsenosides. Similarly, jasmonates have been used to elicit higher accumulation of metabolites in cell cultures of *Taxus chinensis* [54] and *Panax notoginseng* [55].

This study indicates that MJ could increase the accumulation of individual ginsenosides and significantly modify the Rb/Rg group ratio. The strategy developed here,

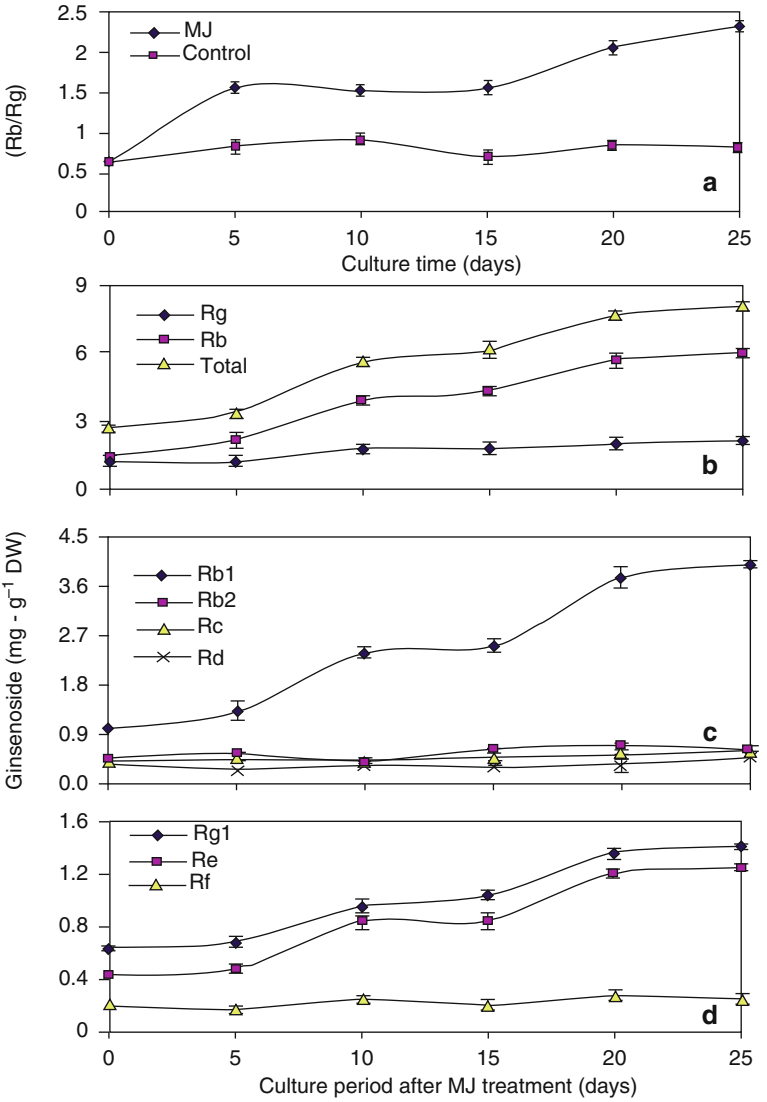

Fig. 6.8 Dynamic changes in Rb and Rg ginsenosides in ginseng cells grown for 25 days after methyl jasmonate (*MJ*) treatment (200 μM) in bioreactors (**a**). Accumulation of total (**b**), Rb (**c**) and Rg (**d**) group ginsenosides in ginseng cells grown for 10 days after MJ treatment (200 μM) in bioreactors. The cells were grown for 15 days without MJ

i.e., initial culturing of cells without elicitors and subsequent treatment of cell cultures with 200 μM MJ is useful for enhancing ginseng cell biomass as well as simultaneously enhancing ginsenoside production. Initial culturing of cells without elicitors and subsequent treatment of cell cultures with 200 μM MJ, is useful for enhancing ginseng cell biomass as well as the ginsenoside production simultaneously.

6.5 Scale up of Ginseng Production in Bioreactors

Based on the above results of 5 and 10 L bioreactor cultures (Fig. 6.9a, b), we cultivated pilot-scale cultures of ginseng cells in 500 and 1,000 L airlift bioreactors (Table 6.10 and Fig. 6.9c). The total biomass of 187 kg fresh weight and 6.2 kg dry weight with a total saponin production of 7.86 mg g^{-1} DW was obtained in 500 L drum bioreactor. Similarly, 400 kg fresh weight (Fig. 6.9d) and 13.2 kg dry weight with a total saponin production of 7.75 mg g^{-1} dry weight were also obtained in 1,000 L balloon type bioreactor. These results are comparable to that of ginseng cell cultures in

Fig. 6.9 *Panax ginseng* cell cultures in bioreactors. (**a**) Cell suspension cultures in 5 L balloon type airlift bioreactors. (**b**) 500 L drum type airlift bioreactor. (**c**) 1,000 L airlift bioreactor. (**d**) Biomass harvested from 1,000 L bioreactor

Table 6.10 Ginseng cell cultures in bioreactors

	Biomass				
Bioreactor type	FW	DW	DW (g L^{-1})	% dry wt.	Total saponin (mg g^{-1} DW)
5 L balloon	1.28 kg	54 g	13.1	4.22	8.82
500 L drum	187 kg	6.2 kg	12.4	3.32	7.86
1,000 L balloon	400 kg	13.3 kg	13.3	3.33	7.75

5 L bioreactor (Table 6.6). In the previous studies, stirred tank bioreactors have been used and taken to scale up process also [19]. Shamakov et al. [20] and Strogov et al. [21] determined the suitable agitation speed for efficient mixing and oxygen transfer. Asaka et al. [56] used airlift bioreactors and achieved higher biomass and ginsenoside productivity than stirred tank bioreactors. However, all these were batch cultures. In order to improve the productivity of biomass and secondary metabolites, fed-batch and high density cell cultures were used by Zhang and Zhong [14] and reported a cell concentration as high as 35 g dry cell L^{-1}. 2.8 and 3.4 fold increment saponin and polysaccharide productivity was revealed in high density fed-batch cultures. By adopting fed-batch, high density cell cultures in Korean ginseng it is possible to achieve improved productivity and research work is in progress in this direction.

6.6 Conclusions and Future Perspectives

Ginseng cell culture has been evolved as an alternative for field cultivation as it has the advantages of higher biomass accumulation and ginsenosides production. Extensive research work has been carried out on ginseng cell and tissue cultures, such as optimization of culture medium, physical conditions for the production of biomass and ginsenosides productivity [1]. In the current studies, cell cultures were established in Korean ginseng and various physiological and physical parameters which affect the biomass and metabolite accumulation have been optimized. Elicitation technology has been achieved for enhanced accumulation of ginsenosides. Cell cultures have been also established in large scale airlift bioreactors (500 and 1,000 L) to obtain voluminous biomass and metabolite productivity. Recently, various bioengineering parameters like fed-batch cultures [14], high density cell cultures [22, 23] have been adopted in *Panax notoginseng* cell cultures and obtained improved productivity of metabolites. Elicitation of ginseng cell cultures with vandate [57] and *N*, *N*'-dicyclohexylcarbodiimide [58] have also been reported for enhanced saponin productivity. There is scope for improvement of secondary metabolite production in Korean ginseng with the adoption of these techniques. Further, research in the improvement of the ginseng cell culture technologies may be useful for commercial production of ginseng raw material.

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