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ENHANCEMENT OF CELL GROWTH AND SAPONIN PRODUCTION IN *Panax ginseng* CELL CULTURE BY NUTRIENT FEEDING AND ELICITATION

by

WONG Kong

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for the Degree of Master of Philosophy

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CERTIFICATE OF ORIGINALITY

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Abstract to thesis entitled

ENHANCEMENT OF CELL GROWTH AND SAPONIN PRODUCTION IN *Panax ginseng* CELL CULTURE BY NUTRIENT FEEDING AND ELICITATION

Plants are the most important sources of natural products, such as food flavors, dyes, fragrances and pharmaceuticals, which are mostly secondary metabolites of plants. Plant tissue and cell cultures are feasible and promising alternatives for the production of secondary metabolites of plants, particularly those naturally rare and difficult-to-cultivate plant species. *Panax ginseng* is a famous and valuable oriental herb, and its root is widely used in traditional medicines and health foods. Although ginseng can be cultivated on farms, the cultivation process takes 5-7 years from seeding to final harvest of the mature ginseng roots. Therefore, plant tissue and cell cultures may be more efficient processes for the production of ginseng root and its components.

The main objectives of this research project are to study the kinetics of *P. ginseng* cell suspension culture and the strategies for enhancement of ginseng cell biomass and secondary metabolite (ginseng saponins or ginsenosides) production in cell suspension cultures. The main factors and strategies being investigated included medium composition, nutrient feeding, and stimulation with osmotic stress and chemical elicitors. Statistic methods were applied in some parts of the project for the design and analysis of experiments and for the identification of optimal culture conditions.

The results showed that the basic kinetics of growth and nutrient metabolism of the *P. ginseng* cells in suspension culture was basically similar to that of most other plant cell cultures. The feeding of sucrose and some other nutrients to the culture near the stationary growth phase extended the growth period and increased the biomass growth index by about 100%. The introduction of osmotic stress (by increasing medium osmolality) to the culture at the time of inoculation (day 0) with osmotica such as sorbitol and sodium chloride stimulated the secondary metabolite biosynthesis, but depressed the cell growth. As the osmotica were fed together with the limiting growth nutrients, sucrose and casein hydrolysate, to the culture around the stationary growth phase, secondary metabolite biosynthesis could be improved without significant cell growth depression.

Statistical design and analysis of the nutrient feeding and osmotic stress experiments indicted that nutrient (sucrose) feeding time is a major factor affecting the biomass productivity and sorbitol concentration is a major factor affecting both biomass productivity and saponin production. A 4-fold increase in the volumetric saponin yield was achieved with the feeding of 45 g/l sucrose (+1.5 g/l casein hydrolysate) and 0.2 M sorbitol to the culture at day 10 post inoculation.

The feeding of chemical elicitors to the culture, such as methyl jasmonate (MJ) and yeast extract (YE) was even more effective than the application of osmotic stress to stimulate the saponin production. With 120 μM MJ, for example, the saponin content of cell was increased by more than 4-fold. When MJ was fed together with sucrose to the culture, the volumetric saponin yield was increased by more than 10-fold. The chemical elicitors,

which were fed to the culture around the stationary growth phase, caused only minor inhibiting effect on the cell growth.

Further investigation showed that the osmotic stress and elicitor treatment induced the general plant defense responses to biotic and abiotic stresses in the ginseng cell cultures, i.e., the increase in the activity of phenylalanine ammonium-lyase (PAL), a key enzyme in plant secondary metabolic pathways, and the transient production of active oxygen species, H_2O_2, known as the oxidative burst which is an early and signal event in plant defense responses. Therefore, the enhanced saponin biosynthesis of ginseng cells may be a result of the defense responses of plant cells induced by the osmotic stress and chemical elicitors.

In conclusion, the application of nutrient feeding and elicitation is a simple and effective strategy for enhancement of ginseng biomass and saponin production in plant cell cultures.

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxy acetic acid
CH	casein hydrolysate
dw	dry weight
FPP	farnesyl pyrophosphate
fw	fresh weight
GI_{max}	maximum growth Index
GPP	geranyl pyrophosphate
H_2O_2	hydrogen peroxide
HMG-CoA	β-Hydroxy-β-Methylgluyaryl-CoA
IBA	indole-3-butyric acid
IPP	isopentenyl pyrophosphate
KT	kinetin
MJ	methyl jasmonate
MS	Murashige and Skoog
MVA	mevalonic acid
NAA	naphthaleneacetic acid
OB	oxygen burst
PAL	phenylalanine ammonia-lyase
Pr	biomass productivity
$Y_{x/s}$	biomass yield
YE	yeast extract

CHAPTER 1 INTRODUCTION

1.1 General introduction

Plants synthesize a wide range of secondary metabolites, which are used as pharmaceuticals, fine chemicals, flavors, fragrances, colours, biopesticides and food additives (Stockigt et al., 1995). These compounds are not involved in the basic metabolic processes of the living cells, but are involved in the interaction of the producing plant with its environment, such as the attraction of pollinators (color, smell), and the resistance against insects (e.g., antifeedant) or microbial infections (phytoalexins). Each plant species has its own specific set of secondary metabolites. Nowadays, about 100,000 compounds are known from plants (Verpoorte et al., 1999). The spectrum of chemical structures, e.g. phenylpropanoids, alkaloids, terpenoids, quinines and steroids, synthesized by the plant kingdom is broader than that of perhaps any other group of organisms (Rao and Ravishankar, 2002). The possibility of harnessing this synthetic capacity to commercial and social benefits has been a main driving force for the present developments in the plant-cell-culture technology.

In spite of the advance in synthetic organic chemistry, the plant kingdom still contributes significantly in both quantity and product range to the specialty chemicals used by a number of industries because their complex structural features are difficult to synthesize by these industrial processes. Thus, the major limitation to the commercial use of these secondary metabolites is their very limited supply from the whole plants. The production of these useful secondary metabolites from plant cell culture has been regarded as a

potential solution to this supply limitation. Plant cells are biosynthetically totipotent, which means that each cell in culture retains complete genetic information to produce the range of chemicals found in the parent plant. Plant cell culture system can be manipulated by sound culture strategies leading to high flux into secondary metabolic pathways, and then can be carried out in large scale bioreactors.

1.2 History of plant cell culture

Blumental and Meyer (1924) obtained voluminous callus on carrot root explants treated with cacti acid. Muir et al.(1954) reported the establishment of a culture of suspended plant cells in an artificial medium. Production of secondary metabolites by means of plant cell, tissue or organ cultures originated in 1947, when James (1947) reported that the occurrence of alkaloids in meristem cultures of solanaceous plants. *In vitro* root cultures for the production of tropane alkaloids were early described by Telle and Gantheret (1947) (*Hyoscyamus niger)*, and Stienstral (1954) (*Dutura stramonium*).

Callus is first induced from certain parts of a plant, growing on a solidified medium. Callus cultured in a liquid medium is called cell suspension culture. Suspension cell culture in shake flask level is commonly used to characterize the growth, nutrient uptake and biosynthesis pattern of the cells. Usually, cells are cultured in the shake flask level before scaling up to bioreactor level. This allows for better understanding and definition of the culture protocols to improve productivity before scaling up to bioreactor level. A large number of plant species have been cultivated successfully in bioreactors as cell suspensions (Fowler, 1984; Mavituna, 1992).

1.3 Secondary metabolites from plant cell culture

The results from previous studies have shown the feasibility of secondary metabolite production by large scale cultures of plant cells or organs as an interesting alternative to the classic production by processing of plant material from agricultural sources. This system possesses a number of advantages. For examples, it is not subjected to the limitation of soil, season and environmental conditions and the cells can grow at a relatively fast rate. Successful cultivation of plant cells in larger bioreactors was reported in 1959 by Tulecke and Nickel. They developed a 10-L production system in carboys. Noguchi et al. (1977) described a 20,000-L culture of tobacco cells in a standard stirred vessel. It is now possible to industrially produce pharmaceuticals by plant culture system.

1.4 Limitations of plant cell culture

Although large-scale production of secondary metabolites by plant cell and tissue culture system is technologically feasible, commercial production of secondary metabolites by large scale plant cell culture is still rare (Becker and Sauerwein, 1990). Considerable research effort has been devoted to the various fields of plant cell culture and secondary metabolite production, e.g. production of flavonoids, anothocyanins, naphthoqunones, anthruquinones, saponins, and tropanes, and, providing many interesting fundamental results. The acceptance of plant cell culture system for producing various chemicals on an industrial scale depends on the economics of the process, which in turn, depends on the ability to grow the particular cells rapidly in large quantities, and to obtain a high yield of product. Apart from a few specialized cases such as tobacco biomass, the products of value

in plant cell cultures are "secondary metabolites" that are normally present at very low levels.

1.5 Methods to enhance secondary metabolite production

Various approaches have been studied to increase the yields of secondary metabolites, including screening and selection of high producing cell lines, improving culture strategy, media optimization, elicitation, culturing of differentiated cells (organ cultures), and immobilization. In recent years, genetic engineering and metabolic engineering has opened a new promising perspective for improved production in a plant or plant cell culture (Verpoorte et al., 1999). Nevertheless, with the development of new nutrient media, culture strategy, techniques for the isolation and selection of cell lines, and genetic engineering, a number of secondary products has been obtained at yields above those found in whole, or parts of plants (Kieran et al., 1997). This encourages the development of mass culture of plant cells for the production of secondary products. Mitsui Petrochemical Industries Limited has designed a process for the mass production of shikonin, which is a dye and pharmaceutical (Curtin, 1983). Nitto Denko Co. has derived the commercial products from *P. ginseng* cell culture since 1990 with a net sale of $3 million in 1995 (Ushiyama, 1996).

1.6 *Panax ginseng* cell culture

Panax ginseng C.A. Meyer is one of the medicinal plants that are well recorded in the traditional Chinese medical books. The name of ginseng is derived from the Chinese "Jen-sheng", meaning, "image of man". Its activities of homeostatic, promoting blood circulation, relieving pain and cure of bleeding, wound and trauma are also well recorded. The major compounds of pharmaceutical importance in ginseng have been identified as ginseng saponins, a group of high molecular-weight glycosides, consisting of a sugar moiety linked to a triterpene or steroid aglycone. These chemical compounds exhibit such characteristics as foaming in aqueous solutions, from which the name saponin is derived. Recently, saponins have also been identified as one of the most effective inhibitors of tumor promotors. The nutritional and biological significance of saponins has been reviewed by Milgate and Roberts (1995).

Ginseng root contains 0.5-3.0 % of ginsenosides (or "panaxosides"), a complex mixture of dammarane glycosides. Many of these are based on protopanaxadiol or protopanaxatriol skeletons. Ginsenosides Rb_1 and Rg_1 are found in the largest amounts (Table 1-1). In addition, certain oleanolic acid glycosides are present. These ginsenosides have been used for treatment of diseases in cardiovascular, cerebrovascular, central nervous, as well as blood and hematopoietic systems (Hostetmann and Marston, 1995a).

Table 1-1. Ginsenoside content of ginseng preparations from different sources (Adapted from Hostettmann and Marston, 1995b).

	Total content	Distribution (relative % to total content)					
	(%)	Rg_1+Rf	Re	Rd	Ro	Rb_2	Rb_1
White ginseng (Korea, Sam Geon Sam)	2.09	18.8	15.4	3.7	13.3	15.6	34.1
White ginseng (Korea, Kiboshi)	1.86	15.1	37.3	1.6	9.1	9.7	26.9
Red ginseng (Korea)	1.02	17.5	7.8	1.7	19.3	24	29.6
White ginseng ("Slender tails")	10.6	4.9	13	3.9	22.7	22.8	32.5
American ginseng	6.01	3.4	18.8	6.3	6.8	0	64.7

1.7 Objectives of the study

In this study, *P. ginseng* cell culture was used as a model system to study the enhancement of cell growth and secondary metabolite biosynthesis by manipulating the culture strategy and the use of physical and biochemical stimuli.

The main objectives of this research project are to study the kinetics of *P. ginseng* cell suspension culture and the strategies for enhancement of ginseng cell biomass and secondary metabolite (ginseng saponins or ginsenosides) production in cell suspension cultures. The main factors and strategies being investigated included medium composition, nutrient feeding, and stimulation with osmotic stress and chemical elicitors. Statistic methods were applied in some parts of the project for the design and analysis of experiments and for the identification of optimal culture conditions. The following

approaches were applied to improve the cell growth and secondary metabolite (saponin) production of *P. ginseng* cell cultures.

1. The effect of specific nutrients on cell growth and secondary metabolite biosynthesis will be characterized. Based on the results, the nutrient composition and nutrient feeding strategy will be optimized to enhance the cell growth and secondary metabolite biosynthesis.

2. It has been reported that the osmotic stress has strong effect on the secondary metabolite biosynthesis, and the increase of osmotic stress enhanced secondary metabolite synthesis. However, this stress may also result in the decrease of biomass growth and the total secondary metabolite productivity. The application of osmotic stress will be modified in this investigation in order to promote biomass growth and secondary metabolite (ginsenoside) biosynthesis simultaneously.

3. It is well known that chemical elicitors applied to the cell culture can induce high level secondary metabolite biosynthesis. The effect of these elicitors on cell growth and secondary metabolite production will be evaluated.

4. There are numerous factors which may affect the biomass growth and secondary metabolite biosynthesis of plant cell culture. In order to optimize these factors comprehensively, statistical methods will be used in the design and analysis of the experiments.

CHAPTER 2 LITERATURE REVIEW

2.1 Plant cell culture as a source of secondary metabolites

2.1.1 Plant cell culture

The plant kingdom provides a wide variety of natural products with diverse chemical structures and a vast array of biological activities, many of which have found applications in health care. These compounds are usually products of secondary metabolism of plant cells. But, due to structural complexity, the resulting multistep syntheses are rarely applied in large scale production. Thus, these useful compounds are still generally obtained from whole living plants. This route of production has some major problems: (a) the targeted compounds are usually present in minute amounts in the plant extract; (b) separation of the compounds are generally difficult and thus expensive; (c) the concentrations of the targeted compounds in plants fluctuate with season, cultivation environment, and geographical locations; (d) the cultivation periods between planting and harvesting are usually long. Because of these problems, many plant secondary products are expensive and facing a shortage of supply. Plant cell culture provides an appropriate solution and alternative approach to the problem.

Plant cell suspension cultures are normally derived from callus culture cultivated on solidified media. Callus is derived from the explants of the whole plants, and then kept alive on a solid medium. Callus can undergo somaclonal variation, usually in several subculture cycles. Owing to this variation, secondary metabolite production is often

variable from one culture cycle to another. After a period of time, genetic stability can be achieved and each callus can be considered as homogenous cell aggregate. The friable callus clumps are then transferred to liquid medium under constant agitation on rotary shakers or stirred vessels, resulting in cultures of single cells or small aggregates of cells. Like microbial cultures, a plant cell suspension culture undergoes mainly four phases of growth, the lag phase, log phase, stationary phase and death phase. The complete growth cycle is usually several days to weeks.

Most of the fermentation techniques developed from the microbial culture can be applied to the plant cell culture. It is possible to cultivate plant cell suspensions using conventional fermenter equipment with minor adjustments and to apply standard modes such as batch, fed-batch, perfusion and continuous fermentations. Thus, the culture process can be scaled up to large values for massive production. The advantages of this technology over the conventional agricultural production are: (a) it is independent of geographical, seasonal and various environmental factors; (b) it offers a defined production system, which ensures the continuous supply of products, uniform quality and yield; (c) it is possible to produce novel compounds that are not found in the parent plant.

There are a number of plant cell cultures producing a higher amount of secondary metabolites than in intact plants. However, there are still problems in the production of metabolites by cell cultures, such as the instability of cell lines, low yields, slow growth and scale-up problems (Ravishankar and Venkataraman, 1993).

2.1.2 Secondary metabolites

Metabolism pathways are networks of biochemical reactions in organisms that are catalyzed by enzymes. In the pathways, the synthesis and utilization of compounds are achieved, which have a direct impact on the vital functions of the organism. This kind of metabolic process is known as primary metabolism. Primary metabolism is basically the same in all types of cells and organisms. Most organisms are able to produce compounds that are not of direct use for their survival, but help the organisms to function better in their environment (Bennet and Bentley, 1989). This type of process is called secondary metabolism. Secondary metabolites can be defined as compounds with a restricted occurrence in taxonomic groups, and that are not essential for a cell (organism) to live, but play a role in the interaction of the cell (organism) with its environment, ensuring the survival of the organism in its ecosystem. Secondary metabolism pathways are restricted to an individual species or genus and might only be activated during particular stages of growth and development (Verpoorte, 2000). There is close relationship between primary and secondary metabolism. Secondary metabolism starts with the end products of primary metabolism. If some crucial steps for the biosynthesis of secondary metabolism can be controlled, the productivity of the secondary metabolites can be enhanced.

Plant secondary compounds are usually classified according to their biosynthetic pathways. Three large molecule families are generally considered: phenolics, terpenes and steroids, and alkaloids. Apart from phenolics that are involved in lignin synthesis, other compounds such as alkaloids are sparsely distributed in the plant kingdom and are much more specific to defined plant genus and species. Presently about 100,000

secondary metabolites have been isolated from plants with terpenoids and alkaloids as major groups. Most plants make similar basic skeletons of compounds, e.g. terpenoids, but differ in the various functional groups (Verpoorte, 2000).

The secondary metabolites which are the most important constituents of the ginseng cells are the ginsenosides, or ginseng saponins. Saponins are high-molecular-weight glycosides, consisting of a sugar moiety linked to a triterpene or steroid aglycone (with 30 carbons). By 1987, the structure of over 360 sapogenins and 750 triterpene glycosides had been elucidated (Bader and Hiller, 1987). The aglycone or non-saccharide portion of the saponin molecule is called the genin or sapogenin. Depending on the type of genin present, saponins can be divided into three major classes: (I) triterpene glycosides, (II) steroid glycosides and (III) steroid alkaloid glycosides (Hostettmann and Marston, 1995a).

Recent investigation has revealed that the mevalonate pathway is not the only biosynthesis process for isoprenic units. The existence of a non-mevalonate pathway for the biosynthesis of certain terpenoids has been demonstrated based on C-labelling patterns that are incompatible with the operation of the mevalonate pathway (Seto et al., 1996; Rohmer et al., 1993). The non-mevalonate pathway starts with glyceraldehyde-3-phosphate and pyruvate. The 5-C isoprenoid precursor unit, IPP is formed by further decarboxylation and rearrangement (Disch and Rohmer, 1998; Knöss et al., 1998; McCaskill and Crotenn, 1998). Based on this pathway, pyruvate and glyceraldehyde-3-phosphate can also be precursors of saponin.

2.2 Strategies for enhancing secondary metabolite production in plant cell culture

2.2.1 Medium modification

The development of media to meet the nutrient requirements of plant cells in culture spans several decades. Although the basic nutritional requirements of cultured plant cells are very similar to those normally utilized by whole plants, the nutrient media for cells, tissues and organs were devised to meet particular requirements (Murashige and Skoog, 1962; Gamborg et al., 1968; Schenk and Hildebrandt, 1972; Philis and Collins, 1980). Typically, a plant cell culture medium contains major inorganic salts, including nitrate, ammonium, phosphate, magnesium, calcium, and trace elements, such as manganese, copper and cobalt, vitamins, carbon sources, and plant growth regulators. In some cases, in order to initiate growth, it is necessary to add undefined components such as coconut milk (Scragg and Fowler, 1985).

The culture medium components mentioned above are basic plant cell nutrients rather than the optimal for different plant species. Thus, the medium composition needs to be modified with respect to the cell strain and culture environment in order to improve the cell growth and secondary metabolite biosynthesis. A complete plant cell medium contains more than 20 components, and it is difficult to manipulate all of them. Previous studies on medium composition have focused on a few major components, including carbon and nitrogen sources, inorganic phosphate and plant growth regulators. The modification of the medium constituents for *Catharanthus roseus* has led to the

development of several media that induce the accumulation of indole alkaloids (Knobloch and Berlin, 1980; Zenk et al., 1977). Smith et al. (1987) also obtained a medium that promoted growth and alkaloid production in a single-stage culture by modification of the standard Murashige and Skoog (MS) media.

2.2.1.1 Carbon source

Plant cell cultures are usually cultivated heterotrophically and carbon must be added in the form of carbohydrates. The most common carbon sources are sucrose and glucose, though plant cells can metabolize other sugars as well (e.g. lactose, fructose, maltose, and galactose). Carbohydrate supplies not only the carbon but also the energy source to the cells. Production of secondary metabolites by plant cells was found to be affected by initial sugar concentration in many plant cell cultures. Sucrose concentrations of 2.5% (w/v) and 7.5% (w/v) in *Coleus blumei* media brought about rosmarinic acid yields of 0.8 and 3.3 g/l, respectively (Misawa, 1985). The yields of benzophenanthridine alkaloids from suspension cultures of *Eschscholtzia californica* was increased 10-fold by increasing the sucrose concentration to 8% (w/v) (Berlin et al., 1983). However, higher concentrations of sucrose at 5% (w/v) reduced the anthocyanin production in cell suspension cultures of *Aralia cordata*, where 3% (w/v) favoured the anthocyanin accumulation (Sakamoto et al., 1993). Generally, sucrose at a concentration of 2-3 %, is most commonly used in plant culture media.

In fact, both the nature and quantity of the carbon sources have been shown to affect product yields. Increasing the sucrose level tends to improve the level of product

synthesis, whereas glucose, although having the same effect on biomass productivity at equimolar concentrations, either does not affect product synthesis or may even be inhibitory (Mantell and Smith, 1983). Zenk et al. (1977) observed that the level of indole alkaloids in cultures of *Catharanthus roseus* was enhanced with increasing sucrose concentrations. Zhang et al. (1996a) studied the effect of initial sucrose concentration on the cell and secondary metabolism of *P. notoginseng*. It was found the final dry cell weight increased with increased initial sucrose concentration. However, too high a sucrose concentration repressed cell growth. It was also found that high sugar level was favorite to the synthesis of ginseng saponin, which may be due to the high osmotic pressure and reduced nutrient uptake. Furuya et al. (1984) reported that MS medium with 0.5% glucose and 2.5% sucrose showed a higher growth ratio than with 3% sucrose. The effect of sugar on the cell growth is complicated. It is still not known why sucrose combined with a small amount of glucose could enhance cell growth. Perhaps, glucose can be used directly by the cells, so there is direct carbohydrate available for cell metabolism before sucrose is hydrolyzed to glucose and fructose (Martine and Park, 1993).

2.2.1.2 Other major nutrients

The other major nutrients for plant cells in culture include nitrogen, phosphorus, sulphur, potassium, magnesium and calcium. In general, a decrease in the major nutrients results in increased secondary product but decrease in the biomass growth rate (Dongall and Weyranch, 1980). Panda et al. (1991) conducted a comprehensive study on the effect of major nutrients on the alkaloid production by suspension cultures of *Horarrhena*

antidysenterica cells. The content of major nutrients in the standard MS medium was modified. It was found that alkaloid synthesis in modified medium was 4.25-fold over that obtained in the standard MS medium. Dongall and Weyranch (1980) were able to control anthocyanin accumulation in carrot cell suspension cultures by using phosphate as the limiting nutrient.

Nitrogen concentration has been found to affect the level of proteinaceous or amino acid products in cell suspension cultures. The general plant tissue culture medium has both nitrate and ammonium as sources of nitrogen. In the culture medium, a variety of nitrogen sources have been used in plant cell culture medium, ranging from inorganic sources such as nitrate and ammonium, to a variety of amino acids. The ratio of the ammonium to nitrate and total nitrogen has shown to markedly affect the production of secondary products. Ammonium ion as the sole nitrogen source is usually undesirable, probably because under such situation, the pH of the medium has a tendency to fall below 5 during culture. This drop in pH may restrict the availability of nitrogen (Wetherell and Dongall, 1976; Zhang et al., 1996b). The inclusion of 20-40 mM potassium nitrate can prevent the extreme fluctuation of pH. Ammonium may be essential for good cell growth. Panda et al. (1992) reported that dry cell weight and alkaloid production were increased with an ammonium:nitrate ratio of 5:1 and 60 mM total nitrogen. Kaul and Hoffman (1993) also found that ammonium as the sole nitrogen source inhibited *Pinus strobes* L. callus growth.

Phosphorus is commonly added to the culture medium as phosphate (PO_4^{3-}). Inorganic phosphate is one of the most important nutrients for cell growth although the absolute

amounts required for cell growth is not high (generally 1.25 mmol/l in standard culture medium). Phosphate is incorporated in molecules such as nucleic acids, phospholipids, and sugar phosphate, and plays an essential role in the energy metabolism (Schlatmann et al., 1996). The phosphate concentration in the medium can have a major effect on the production of secondary metabolites in plant cell cultures. Higher levels of phosphate were found to enhance the cell growth, but have negative influence on secondary product accumulation. Reduced phosphate levels induced the production of ajmalicine and phenolics in *C. roseus* cell culture of caffeoyl putrescines in *Nicotiana tabacum* and Harman alkaloids in *Peganum harmala* (Sasse et al., 1982).

The cations, such as magnesium, potassium and calcium, play an essential role in cell metabolism. For example, magnesium ion is one of the essential factors in translation. It may act as cofactor and activator of various enzymes. Calcium ion and potassium ion inhibit enzymes such as the glycolysis enzyme pyruvate kinase (Endress, 1994). Liu and Zhong (1996) reported that more soluble sugar was stored within the cells under potassium ion deficiency, and a curvilinear relationship between initial potassium ion concentration and the active biomass (the total cell biomass minus intracellular soluble sugar) accumulation. The saponin content was remarkably enhanced with the decrease of initial potassium ion within 20-60 mM. The highest saponin concentration was obtained at an initial potassium ion of 60 mM. Bramble and Graves (1990) observed that there was a characteristic relationship between the concentration of calcium in the external medium and the concentration of extracellular and intracellular phosphate. The intracellular phosphate level was, in turn, related to the production of alkaloids. Calcium is also a

crucial ion for signal transduction in the elicitation of plant defense responses and secondary metabolism (Low and Merida, 1996).

2.2.1.3 Minor nutrients

The micronutrients, Fe, Mn, Zn, Cu, Mo, B, Co and Ni, act as cofactors and inducers of enzyme synthesis. For example, nickle increases synthesis in tobacco, rice and soybean cell suspension cultures. Boron is essential for membrane function, permeability and integrity. In order to maintain a minimum supply of Fe, it is usually added in complexes with EDTA or sequestrin. Previous studies have shown that iron deficiency reduced the growth of rice suspensions dramatically, Zn, Cu, B, Mn and Mo deficiency also had an inhibitory effect (Ohira et al., 1975). Zhong and Wang (1996) reported that with an increase in initial Cu^{2+} concentration within the range of 0-6.0 mM, the growth of *Panax notoginseng* was greatly improved. The maximum saponin content did not change significantly in the range of Cu^{2+} concentrations investigated, but a relative higher saponin production was obtained at 10 mM of initial Cu^{2+}.

2.2.1.4 Phytohormones

The type and quantity of growth hormones usually exert significant effect on the growth of plant tissues. The growth and viability of cultures are usually ensured by adding synthetic or naturally occurring auxins. Plant growth regulators affect growth and differentiation and thus affect secondary metabolism of cultured cells. The effects vary with the type and the quantity of phytohormone. It is believed that they induce specific

enzymes, possibly with the involvement of RNA synthesis. The most important phytohormones are auxins, cytokinins, abscisic acid, giberellins and ethylene.

Several studies have been carried out on the influence of various growth regulators, especially auxins, on alkaloid production by *C. roseus* cultures (Lounasmaa and Galambos, 1989; Ganapathi and Kargi, 1990). Zheng et al. (1982) studied the effect of hormone regulation on cell suspension culture of *Anisodus acutangulus*. The results showed that, when used separately, both (2,4-dichlorophenoxy acetic acid) 2,4-D at 0.1 mg/l and Naphthaleneacetic acid (NAA) at 0.6 mg/l were satisfactory, while the effect of kinetin, gibberellin, and etheral was insignificant; the combination of 2,4-D, NAA and kinetin (KT) was found most effective in promoting the cell growth in suspension. Zhong et al. (1996) investigated the effect of growth regulators, i.e. 2,4-D, indole-3-butyric acid (IBA), and KT, on the cell growth, saponin production and nutrient utilization of *P. quinquefolium* cell culture. The highest content of ginsenoside saponin was reached with a combination of the growth regulators of 2.5 mg/l IBA and 0.1 mg/l KT without addition of 2,4-D.

2.2.2 Optimization of culture environment

Environmental conditions such as light, temperature, medium pH and oxygen also greatly affect the cell growth and secondary metabolite accumulation in plant tissue cultures.

2.2.2.1 Temperature

There is little information in the literature about the temperature optima for the growth of cell cultures, let alone for secondary metabolite production in the cultures. This is probably due to the fact that, traditionally, *in vitro* studies have been carried as a matter of routine at temperatures of around 25 °C. In general, a temperature range of 26±3 °C has been found to be optimal for plant cell growth. Above 30 °C, especially above 33 °C, and below 21 °C, growth usually diminishes rapidly. Extreme temperature will inhibit the activities of the enzymes which in turn affects cell growth. Contrasting results have been reported of alkaloid accumulation at lower temperatures, inhibition (Morris,1986), stimulation (Courtois and Guern, 1980) or no effect (Toivonen et al., 1992) on alkaloid production. The effect of temperature on alkaloid production seems to be cell line dependent.

2.2.2.2 Illumination

Light exerts an essential and specific influence on the development of plants. Effects of light quality on the cell morphology, growth and development, chemical and enzymatic reactions have been extensively investigated (Stickland, 1972; Grisebach and Hahlbrock, 1974; Seibert and Kadkade, 1980; Hahbrock et al., 1980). It has been shown that light influences the ajmalicine/serpintine accumulation ratio in *Catahranthus roseus* culture (van der Heijden et al., 1989; Lounasmaa and Galambos, 1989). The effect of light depends on the wavelength and intensity, and varies in heterotrophic, photomixotrophic and photoautotrophic cultures. Light intensity affects metabolism via a direct influence on concentration of phytohormones, and at the same time, it affects the uptake of

nutrients, e.g. the oxygen-dependent uptake of sugar and that of nitrate, both transmitted by a carrier.

Li and Zhu (1990) investigated the effect of light density and quality on the production of anthocyanin in suspension culture of *Panax ginseng* cells. Suspension cell cultures were illuminated for 16 hours each day. It was found that large proportions of cells were pigmented owing to even illumination under suspension cell culture. Moreover, anthocyanin obtained from cells under white and blue light illumination was more comparable than that of red and yellow light.

There is an acceptor called UV-β receptor which absorbs light and promoted flavonoid synthesis (Beggs et al., 1986). The ability to form flavonoids appears to be a way of plant's defense against UV radiation (Salisbury and Ross, 1992). In dark culture, intimidation from UV-light is removed; there is no negative effect on ginseng cell growth even under darkness. In dark culture, biosynthesis of saponin will be more promoted. Li and Zhu (1990) also reported that ginseng saponin was from 0.3-0.7 % in light culture which was much lower than those recorded without illumination.

2.2.2.3 pH

The optimal medium pH is usually adjusted between 5 and 6, and higher pH will affect the solubility of the salts in the medium and thus their availability to the cells. The concentration of hydrogen ions in the medium changes during the development of the culture. The medium pH decreases during ammonia assimilation and increases during

nitrate uptake. The pH of a culture is determined by the contents of ammonium, nitrate and phosphate as well as the concentrations of excreted acids in the medium. Since these salts are also essential nutrients, their concentration decreases as the cells grow so that the buffering capacity of the medium is reduced.

Kovacs et al. (1995) studied the growth of haploid maize cells in suspension culture at different initial pH. From pH 5.8, the pH profile showed a very rapid decrease during the lag phase and a steady increase during the exponential growth phase. This kind of pH profile was reported to be typical of certain somatic plant cell cultures (McDonald and Jackmann, 1989; Ryu et al., 1990). As the initial pH decreased to 4.7, the lag phase seemed to be eliminated and the exponential growth phase was longer with approximately the same maximum specific growth rate, as those starting at pH 5.8, resulting in a significantly higher biomass production. The pH can also be applied to control the content of secondary metabolites in a process. It was reported that the change in medium pH between low and high values was used to release intracellular alkaloids into the culture medium (Payne et al., 1988; Asada and Shuler, 1989).

2.2.3 Other strategies

Nutrient feeding and elicitation, with biotic or abiotic elicitors has been reported to promote cell growth and enhance secondary metabolite biosynthesis. The effects of these methods on *P. ginseng* cell culture will be investigated in later experiment. Literature on these topics will be reviewed in each chapter separately.

2.3 *Panax ginseng*

Ginseng, a medicinal herb, has long been used in the Far East, particularly in Korea and China as a respected herbal medicine in maintaining physical vitality. The genus name Panax (Pan=all + axos=medicine) means 'cure all' in Greek. The herbal root is so named as ginseng, because it is shaped like a man, and is believed to embody his three essences (i.e. body, mind and spirit). According to Bencao Gangmu (Encyclopedia of Herbs) written by Li Shizhen in China, 1596 A.D., ginseng had been used mainly as a tonic to invigorate weak bodies, but only rarely as a curative medicine. *Panax ginseng* C.A. Meyer (named by the Russian scientist Carl Anton Meyer in 1843), which is also referred to as Asian ginseng, is distributed primarily in northern Asia, e.g. China and Korea.

Demand for ginseng-based substances is growing both for medical and prevention purposes. Ginseng-based agents are used for manufacturing of food products, various additives, including beverages (tinctures, balsams, non-alcoholic beverages, etc.), teas, perfumery and cosmetic products - creams, shampoos, gels, etc. Its highly prized medicinal properties have led to excessive exploitation resulting in the virtual extinction of the plant in its native habitat both in Asia and North America. As a result, ginseng is being developed as a horticultural crop on China, Korea, Japan, Russia, and Germany. Ginseng's high price on the world markets, despite increased production, has created worldwide interest in this crop. However, ginseng is difficult to grow, and needs between six and seven years of growth before the roots reach the certain weight associated with a higher level of active ingredients. It is then that the root is harvested. As the plant's age increases so does its value and price. Therefore, it is expected that the concentrations of

the effective components fluctuate greatly in response to places of production and the harvest times. Moreover, the growing of same species at different place also has different medicinal properties (Sticher, 1998). The development of alternative production techniques using tissue culture is required to meet commercial demand and perhaps keep the high quality of the product because a consistent culture environment can be ensured.

The study of ginseng tissue culture might be first documented by Lou et al. at a Chinese journal in 1964. Then, Butenco of the Soviet Union reported the studies of tissue culture of ginseng in 1968. It was starting in 1972 that Furuya et al. of Kitasato University obtained cultured tissues that contained the main effective components, ginsenosides, of ginseng. Similar result was also reported by Staba et al. of Minnesota State University. Later, Furuya et al. (1983) reported that they succeeded in obtaining cultured ginseng tissues which accumulated as much ginsenosides as did cultivated roots. Nitto Denko Corporation constructed a 20 kL fermenter based on 30 L results to scale-up cultivation of ginseng cells in collaboration with Furuya in 1985 resulted in maximum productivity of 19 g/l, approximately 700 mg/l. The cultured product was confirmed to be fundamentally of the same constitution as that in the origin plant. In 1988, Nitto Denko Corporation was approved by the Ministry of Welfare and Health in Japan to market the cultured ginseng mass as food additives. The products is used as an adjuvant with valuable physiological activities and is added to wines, tonic dinks, soups, herbal liqueurs and other preparations. (Ushiyama, 1991; Furuya and Ushiyama, 1994).

2.4 Ginseng saponins

Chemical analysis of ginseng revealed the presence of many ingredients, including organic acids, vitamins, sugars, inorganic salts, sterols, oligopeptides, polysaccharides, volatile oils, and saponins. Of these, the saponins (commonly known as ginsenosides) are well studied for their biological properties.

Saponins, a sweet-bitter material, usually exist in plants in the form of glycosides known as "saponin glycosides." Saponin glycosides are giant molecules extractable from the plant materials with hot water or alcohol, consisting of a sugar moiety (glycone) linked to a non-sugar portion (aglycone). The aglycone is also called genin or sapogenins. Depending on the type of genin present, the saponins can be divided into three major classes: (1) triterpene glycosides e.g. ginsenosides (ginseng), glycyrrhizin (licorice), saikosides (bupleurum root); (2) steroid glycosides; (3) steroid alkaloid glycosides. By 1987, the structure of over 360 sapogenins and 750 triterpene glycosides had been elucidated. This rapid progress is the result of the many dramatic advances in isolation and structure elucidation techniques (Hostetmann and Marston, 1995a).

To date, more than 30 ginsenosides have been found in the roots and other parts of *P. ginseng*, and a total of over 60 ginsenosides were isolated from members of the *Panax* genus, of which Rb1, Rb2, Rc, Rd, Rf, Re, and Rg1 are the major ones. Many of these compounds are responsible for the wide range of medicinal effects of ginseng (according to Liu et al., 2000).

Traditionally, biosynthesis of saponins is thought to be via the mevalonate pathway (Hostetmann and Marston, 1995a; Lehninger et al., 1995; Bianchini, et al., 1996; Seto et al., 1996; Knöss and Reuter, 1998). Mevalonate pathway starts from acetyl-CoA. 3 acetyl-CoA are condensed to form the important intermediate, β-Hdroxy-β-Methylgluyaryl-CoA (HMG-CoA). The enzyme HMG-CoA reductase catalyses the formation of mevalonic acid (MVA) and is known to be a key enzyme of the mevalonate pathway (Bianchini et al., 1996). Subsequently, isopentenyl pyrophosphate (IPP) is biosynthesized by subsequent phosphorylation and carboxylation. IPP and its isomer dimethylally pyrophosphate are activated biogenetic precursors of the linear branching point molecules in isoprenoid biosynthesis, geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP). The triterpenes and steroids are built up of isoprene units and have a common biosynthetic origin that they are all derived from squlene. Based on the mevalonate pathway, it is found sodium acetate, mevalonatic acid, geraniol , faresol, squlene can be used and added to the culture medium as the precursors for biosynthesis of triterpenes and steroids. Furuya et al. (1983) investigated the regulation of saponin production in callus culture of *P. ginseng* by using the above-mentioned precursors of saponin. Saponin contents were increased as compared with the control sample.

Investigations have also revealed that the mevalonate pathway is not the only biosynthesis process for isoprenic units. The existence of a non-mevalonate pathway for the biosynthesis of certain terpenoids has been demonstrated based on C-labelling patterns that are incompatible with the operation of the mevalonate pathway (Rohmer et al., 1993; Seto et al., 1996). The alternative route is called non-mevalonate pathway that starts with glyceraldehyde-3-phosphate and pyruvate. The 5-C isoprenoid precursor unit,

isopentenyl pryophosphate (IPP) is formed by further decarboxylation and rearrangement (Rohmer et al., 1993; Seto et al., 1996; Knöss et al., 1998; McCaskill and Crotenn, 1998). Based on this pathway, it is found pyruvate and glyceraldehyde-3-phosphate can also be added to the medium as precursors of saponin.

CHAPTER 3 MATERIALS AND METHODS

3.1 Cell line and culture maintenance

The ginseng cell strain used in this study was induced from the root of a six-year-old plant, *Panax ginseng* C.A. Meyer, which was harvested in northern China.

The basal medium for suspension culture was the MS medium (Murashige and Skoog, 1962) (Appendix 1) (Life Technologies, Grand Island, NY, USA, Cat. No. 10632). Complete medium was supplemented with 1 mg/l 2,4-D and 30 g/l sucrose as the carbon source, and 500 mg/l casein hydrolysate (chemicals from SIGMA, St. Louis, MO, USA). All the medium components were dissolved in deionized water and the solution pH was adjusted to 5.8, and then sterilized by autoclaving at 121 °C for 20 minutes.

Suspension cultures were obtained by transferring small portions of callus to liquid medium. The suspension culture (Stock) was propagated in shake flasks, with 125 ml and 250 ml Erlenmeyer flasks, each containing 25 ml and 50 ml medium respectively, on an orbital shaker shaking at 110-120 rpm. The shake flask culture was kept in dark at 25 °C, subcultured every 2 weeks.

3.2 Determination of cell growth

Fresh cell weight (fw) was obtained by filtering the cell suspension through a preweighed dry filter paper (Whatman, 55 mm × 100 circles, Cat N. 1001055) under reduced pressure. The filter paper with cells was then dried at 50 °C in oven until constant weight. Then the dry weight (dw) of the cells was determined. The supernatant collected was used for the analysis of medium nutrients.

3.3 Measurement of medium nutrients and properties

3.3.1 Major nutrients

Residual sugar (sucrose and fructose) concentration in the culture medium was determined according to the methods described in Pollard and Walker (1990). Glucose concentration was measured by using the toluidine reagent from Sigma. Procedures for the determination of glucose, fructose, and sucrose are listed at Appendix 2. Total sugar concentration was determined by phenol-sulfuric acid assay (Chaplin and Kennedy, 1996) (Appendix 3). Nitrate concentration was determined by the method described by Sawicki and Scaringelli (1971, cited in Alfermann et al., 1997) in which nitrate is reduced to nitrite; nitrite reacts with a diazo reagent to give a coloured product (Appendix 4). Determination of ammonium concentration was based on the method described by Fawcett and Scott (1960, cited in Alfermann et al., 1997), which is based on the complex reaction between ammonium ions and hypochlorite, phenol and nitroprusside to form a blue indophenol anion (Appendix 5). Inorganic phosphate concentration was determined

by the method of Martland and Robinson (1926, cited in Alfermann et al., 1997) (Appendix 6).

3.3.2 pH, osmotic pressure and conductivity

Medium pH was measured with a glass pH electrode (GC-5011C, TOA Electrons, Ltd.). Osmotic pressure of the culture medium was determined by measuring the osmolality of the culture filtrate by a vapor pressure osmometer (Wescor Inc., Logan, Utah, USA). Conductivity of the culture medium was measured by a conductivity meter (Lutron CD-4303).

3.3.3 Extraction and analysis of saponin content of cell

Ginseng saponin (total saponin) was extracted and purified by thin-layer-chromatography, and then quantified by colorimetric method (Wang et al., 1979; Zhang et al., 1980 and 1983) (Appendix 7). The saponin content of cell was expressed as weight percentage (%) and the total yield in the culture as mg/l.

3.3.4 Phenylalanine ammonia-lyase (PAL) activity and hydrogen peroxide

PAL activity in plant cell was measured by detecting the change in absorbance (Dörnenburg and Knorr, 1995) (Appendix 8). Production of H_2O_2 was detected by oxidative quenching of a fluorescent peroxidase substrate, pyranine (8-hydroxypyrene-

1,3,6-sulfonic acid trisodium salt, λ_{ex} 405 nm, λ_{em} 512 nm) in medium. 25 ml medium was treated with 50 µl of a 1 mg/ml pyranine solution to give a concentration of 2 µg/ml. The generation of H_2O_2 was monitored by the decrease in fluorescence due to the peroxidase-mediated oxidation of pyranine with a luminescence spectrometer (Model LS50B, Perkin Elmer, Shelton, CT, USA) (Levine et al., 1994) (Appendix 9).

3.4 Quantification of growth characteristics

The growth parameters of cell culture were calculated by using the following formulae (Pirt, 1975 ; Strogov et al., 1991).

- Maximum growth index: $GI_{max} = X_{max}/X_o$
- Biomass productivity or average growth rate (g dw/l-d): $Pr = (X_{max} - X_o)/t$
- Biomass yield (g dw/g on sucrose consumed): $Y_{x/s} = (X_{max} - X_o) / (S_o - S)$

  X = Biomass concentration (X_{max}, maximum; X_o, initial) (g dw/l)

  S = Sugar concentration (g/l)

CHAPTER 4 BASIC CULTURE CHARACTERISTICS

Characterization of the cell growth and nutrient metabolism is the first important step of optimization. The growth pattern and behaviour can be obtained by studying the biomass growth, nutrient uptake, and the change of the cell culture environment. The results will be helpful for the selection of a culture strategy to achieve higher biomass and saponin yields.

Cell growth showed the typical pattern of microbial growth (Pirt, 1975) and plant cell suspension growth (Figure 4-1).

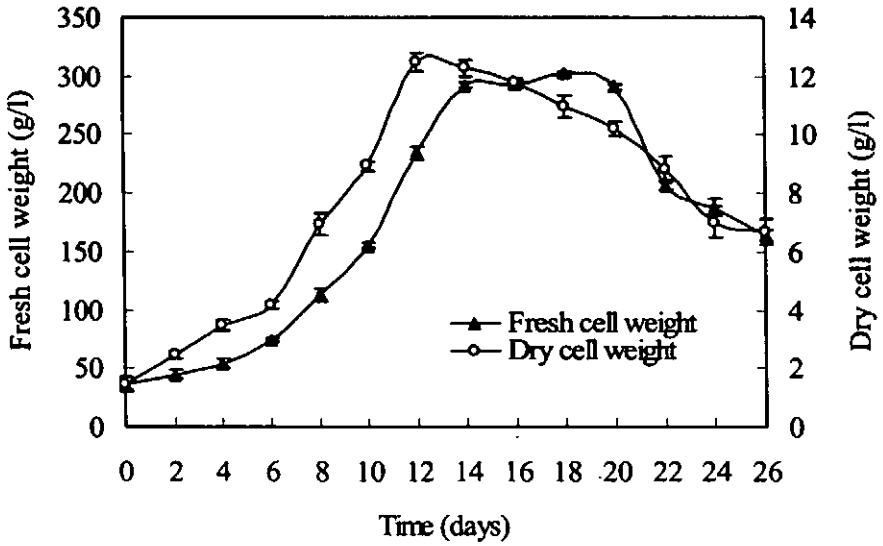

Figure 4-1. Time profile of the growth of *P.ginseng* cells in terms of fresh cell weight and dry cell weight.

The lag phase can be seen in the first 2 days. This indicates that the cells need a short time to adapt to the new environment. Then, the cells entered the exponential growth phase on day 12-14. During this period, biomass concentration increased from 1.46 g/l to

12.49 g/l, GI_{max} = 8.55. The biomass remained nearly constant from day 12 to day 14 and decreased gradually thereafter. Under microscopic observation, more and more cell debris were found after the stationary phase. This may be due to cell autolysis by digestive enzymes in the cells. Similar growth trend was also seen in the other plant cell cultures (Snape et al., 1989; Srinivasan and Ryu, 1993; Pépin et al., 1995; Zhang et al., 1996a and 1997).

Figure 4-2 showed the change of dry cell weight to fresh cell weight ratio (dw/fw) during the culture process. The ratio increased gradually from the lag growth phase to the early stage of exponential growth phase and then kept relatively constant during the exponential growth phase. Then it decreased rapidly from the late exponential phase and then remained constant during the stationary growth phase.

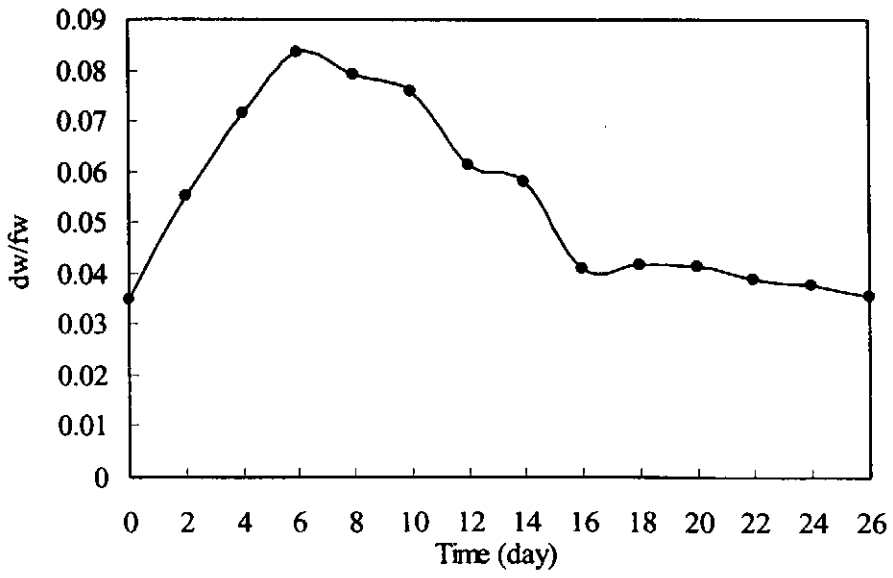

Figure 4-2 Ratio of dry cell weight to fresh cell weight.

The change in dw/fw can be observed in the cell morphology (Figure 4-3). The cell size decreased as the culture changed from the lag growth phase due perhaps to the rapid growth in this period. The decrease in cell size accompanied with the decrease in water holding capacity resulting in a drop in cell fw. However, the biomass increased dramatically during this period. Thus, this phenomenon caused a net increase in the ratio of dw/fw.

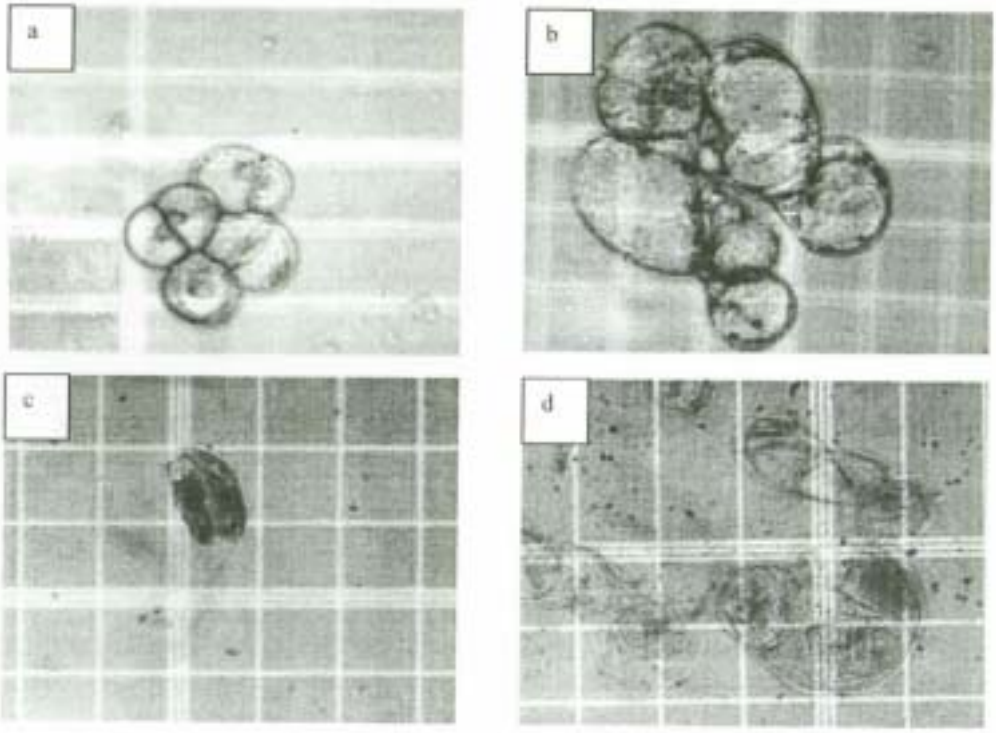

Figure 4-3. Morphology of the *P. ginseng* cells at different growth stages observed under microscope. (a) Cell division was observed during exponential growth phase. The cell size was rather small. (b) At stationary growth phase, the cellular size increased. (c and d) Cellular autolysis and the remained cell debris were observed at the late stationary growth.

As the cells entered stationary growth phase, rapid cellular division ceased but the cell increased in size (Figure 4-3, photo b). This resulted in an increase in water holding capacity. Since the cells no longer grew and the water holding capacity increased, the dw/fw decreased eventually. The increase in cell volume and water content could be related to the drop in osmotic stress during the exponential growth phase. The cellular size and morphology at late exponential growth phase was similar to that at the early lag phase, when the cells were just inoculated into the fresh medium. At the late stationary growth phase and death phase, cellular autolysis and cell debris could be observed (Figure 4-3, photo c and d).

The time course for the formation of ginseng saponin during the culture process is shown in Figure 4-4. The saponin content decreased gradually from day 0 to day 4 and then increased gradually from day 6 to day 10. After day 10, the saponin content decreased rapidly until day 14, while the dry cell weight increased 3.54 g/l. This may be due to the increase in biomass concentration outweighing the increase in saponin content, so saponin in the biomass was diluted during cell division resulting in lower saponin content of cell. The other reasons for this phenomenon include the diversion of carbon flux from the secondary metabolic pathways, low activity of key enzymes, lack of appropriate storage sites or transport mechanisms, and unregulated catabolism of the product. The cells stepped to stationary phase after day 12 and cell dw decreased gradually from day 12 to day 20. Saponin content increased rapidly from day 14 to day 16 and then gradually from day 16 to day 22. When the cells entered stationary phase, cell division stopped for the accumulation of saponin in the cells (from day 12 to day 16). After day 22, saponin content decreased gradually because cells began to autolysis and thus saponin

leaked out from the cells into the medium, resulting in lower saponin content of cells. Apart from the microscopic observation of cell debris, cell lysis could also be reflected by the darkening of the culture medium due to the release and oxidation of phenolic compounds produced by the cells. Similar phenomenon was also reported in the case of *Panax notoginseng* cell cultures (Zhang et al., 1997), *Taxus cuspidate* cell cultures (Fett-Neto and Dicosmo, 1992; Fett-Neto et al., 1993 and 1994), *Anchusa officinalis* cell cultures (Snape et al., 1989) and other species of plant cell cultures (Charlwood et al., 1990).

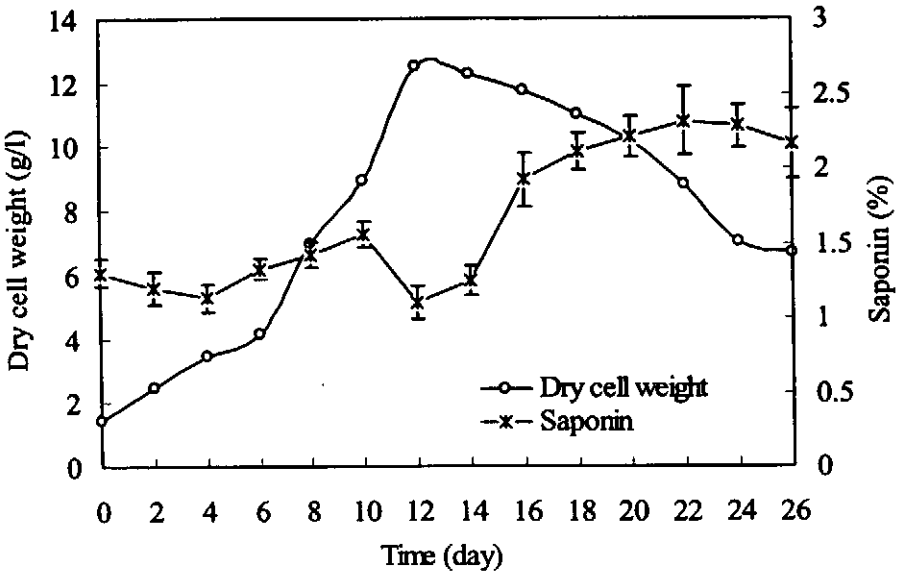

Figure 4-4. Time profile of dry cell weight and saponin content in suspension culture.

The change in cell biomass was closely linked with the concentration of sugars in the culture medium, and the biomass growth leveled off with the depletion of sugars in the medium on day 14 (Figure 4-5). Less sugar was consumed during the first 2 days, and most sugar was consumed from day 2 to day 13, the period of exponential growth phase.

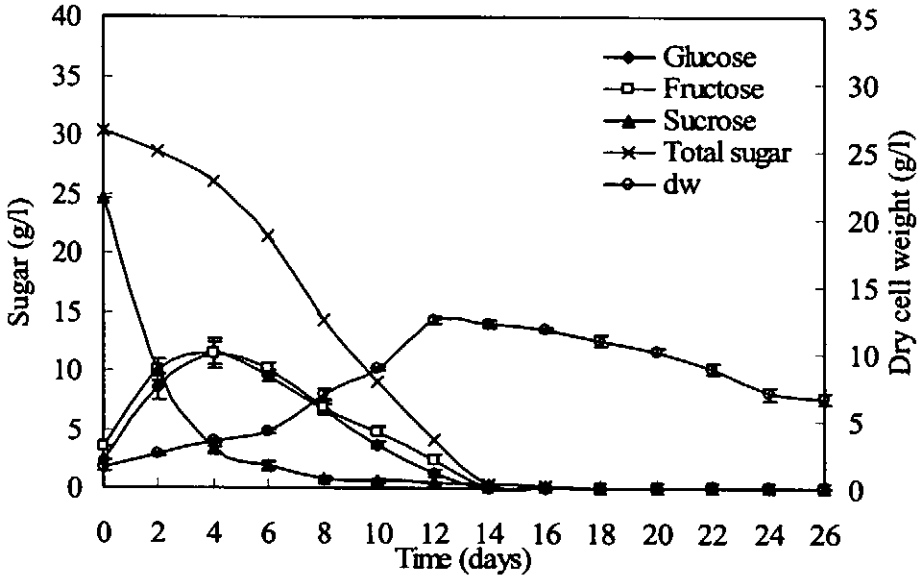

Figure 4-5.Time profile of sugar concentration and cell growth in the suspension culture.

The close relationship between the increase in biomass and the consumption of sugar suggests that sugar was used for rapid metabolism rather than storage within the cells. This could also be reflected by the sugar conversion rate. During the first four days, sugar conversion rate was above 40%, and then above 30% till day 14, indicating that less than half of the sugar consumed was used for the increase in cell biomass. Sugar is the major carbon source of cell biomass, and its sugar availability will affect the biomass growth. Although sucrose was the only sugar added to the culture medium, glucose and fructose were also detected in the culture medium. Obviously, glucose and fructose were originated from hydrolysis of sucrose. During autoclave, sucrose is hydrolyzed to glucose and fructose under high temperature. In the culture medium, sucrose is hydrolyzed to fructose and glucose by the enzymes secreted from the cells. The results showed that *P. ginseng* consumed glucose and fructose nearly simultaneously although glucose was

taken up at high rate. Fett-Neto et al. (1994) reported the different results in the cell suspension of *Taxus cuspidata*. It was found that fructose concentration was higher than glucose in the culture medium. Glucose was more preferred by the cells than fructose. This may be related to the availability of enzymes in the cells.

Figure 4-6 shows the change in nitrogen uptake during the suspension cell culture. The uptake of nitrate was more rapid than that of ammonium during the first few days. After day 7, the situation was reversed. The change of nitrogen concentration in the medium will affect the pH value, which was also confirmed by measurement. The result was different from that reported in another plant cell culture in which ammonium ions were used rapidly during the first several days and nitrate ions were used gradually (Pépin et al., 1995). Ammonium ions were detected again in the medium on day 13 and increased slowly. This phenomenon might be due to cell lysis after day 13, resulting in the release of ammonium into the medium. The decrease in biomass after day 13 also indicated the occurrence of cell lysis. The rapid nitrogen uptake during the early culture indicated a high demand of nitrate in the growth-related metabolism. The rapid nitrate uptake in the initial phase may be important to support protein synthesis for the cell growth. (Zhong et al., 1996; McDonald and Jackmann, 1989). Generally, the uptake and reduction of nitrate (due to the formation of OH^- equivalent) increases the alkalinity of the medium while the uptake of ammonium (exchanged by H^+) increases the acidity of the culture medium. The pattern of nitrate and ammonium ion uptake could also be observed by referring to the pH profile. The pH increased during the first two days and then decreased rapidly until day 6. pH value increased rapidly during the exponential growth phase, and remained steady during the stationary growth phase. The pattern of pH change was quite different from

other suspension cell cultures at which pH dropped initially. The initial increase in pH is probably due to rapid uptake of nitrate, and then intracellular nitrate reduction combined with a cotransport of protons during sugar uptake (Zamski and Wyse, 1985). Then, the drop in pH is probably due to uptake of ammonium ions following nitrate (McDonald and Jackmann, 1989).

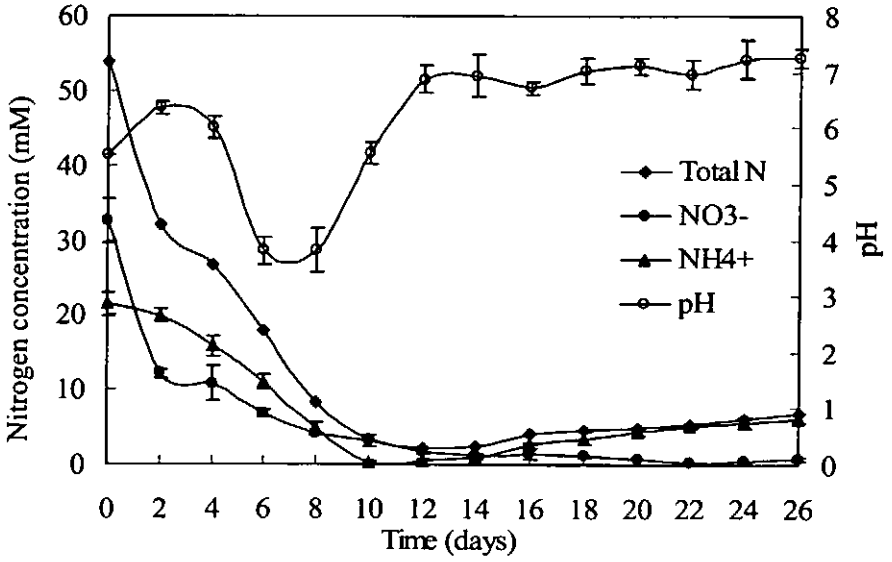

Figure 4-6.Time profile of nitrate, ammonium concentration and pH value in the culture medium.

In *P. ginseng* cell cultures, phosphate was taken up completely during the first several days (Figure 4-7). The same pattern of phosphate uptake was also reported in other plant cell cultures (Zhong et al., 1995; Pépin et al., 1995). Rapid cell growth continued until day 13 even phosphate depletion occurred on day 5 suggesting that phosphate was up-taken and stored inside the cells and then distributed to the other cells during cell division. The metabolites in the glycolysis and tricarboxylic acid cycle can be partly used to synthesize large molecules, e.g. lipids, proteins, and nucleic acids, which are needed for

growth. The effectiveness of formation of these large molecules depends on the supply of ATP which is in turn controlled by the phosphate. Thus, a high intracellular phosphate may be necessary to support the growth and metabolism of the cells.

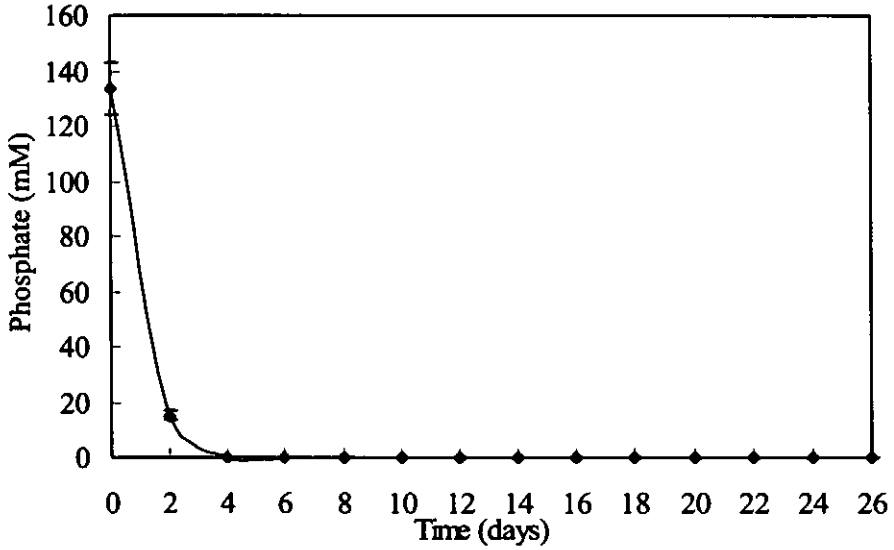

Figure 4-7.Time profile of phosphate concentration in the culture medium.

The dry cell weight and the conductivity of the medium are shown in Fig. 4-8. Generally, the increase in cell was correlated with the decrease in conductivity. This result suggests that the cellular uptake of ionic nutrients is consistent with the cell growth. The conductivity is directly proportional to the concentration of ionic nutrients in the medium. The conductivity of medium is mainly attributed to the ionic substances, such as nitrate and phosphate. Thus, the change in medium conductivity was due to the change in the concentration of ionic components. Ryu et al. (1990) reported that the decrease in conductivity during plant cell cultivation is directly proportional to the decrease in nitrate concentration due to the cellular uptake. This means that the conductivity is inversely

proportional to the cell growth (Suresh et al., 2001). Our results seem to agree with point as the dry cell weight increased while the conductivity declined.

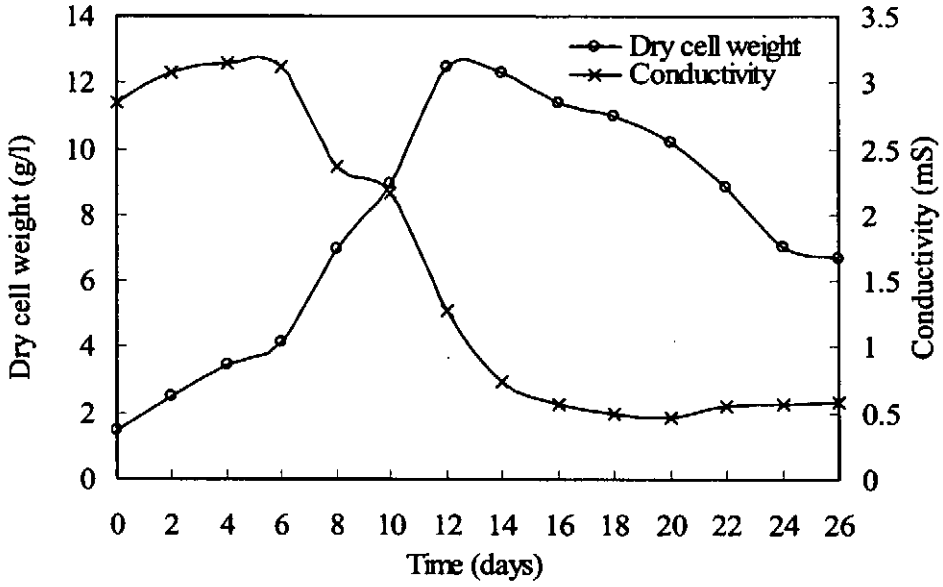

Figure 4-8. Time profile of conductivity and dry cell weight of the suspension culture medium.

Figure 4-9 shows that the osmolality of the medium first increased at the first 6 days and then decreased rapidly between day 6 and day 13, and finally kept constant until the end of the cultivation period. Subsequently, the osmolality decreased linearly with an increase in the cell during exponential growth phase. Osmolality is equal to the sum of osmolalities of the medium components. It is interesting to note that the pattern of osmolality change in the culture medium is similar to the change of conductivity. Therefore, osmolality may be dependent on total ion concentration in the medium.

Suresh et al. (2001) has tested the effect of other environmental changes on the osmolality of the medium without biomass. The pH and temperature did not have any

effect on the medium osmolality. Increase in glucose and sucrose concentration increased the osmolality significantly unlike in the case of conductivity where the change in sucrose and glucose concentration showed little effect in medium conductivity.

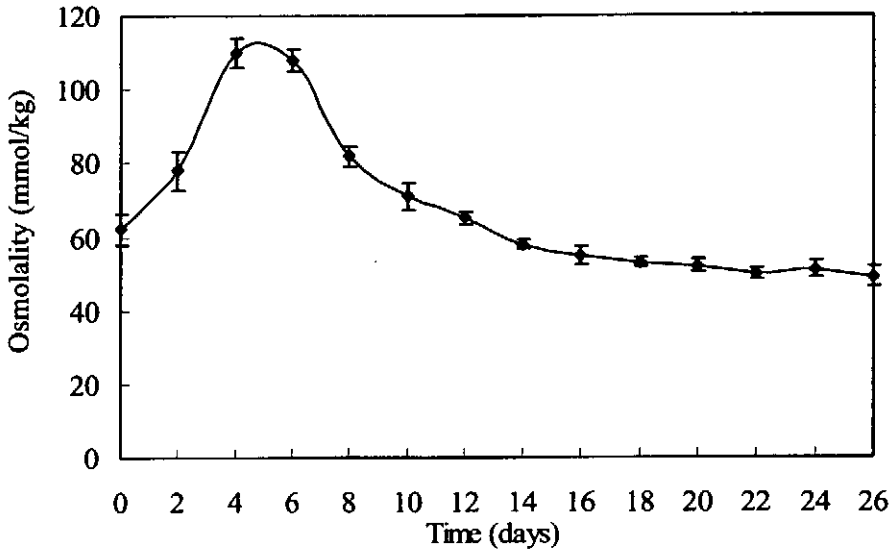

Figure 4-9. Time profile of osmolality in the suspension culture medium.

In summary, the highest biomass concentration was obtained on day 12, while the highest saponin content was obtained at day 22, and the highest saponin yield on day 16. Saponin production came after biomass growth, and saponin production increased as cell growth decreased. The culture characteristics of *P. ginseng* cell growth are summarized in Table 4-1.

Table 4-1. The characteristics of *P. ginseng* cell culture.

Fresh weight (g/l)	Dry weight (g/l)	Maximum growth index	Average growth rate (g/l-d)
312 (16)	12.5 (12)	8.55	0.92
Biomass yield on sucrose (g cell/g sucrose)		Saponin content (% of dw)	Saponin yield in culture (mg/l)
0.43		2.71(22)	281(16)

The data in parentheses indicate the time when the maximum value was attained.

CHAPTER 5 EFFECT OF NUTRIENT FEEDING ON GINSENG CELL CULTURE

5.1 Introduction

The growth of ginseng cell suspension cultures takes the generalized form of sigmoid curve (Figure 4-1). There is a period of little or no cell division (the lag phase) and a period of exponential cell division (the log phase), and then a gradual cessation of cell division as nutrients become depleted (the stationary phase). Each of these phases has their specific nature. By manipulating the nature of the cell growth cycle, it is possible to control the pattern of cell growth and secondary metabolite biosynthesis.

In this chapter, the investigation will concentrate on the effect of nutrient feeding on ginseng cell growth and secondary metabolite biosynthesis. Many previous studies have shown that the increase in initial nutrient concentration can promote biomass growth and secondary metabolite biosynthesis. There was a general trend of increasing volumetric productivity of shikonin and cell concentration of *Lithspermum erythrorhizon* culture by increasing initial sucrose concentrations (Srinvasan and Ryu, 1993). Similar result was also shown with taxane production in suspension cultures of *Taxus chinensis* (Wang et al., 1999). However, too high an initial nutrient concentration will lead to depression of the cell growth and secondary metabolism. Previous studies have shown that cell growth increases with the increase in sucrose concentration from 10 g/l up to 50 g/l. Further increase in sucrose concentration would lead to growth depression (Wu and Ho, 1999). In

the case of *Vitis vinifera* cell cultures, a reduced cell concentration was observed at sucrose concentration of 50 g/l (Do and Cormier, 1990).

Nutrient feeding can alleviate the growth inhibiting problems at high initial nutrient concentrations and enhance both biomass growth and secondary metabolite biosynthesis. The concept of nutrient feeding can be taken as fed-batch culture. Fresh nutrients were fed into the culture systems at a time when the nutrients were used up. Generally, secondary product formation was induced after feeding of nutrients, often by application of high sucrose concentration (Martinez and Park, 1993). The most suitable moment for the nutrients feeding seems to be the end of the growth, when maximum cell biomass was attained. Moreover, the cell growth and secondary metabolite biosynthesis were also affected by the kinds of nutrients fed. In *Lithspermum erythrorhizon* suspension culture, feeding of sucrose-rich medium enhanced shikonin production, while feeding of nitrate-rich medium promoted cell growth. It was possible to stimulate shikonin production by sucrose-rich medium by first cultivation in nitrate-rich medium (Srinivasan and Ryu, 1993). Therefore, a comprehensive feeding strategy has to be designed in order to improve the cell growth and secondary metabolite synthesis.

The objective of this investigation is to design a nutrient feeding strategy for increasing the biomass concentration and secondary metabolite yield simultaneously, and to study the effects of individual nutrients and their interactions.

5.2 Methodology

The complete MS medium was used to start the culture and then certain nutrients were fed into the medium before the stationary growth phase. In the preliminary stage, the MS medium and its supplements used at this study were divided into several major groups according to their general constitution. They were carbon sources, macronutrients (major salts), micronutrients (minor salts), growth regulators and vitamins (Table 5-1). Each group of these nutrients has specific effect on the growth of the cell culture (Gamborg, 1984; Gamborg and Phillis, 1995). These nutrients group will be fed to the culture in all possible combinations.

In the first set investigation, a full 2^4 factorial design was employed. Full factorial designs are often used at an early stage of an investigation with a large number of factors (Table 5-2). The cells were harvested on day 22, day 26 and day 30 respectively.

Table 5-1. Classification of the ingredients of the MS medium into several major groups.

Ingredients	**Components (mg/l)**	**Ingredients**	**Components (mg/l)**
Major salts		**Organic Salts**	
NH_4NO_3	16500	Myo-Inositol	100.00
KNO_3	1900.00	Nicotine Acid	0.50
$MgSO_4$	180.70	Pyridoxine·HCl	0.50
KH_2PO_4	170.00	Thiamine·HCl	0.10
$CaCl_2$	332.20	Glycine (Free Base)	2.00
Minor salts		**Growth regulators**	
H_3BO_3	6.20	2,4-D	1.00
$MnSO_4·H_2O$	16.390	**Carbon Source**	
$ZnSO_4·H_2O$	8.60	Sucrose	30000
$Na_2MoO_4·2H_2O$	0.25	**Other Supplements**	
$CuSO_4·6H_2O$	0.025	Casein hydrolysate	500
$CoCl_2$	0.025		
KI	0.83		
$FeSO_4·7H_2O$	27.80		
Na_2EDTA	37.26		

Table 5-2. A 2^4 factorial experimental design for nutrient feeding experiment.

Group	1	2	3	4	Variable
1	-	-	-	-	1. Major salts
2	+	-	-	-	2. Minor salts
3	-	+	-	-	3. 2,4-D
4	+	+	-	-	4. Vitamins and Organics
5	-	-	+	-	
6	+	-	+	-	
7	-	+	+	-	
8	+	+	+	-	
9	-	-	-	+	
10	+	-	-	+	
11	-	+	-	+	
12	+	+	-	+	
13	-	-	+	+	
14	+	-	+	+	
15	-	+	+	+	
16	+	+	+	+	
17	Feeding 25 g/l sucrose only				
18	Adding same amount of distilled water (5 ml)				

Note: Since sucrose is the major carbon source for synthesis of biomass and cell metabolites, 25 g/l sucrose and 500 mg/l casein hydrolysate were added to all the groups except Group 17 and 18 of culture medium. A set of experiment is also run by feeding the sample with the same amount of sterilized distilled water (Group 18). 5 ml, six times concentrated, freshly prepared nutrient solution, was fed into the culture flasks on day 16 to make the total culture volume up to 30 ml, and all nutrients were at the same concentration as in the standard MS medium except sucrose.

5.3 Results and discussion

In the first set of experiments, the complete MS medium with supplements was divided into several major groups and fed into the culture flasks on day 16 at different combinations. The maximum dry cell weight obtained has been shown in Figure 5-1. In the group with nutrient feeding (group 1 to17), the maximum dry cell weight ranged from 18.82 g/l (group 17) to 23.34 g/l (group 2), which were much higher than that obtained in the culture without nutrient feeding, 12.50 g/l (Table 4-1). On average, the highest biomass density was achieved in groups 1, 2, and 3, the feeding of sucrose plus casein hydrolysate (group 1), sucrose plus casein hydrolysate and major salts, and sucrose plus casein hydrolysate and minor salts.

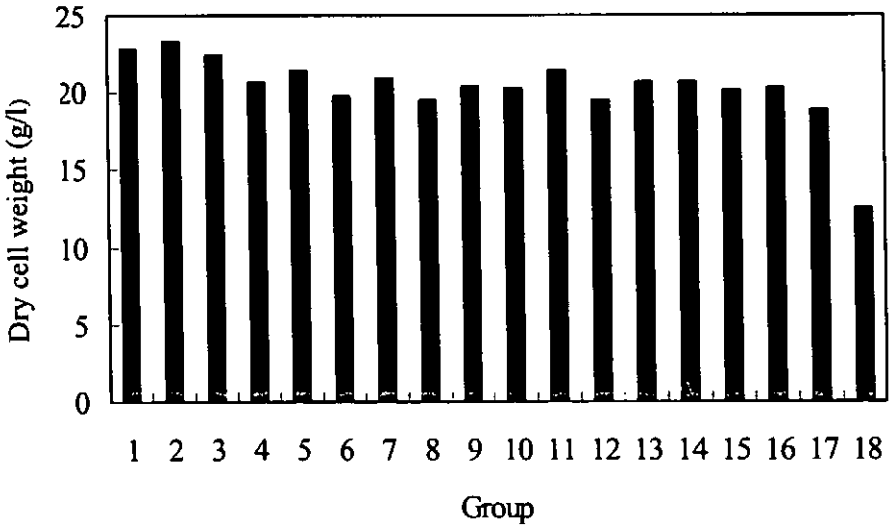

Figure 5-1. The maximum dry cell weight under different nutrient feeding combinations (Initial cell density was 1.30 g/l).

The dry cell weight obtained from group 17, feeding sucrose only, was obviously lower than that from the other groups on day 22, though it increased gradually from day 22 to day 26. This indicates that feeding of sucrose, the carbon source only, could also support rapid cell growth. However, there are other nutrients that limited the further cell growth. The dry cell weight obtained from group 1, feeding sucrose together with casein hydrolysate, was higher than that obtained from group 17, feeding sucrose only. Thus, casein hydrolysate seemed to play an important role for cell growth and development. The groups with either major nutrients or minor nutrients added also resulted in higher dry cell weight than that of the group fed with sucrose only. As compared to sugar consumption, the sugar concentration in all the groups decreased gradually from day 22 to day 30 (Figure 5-2). Our previous study has shown that cell growth was much inhibited in the absence of 2,4-D. In this experiment, however, the feeding of the growth regulator did not improve the biomass yield. This may be due to that 2,4-D in the initial medium still has not been used up at later stage.

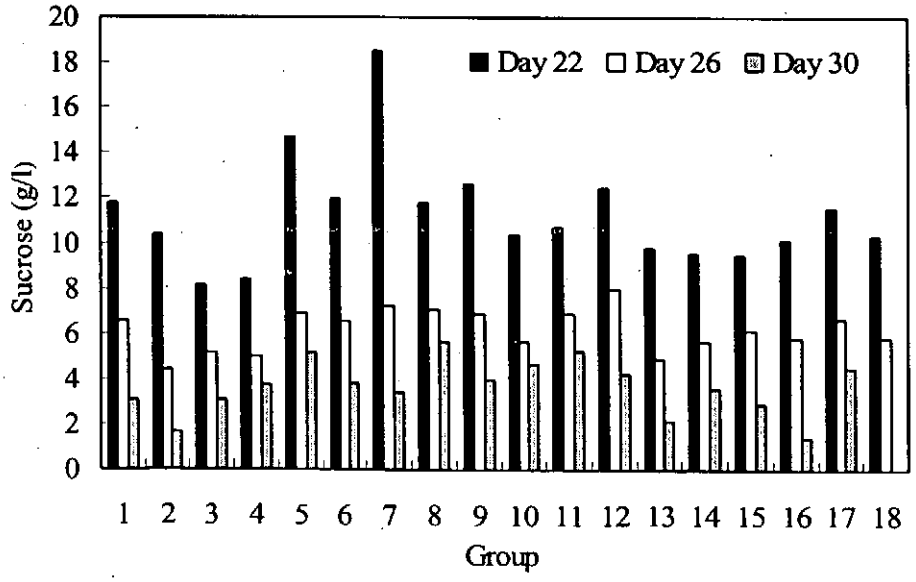

Figure 5-2. Sucrose concentration in cultures with different combinations of nutrient feeding.

The growth characteristics obtained from this set of experiment are summarized at Table 5-3. From Table 5-3, it is seen that maximum growth index of group 1, 2 and 3 was higher than that of other groups. Therefore, feeding of sucrose together with casein hydrolysate or that with either major salts or minor salts could promote cell growth.

Table 5-3. Growth characteristics of the cell cultures with different nutrient feeding solutions.

Group	Maximum growth index	Biomass productivity (g/l-d)
1	17.58 (26)	0.83 (26)
2	17.95 (30)	0.98 (22)
3	17.23 (22)	0.96 (22)
4	15.92 (30)	0.78 (22)
5	16.45 (26)	0.89 (22)
6	15.17 (22)	0.84 (22)
7	16.14 (22)	0.89 (22)
8	15.05 (22)	0.83 (22)
9	15.65 (30)	0.73 (22)
10	15.62 (26)	0.83 (22)
11	16.51 (26)	0.80 (22)
12	15.05 (26)	0.82 (22)
13	15.91 (22)	0.88 (22)
14	15.88 (22)	0.88 (22)
15	15.53 (22)	0.86 (22)
16	15.63 (26)	0.86 (22)
17	14.48 (26)	0.67 (26)
18	12.50 (12)	0.41 (12)

The data in parentheses indicate the time when maximum value was attained. Initial cell density was 1.30 g/l.

Based on the result of the first set of experiments, the second set of experiments was designed. In the second set of experiment, the major groups of nutrients screened out from the first set, together with 25 g/l sucrose, were added into the culture flasks on day 16. The design is shown in Table 5-4.

Table 5-4. Nutrient groups fed into the culture in the second set of experiments.

Group	Nutrient combination
1	Sucrose (Suc.) (25 g/l)
2	Sucrose (25 g/l) + Major salts (Maj. S) + Casein hydrolysate (CH)
3	Sucrose (25 g/l)+ Minor salts (Min. S) + Casein hydrolysate (CH)
4	Sucrose (25 g/l) + Organic salts (OS) + Casein hydrolysate (CH)
5	Sucrose (25 g/l) + Casein hydrolysate (CH)

In the second set of experiments, several nutrient groups (Table 5-4) screened out from the first set were fed into the culture flasks on day 16. The time profile of ginseng cell growth (Figure 5-3) shows that the growth in the group fed with sucrose only always lagged behind that in the other groups. This result was also seen in the first set of experiment. Clearly, casein hydrolysate contains some factors that are crucial for ginseng cell growth, since the groups fed with casein hydrolysate, group 3 and group 4, had better growth than the other groups. In the control group, the saponin content of cell was kept at steady state until day 26. While the saponin content in those nutrient feeding group decreased dramatically from day 16 to day 22 and then increased rapidly from day 22. The saponin contents in the nutrient-feeding groups were even higher than that in the control on day 30.

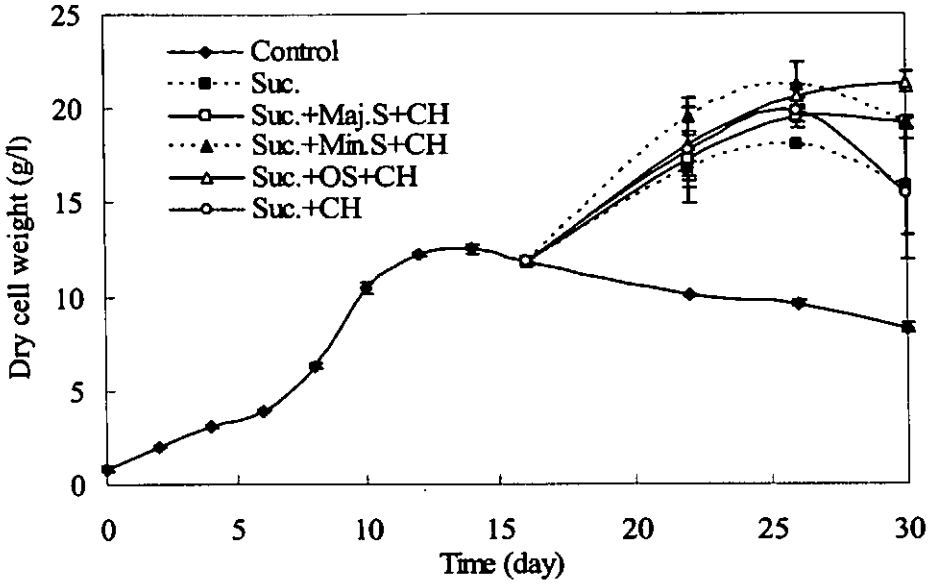

Figure 5-3. The cell growth profile in term of dry cell weight with different combinations of nutrients fed at day 16.

After nutrient feeding, there were plenty of nutrients, promoting rapid cell growth on the primary metabolism of the cells. However, the secondary metabolite biosynthesis decreased, resulting in lower saponin content of cells in the nutrient-feeding groups on day 22. Among the nutrient-feeding groups, the group fed with sucrose and casein hydrolysate (group 5) showed smaller decrease in saponin content on day 22, and the highest yield of saponin on day 26. However, the saponin content in the other nutrient-fed groups caught up with group 5 on day 30 (Figure 5-4). In general, the nutrient feeding did not affect the saponin content of cell. The saponin yield obtained from group 5 was the highest owing to high cell biomass (Figure 5-5).

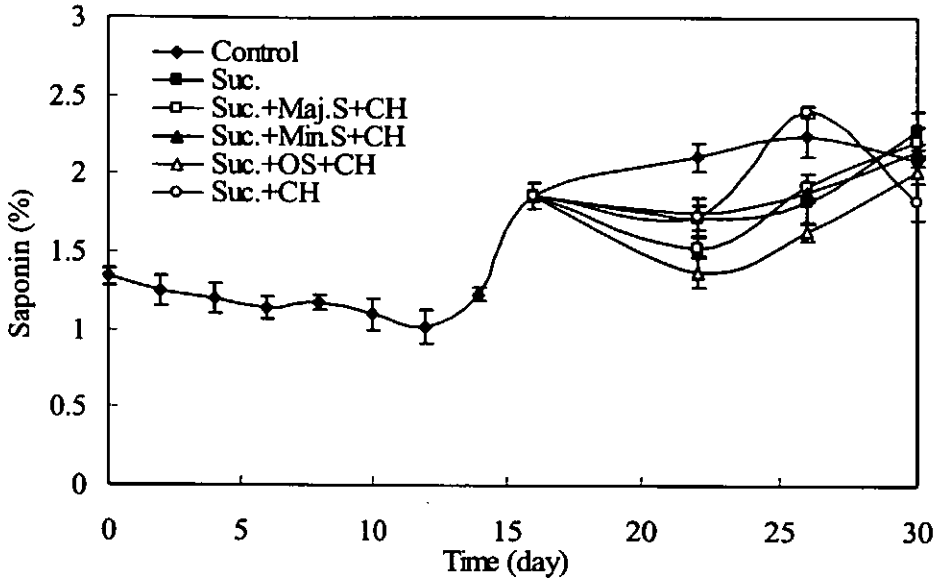

Figure 5-4. The time profile of saponin content in cell culture with different combinations of nutrient feeding.

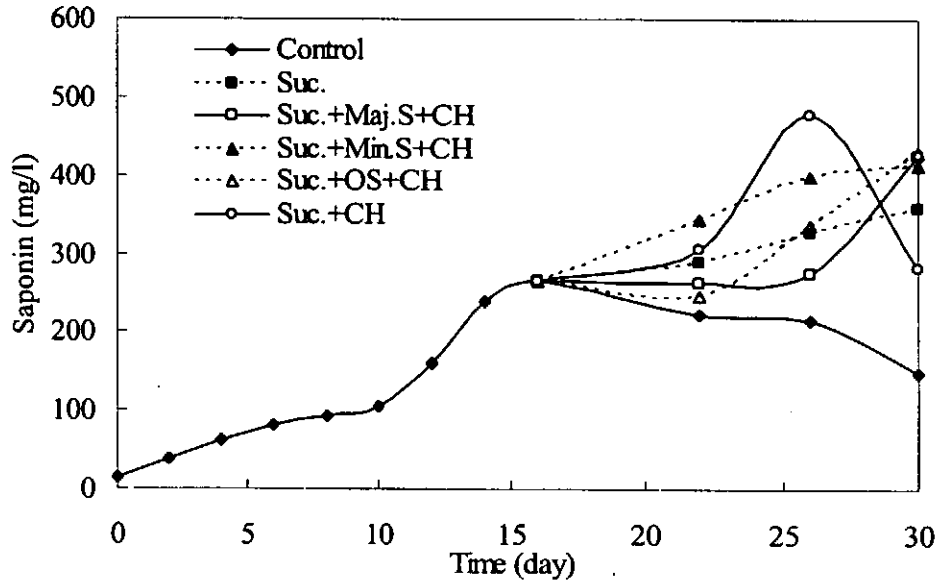

Figure 5-5. The time profile of saponin yield in cell culture with different combinations of nutrient feeding.

The results of the second set of experiments are summarized in Table 5-5. Although both the maximum growth index and saponin yield (mg/l) were all increased by nutrient feeding, the biomass productivity was lower than that without the feeding. This may be due to the late feeding schedule (after the stationary phase).

Table 5-5. Summary of the results from the second set of feeding experiment.

Group	Maximum growth index	Biomass productivity (g/l-d)	Maximum saponin content (%)	Maximum saponin yield (mg/l)
Control	15.2	0.97	2.24	266
1	21.9	0.66	2.28	360
2	23.7	0.72	2.21	425
3	25.8	0.78	2.15	413
4	24.1	0.74	2.02	431
5	24.1	0.73	2.40	478

5.4 Summary

The cell growth was sustained when suitable nutrients were fed into the culture at early stationary growth phase. The culture fed with sucrose and casein hydrolysate had higher dry cell weight than the group with sugar fed only. Casein hydrolysate seemed to play an important role for cell growth and development. The cultures fed with both major inorganic salts and minor trace elements had lower maximum dry cell weight than those fed with either major inorganic salts or minor trace elements. The feeding of growth regulator had no beneficial effect on cell growth. The feeding of nutrients caused a decrease in saponin content at first because of rapid cell growth undermining the saponin

biosynthesis. The maximum saponin content, however, was not significantly affected by the nutrient feeding strategy.

CHAPTER 6 EFFECT OF OSMOTIC STRESS ON GINSENG CELL CULTURE

6.1 Introduction

The effect of osmotic stress on plant growth and metabolism has been recognized for many years (Crafts, 1968). The high osmotic pressure treatment acts as a physical elicitor on the cell growth and biosynthesis. Triggering metabolite pathways with high osmotic pressure may be an alternative strategy leading to the enhancement of secondary metabolite biosynthesis in plants. Kimball et al. (1975) studied the effect of increased osmotic pressure generated by addition of mannitol, sorbitol, glucose or sucrose to the medium on morphology of soybean (*Clycine max* L.) cells. Under high osmotic pressure, cells became smaller and spherical regardless of the osmoticum used while the cells in control were large, elongated and irregularly shaped. Do and Cormier (1991) grew cell suspension of grape, *Vitis vinifera* L. cv Gamay Fréaux, under different degrees of water stress induced by an increase of sucrose concentration or by the addition of mannitol to the culture medium. The increase of osmotic pressure resulted in a significant increase in the accumulation of anthocyanins in the cells. However, the cell growth was repressed under increased osmotic pressure. As a response to the stress, the enzymes of biosynthetic pathways other than the primary metabolism were induced, resulting in an accumulation of secondary products (Moreno et al., 1993). Zhang et al. (1995) claimed that at high initial osmotic pressure, the specific saponin production was increased in the *Panax notoginseng* cell suspension culture, while the consumption rate of major medium components and the specific cell growth rate were decreased.

It is generally agreed that osmotic stress inhibits biomass growth, but enhances secondary metabolite biosynthesis in plant cell culture. Cellular response to osmotic stress is very complicated, so the actual mechanisms involved in the response are not fully understood. However, there is direct evidence showing that plant cells in suspension culture under stress show an increase in hydrogen peroxide synthesis, enzymatic reactions and anthocyanin biosynthesis (Dörnenburg and Knorr, 1995).

This investigation was aimed at the effect of osmotic stress on cell growth and saponin biosynthesis.

6.2 Methodology

In the first set of experiments, the osmoticum, sorbitol, was added to the fresh culture medium initially at two different concentrations (0.1 M and 0.3 M respectively) to achieve different osmotic conditions. Samples were harvested every two days. In the second set of experiments, osmotica, such as sodium chloride and sorbitol, together with sucrose (15 g/l), were fed into the culture flasks on day 14 to make the total culture volume up to 30 ml. The six groups of components fed into the culture are shown in Table 6-1. Sucrose was added to all the feeding groups because the increase in medium sucrose concentration has two fundamentally different effects on the physiology of plant suspension cell cultures. One effect is the physical change in the cellular environment by the increase of osmotic pressure, and the other effect is the increase of available carbohydrate source as biochemical substrate to maintain cell life and to support cell growth.

Table 6-1. The components fed into the culture flasks (on day 14) and the resultant medium osmolality in the second set of experiments.

Group	Feeding solution[a]	Osmolality (mmol/kg)	
		Feeding solution	In culture
6	None (control)[b]	114	47
5	MS + Suc. (15 g/l)	87	136
4	MS + Suc. (15 g/l) + sorbitol 0.4 M	332	391
3	MS + Suc. (15 g/l) + sorbitol 0.2 M	210	270
2	MS + Suc. (15 g/l) + NaCl 0.3 M	419	475
1	MS + Suc. (15 g/l) + NaCl 0.1 M	198	250

a. MS = MS basal salts

b. Measured with fresh medium

6.3 Results and discussion

6.3.1 Addition of sorbitol into fresh medium

The osmotic pressure of fresh culture media was mainly affected by the concentration of sucrose and mannitol, reached maximum at day 4 to day 6 (Figure 6-1), and then decreased gradually. The early increase in osmotic pressure was mainly due to the increase of reducing sugars, glucose and fructose, in the medium. Previous study on the basic growth characteristics has shown that glucose and fructose, which were not added to the medium, were detected in the medium (Figure 4-5).

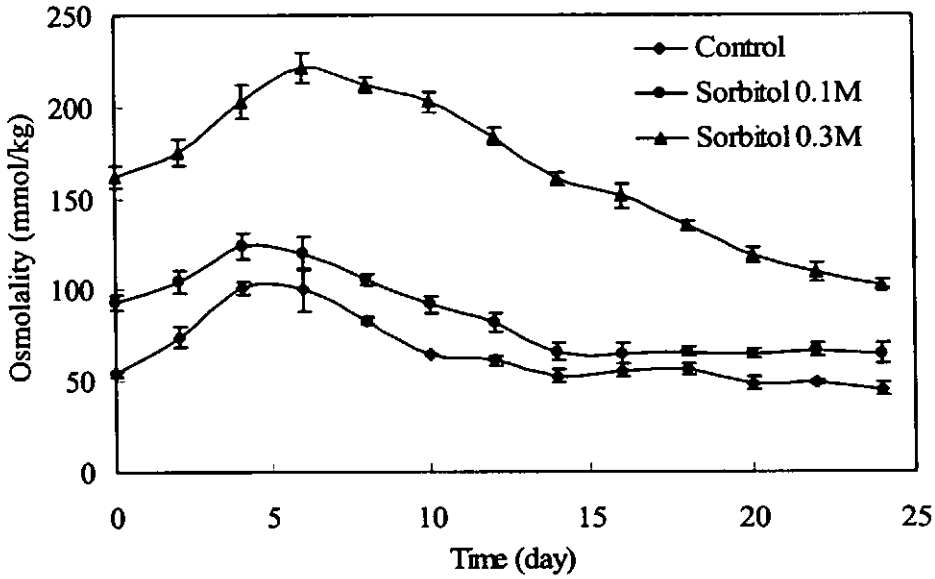

Figure 6-1. Time profile of osmotic pressure in the culture filtrate.

The ginseng cell growth in all the three culture media was similar in the first four days. Then, the cells cultured in normal medium and medium with 0.1M sorbitol grew rapidly until day 10 and day 12 respectively, while the growth in 0.3 M sorbitol medium was much repressed (Figure 6-2). Clearly, cells under severe osmotic stress (0.3 M sorbitol) showed delayed and reduced growth. Microscopic observation showed that the cell size was reduced under high osmotic stress. This is reflected from the ratio of dry cell weight to fresh cell weight which depends on the water holding capacity of the cells (Figure 6-3). Cells cultured under high osmotic stress had relatively small size, low fresh cell weight and thus high dw/fw. The saponin content of cells was not significantly affected by the addition of 0.1 M sorbitol, but increased notably by 0.3 M sorbitol (Figure 6-4).

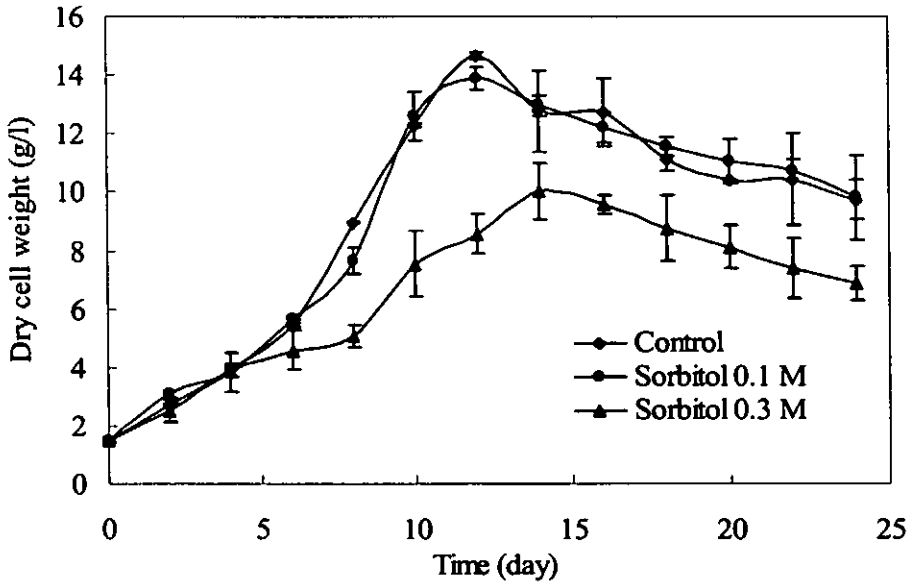

Figure 6-2. Time profile of dry cell weight under different osmotic conditions.

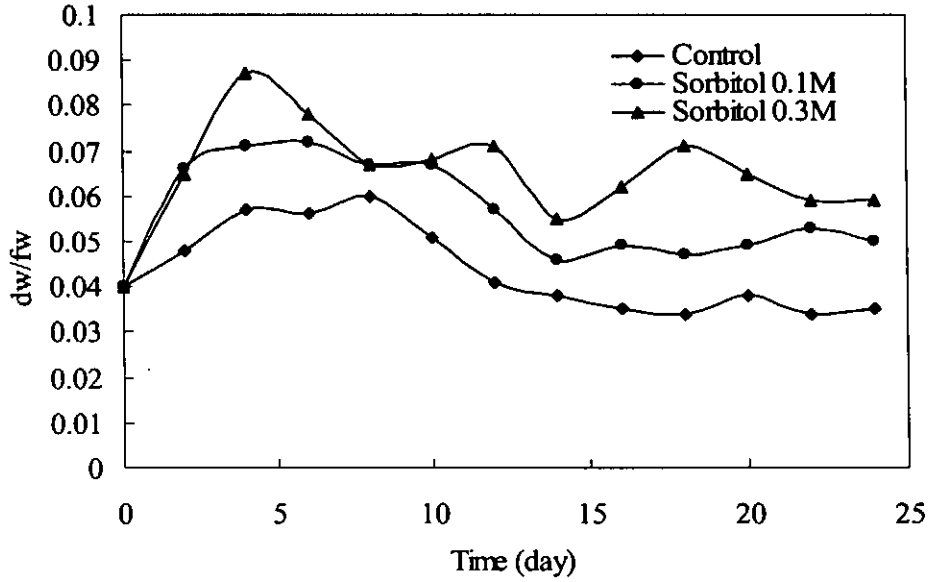

Figure 6-3. Time profile of dry cell weight to fresh cell weight ratio under different osmotic conditions.

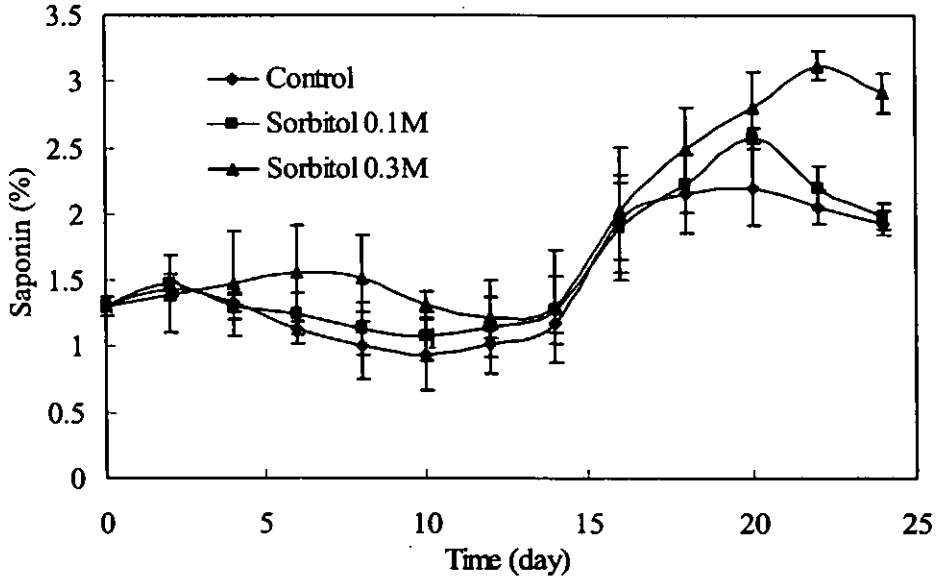

Figure 6-4. Time profile of saponin content of cells under different osmotic conditions.

6.3.2 Feeding nutrients and osmotica in early stationary phase

Figure 6-5 shows the cell growth profile in terms of dry cell weight with sucrose and osmotica fed in the early stationary phase. The maximum biomass was obtained by feeding sucrose (15 g/l) but without the osmotica on day 18. The groups fed with 0.2 M sorbitol and 15 g/l sucrose showed a steady cell concentration over a long period time (around 10 days) although the maximum biomass concentration was slightly lower than that by feeding sucrose alone. The dry cell weight obtained from the groups fed with 0.4 M sorbitol and 0.1 M sodium chloride decreased more rapidly. The feeding of 0.3 M NaCl resulted in a sharp drop in the cell concentration. The difference in the osmotic pressure of the medium with 0.1 M NaCl and that with 0.2 M sorbitol fed is small, 250 mmol/kg versus 270 mmol/kg respectively. However, the cell growth was inhibited by 0.1 M NaCl but not by 0.2 M sorbitol. Therefore, the strong inhibitory effect of NaCl

should not be attributed to the osmotic stress, but to the Na^+ and Cl^- ion toxicity at high concentration.

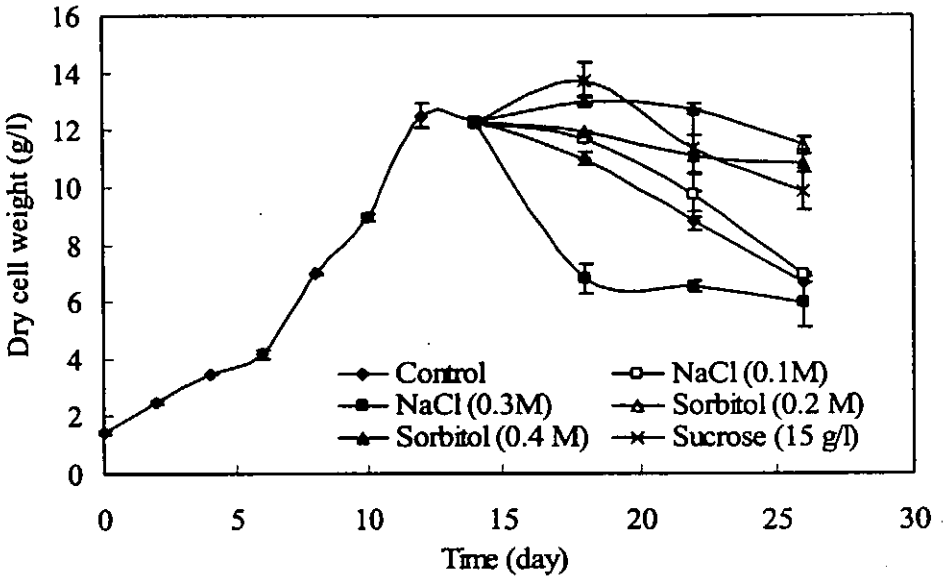

Figure 6-5. Time profile of dry cell weight of *Panax ginseng* cell cultured in medium with different concentrations of osmotica fed at day 14. Sucrose (15 g/l) was also fed, together with the osmotica, into culture medium.

Although osmotic stress caused growth repression, the cell growth could restore as the cells adapted to the osmotic stress of the surrounding environment. Gangopadhyay et al. (1997) reported that the loss of cell viability of the osmotic stress-adapted *Nitotiana tabacum* L. var. Jayasri callus was comparatively less than the unadapted callus even after shock-treatment with 1282 mM sodium chloride and 823 mM mannitol. Their results indicated that the effects of different osmotica on plant tissue varied with the stress-inducing-agents. Osmotic stress can be applied as a means to control the cell size and morphology, and maintain steady cell growth. The cells cultured in the medium with high osmotic stress had higher saponin content (Figure 6-6). The highest saponin content, 3.28%, was attained on day 18 from the group fed with 0.3 M NaCl. The culture fed with

0.4 M sorbitol attained nearly the same saponin content, 3.18%, on day 22. It seemed that the secondary metabolism related to saponin production was stimulated by the osmotica while the primary metabolism related to the biomass growth was inhibited.

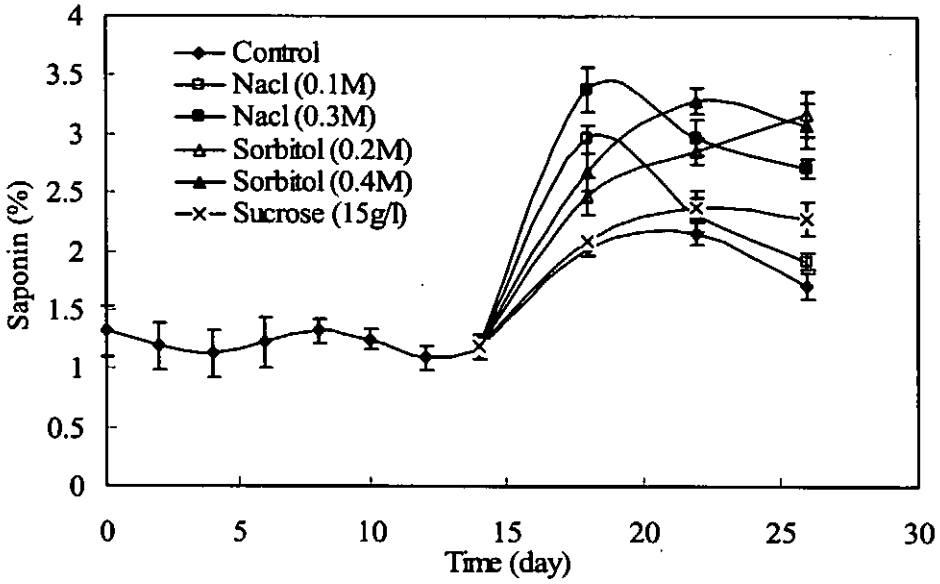

Figure 6-6 Time profile of saponin content of cells with various osmotica fed at day 14.

Although the cells cultured in the medium fed with 0.3 M NaCl had highest saponin content (3.28 %), the strong biomass growth inhibition led to lower total saponin yield (223.7 mg/l on day 18). The highest saponin yield was obtained as sorbitol was used as the osmoticum (0.2 M and 0.4 M) (Figure 6-7). As shown in Figure 6-5, the cell concentration was kept steady after the sorbitol was fed on day 14.

The cellular responses to the osmotic stress and elicitation will be discussed in Chapter 8.

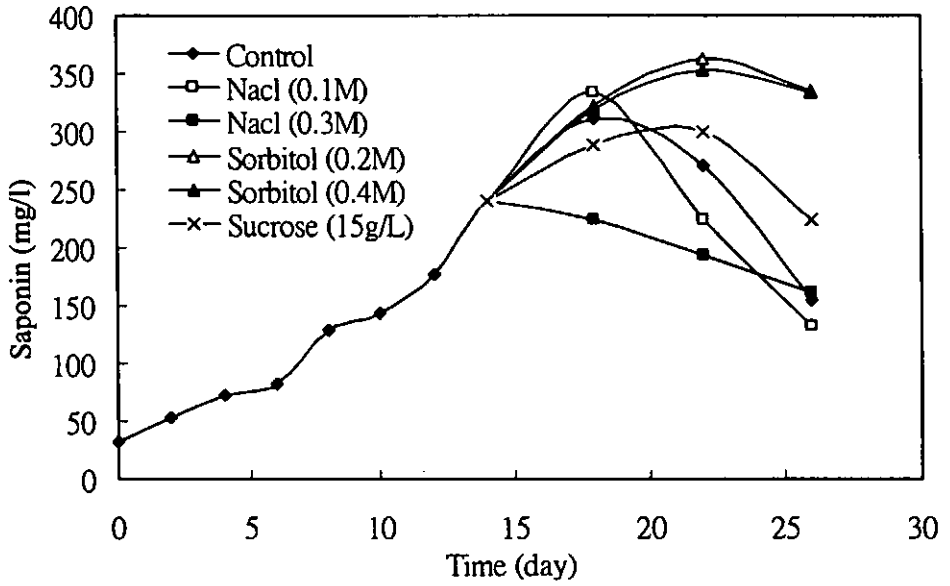

Figure 6-7. Time profile of saponin yield with different osmotica fed at day 14. Sucrose (15 g/l) was also fed, together with the osmotica, into culture medium.

CHAPTER 7 EFFECT OF ELICITORS ON GINSENG CELL GROWT AND SAPONIN BIOSYNTHESIS

7.1 Introduction

An elicitor may be defined as a substance which, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds. Thus, elicitation is the induced or enhanced biosynthesis of metabolites due to addition of trace amounts of elicitors. Elicitors are generally classified as abiotic or biotic. Abiotic elicitors are substances of non-biological origin, predominantly inorganic salts, and physical factors such as heavy metal salts, UV light, heat and high pH. Biotic elicitors are classified as exogenous or endogenous on the basis of their origin. Exogenous elicitors are derived from microorganisms or insects, e.g. chitosan and glycoproteins. Endogenous elicitors originate from plant structural compounds, e.g. lignin-like compounds, jasmonic acids, salicylic acid and pectinase (Radman et al., 2003).

The treatment of plants with elicitors can induce physiological and morphological defense responses in plants, e.g. under drought, exposure of UV-light, and high salinity environment. These responses have been considered as defense response of plants to ensure their survival, persistence and competitiveness. The induced defense responses include suicide of the attacked host cells (hypersensitive response), the production of antimicrobial secondary metabolites called phytoalexins – low molecular mass antimicrobial secondary metabolites.

Cultured plant cells are in principle totipotent and, therefore, any product present in the parent plant should also be synthesized in culture under the right incubation conditions. Thus, application of elicitor can be a way to induce secondary metabolite biosynthesis. Exposure of plant cell cultures to biotic elicitors or abiotic elicitors frequently induces the synthesis of secondary metabolites in plants (Benhamou, 1996). Treatments of plant cell cultures with abiotic elicitors have been widely reported. $AgNO_3$ increased the intracellular accumulation of scopolamine and hyoscyamine in the hairy root cultures of *Brugmansia candida* (Pitta-Albarez et al., 2000). Gontier et al. (1994) found that the addition of $CaCl_2$ increased approximately ten times the intracellular content of scopolamine and hyoscyamine in a suspension culture of *Datura innoxia*.

Jasmonic acid and its derivatives are considered to be involved in a part of the signal transduction pathway that induces particular enzymes catalyzing biochemical reactions to form defense compounds of low molecular weight in *P. ginseng*, such as polyphenols, alkaloids, quinones, terpenoids, and polypeptides (Kushiro et al., 1997; Lee et al., 1995). Hahn et al. (2003) used five kinds of jasmonate for elicitation of ginsenosides biosynthesis in adventitious root cultures of *P. ginseng* and found that the contents increased in all treatments regardless of concentrations, but the fresh weight, dry weight and growth ratio of the roots decreased with increasing jasmonate treatments. Apart from ginseng cell culture, exogenously applied jasmonates have provoked profound effects on accumulation of paclitaxel in *Taxus* cell suspension culture (Yukimune et al., 1996), rosmarinic acid in *Coleus blumei* cultures (Szabo et al., 1999), and anthocyanins in strawberry (Miyanaga et al., 2000). Yeast extracts have also been used to induce secondary metabolism biosynthesis. Yeast extract had slightly positive effect on biomass,

and significantly increased the intracellular content of both scopolamine and hyoscyamine in the hairy root cultures of *Brugmansia candida* (Pitta-Albarez et al. 2000). Application of an yeast elicitor resulted in an overall increase in cryptotanshinone production whereas the constituent level of rosmarinic acid was reduced in the culture of the *Agrobacterium tumefaciens* C58 transformed *Sal6ia miltiorrhiza* cells (Chen and Chen, 2000).

Lu et al. (2001) tested the effect of methyl jasmonate and yeast extract as elicitors on the cell growth and saponin production on *P. ginseng* cell culture. Their result showed that the culture treated with 3 g/l yeast extract on the day of inoculation caused saponin concentration at 20-fold greater than that in the control. The cultures treated with 500 μM methyl jasmonate on the day of inoculation caused maximum saponin production, 28-fold greater than that in the control. The addition of elicitors was not as significant an influence on cell growth as on saponin production. Dong and Zhong (2002) studied the effect of adding methyl jasmonate (100 μM), sucrose feeding (20 g/l), and the combination of both on the cell growth and taxane production in *Taxus chinensis* cell culture and found that the feeding of sucrose and methyl jasmonate at day 7 enhanced both the taxuynnanine C production and productivity.

The two elicitors, methyl jasmonate and yeast extract, were used in this experiment to explore the effect of elicitation on *P. ginseng* cell culture. Methyl jasmonate was used because it have been widely used for elicitation purpose and thus its effect has been well documented. Yeast extract is cheap and easy to prepare and use.

7.2 Methodology

Previous studies showed that addition of elicitors to the suspension culture at the late exponential growth phase could induce secondary metabolite biosynthesis. Our investigation on physical elicitor, osmotic stress, also revealed that application of elicitors in that period could enhance saponin production. Moreover, application of physical elicitor together with sucrose could promote not only cell growth but also saponin production.

In this investigation, ginseng cells were cultured in normal MS medium at first. Elicitors, either methyl jasmonate or yeast extract, were added to the suspension at day 10, the late exponential growth phase. Nutrients, sucrose and casein hydrolysate, were also fed into the suspension at the same time when necessary. Elicitors were added at the late exponential growth phase in order to let the cultures attain enough cell biomass. Cells were harvested at day 20 and day 24. Effect of abiotic elicitor feeding, i.e. sorbitol, and biotic elicitor feeding, i.e. methyl jasmonate and yeast extract, was also compared. The elicitor and nutrient feeding concentrations were summarized at Table 7-1.

Yeast extract was dissolved in deionized water and the pH was adjusted to 5.8 before autoclaving. Methyl jasmonate in alcohol was diluted with pure alcohol to desirable concentrations.

Table 7-1. Group of the elicitors and nutrients in feeding experiments.

Group	Components
1	Control
2	Sucrose (Suc) (30 g/l) + Casein Hydrolysate (CH) (500 mg/l)
3	Suc (30 g/l) + CH (500 mg/l) + Sorbitol (Sor) (0.2M)
4	Methyl jasmonate (MJ) 120 uM
5	Yeast extract (YE) (1g/l)
6	MJ + Group 2
7	YE + Group 2
8	MJ + Group 3

7.3 Results and discussion

7.3.1 Effect on cell growth and saponin biosynthesis

Cell growth

The effect of different culture conditions on *P. ginseng* cells is summarized in Figure 7-1. Cultures fed with elicitors, i.e. methyl jasmonate and yeast extract, only showed slightly decrease in dry cell weight as compared to the control group. This revealed that feeding of elicitors at the late exponential growth phase had some inhibition effect on cell growth. It has also been reported that addition elicitors to cell cultures may inhibit biomass accumulation because elicitation usually switches primary metabolism to secondary metabolism (Zhang et al., 2000; Dong and Zhong, 2002). Lu et al. (2001) also reported

that cell growth was decreased slightly when YE was added 5-15 days after inoculation while cell growth was less influenced by MJ. The result was different from that cell biomass was increased with the addition of elicitors because of the deposition of new materials on the cell wall as a barrier to the invading agent (Robertson et al., 1995; Rijhwani and Shanks, 1998).

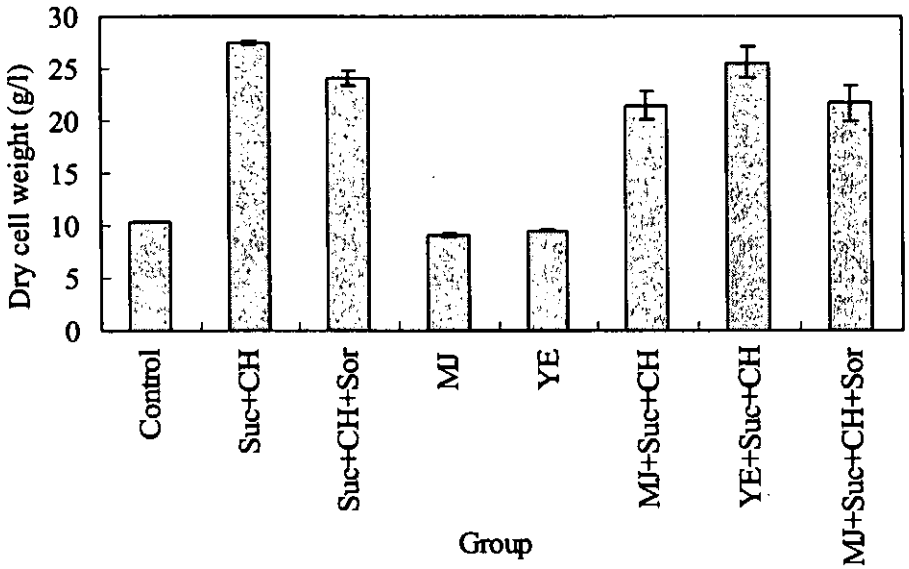

Figure 7-1. Effect of different nutrients fed on cell growth.

For these nutrient feeding groups, cell biomass increased rapidly after feeding. The dry cell weight was 100 % higher than that of control group. The culture fed with nutrients and 0.2 M sorbitol had lower dry cell weight than the culture fed with nutrients only. This result was also seen in previous studies. Under the same nutrient feeding condition, addition of yeast extract (1 g/l) had less inhibition effect on the cell growth than that of MJ (120 μM). As comparing the group fed with MJ plus nutrients and the one fed with 0.2 M sorbitol additionally, their dry cell weights were similar, which indicate that the major inhibition effect came from MJ (120 μM) rather than sorbitol (0.2 M). In fact, the dry cell weight of the culture fed with nutrients and 0.2 M sorbitol was about 3 g/l higher

than that of the culture fed together with MJ. Thus, it might be that the concentration of MJ was too high that caused inhibition to the cell growth. This result was different from Lu et al. (2001)'s that MJ at 500 μM had less significant effect on *P. ginseng* cell growth. On the whole, feeding of either abiotic or biotic elicitors or both would decrease primary metabolism and result in inhibition of cell growth.

Saponin production

The experimental results showed that biotic elicitors were more effective than abiotic elicitors in inducing secondary metabolism, saponin synthesis in this case, in *P. ginseng* cell culture (Figure 7-2). The cultures fed with nutrients and nutrients plus 0.2 M sorbitol had saponin content about 50% higher than that of the control. But, the groups fed with biotic elicitors had much higher saponin content (up to 7 fold) than the control group and the group fed with sorbitol plus nutrients. There was 10 folds increase in the saponin yield in the cultures fed with nutrients and elicitors as compared to the control (Figure 7-3). The degree of enhancement was not as high as that reported by Lu et al. (2001). Their results showed that feeding of 3 g/l yeast extract on the day of inoculation (day 0) caused the increase in saponin concentration by 20-fold while feeding of 500 μM MJ resulting in saponin content 28-fold greater than control. The elicitor concentrations used in this experiment was limited to a specific one and fed at the late exponential phase, so the result could not be compared with Lu et al.'s. The difference in the effect on saponin concentrations might be due to the difference in method of saponin analysis. Lu et at. (2001) used HPLC method to analyze the individual ginsenosides in the cells while we used TLC to measure the total saponin. The analysis may also be affected by the rather

exhausting extraction and sample purification procedure. So, it may be not reliable to compare the saponin contents of ginseng reported from different laboratories (Wu and Zhong, 1999). On the whole, both investigations showed that saponin biosynthesis were much enhanced by the elicitors applied.

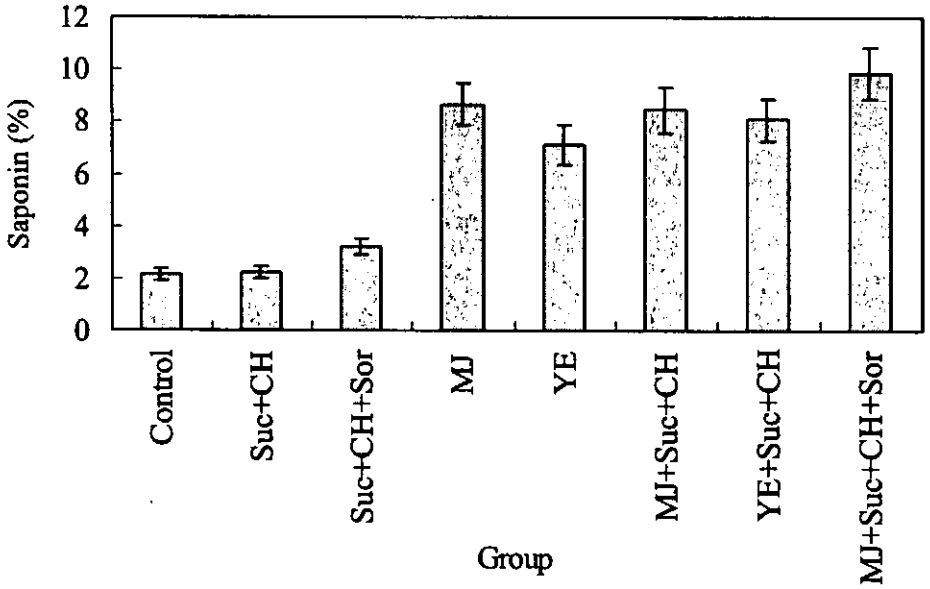

Figure 7-2. Effect of different nutrients fed on saponin content.

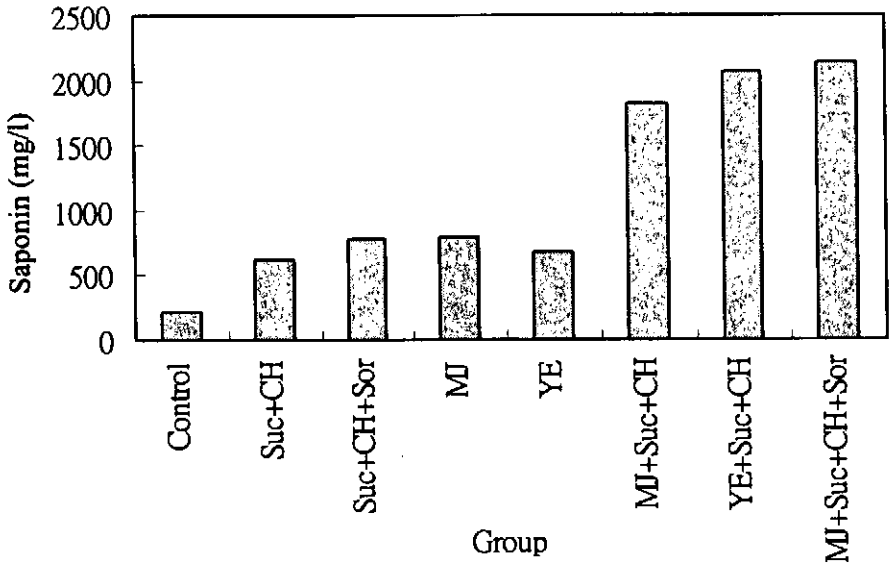

Figure 7-3. Effect of different culture conditions on saponin yield.

The difference in the effect of elicitation on saponin biosynthesis between abiotic and biotic elicitors showed that they might have different inducing mechanism on biosynthesis. It has been reported that JA or its methyl ester MJ is a signal transducer and thus play an important role in genetic regulation and induction of defense system in cells (Sudha and Ravishankar, 2002). Higher concentrations of jasmonic acid would cause growth inhibition and even cell death. Addition of yeast extract to the cell cultures of *Orthosiphon aristatus* enhanced the activities of phenylalanine ammonia lyase and tyrosine aminotransferase which are the key enzymes of rosmarinic acid biosynthesis (Sumaryono et al., 1991). Thus, dosage of jasmonic acid and yeast extract application is an important factor for the cell growth and secondary metabolism (Rijhwani and Shanks, 1998). Our result revealed that 120 μM MJ would have minor inhibition effect on *P. ginseng* cell growth. Similar result was also observed at 1 g/l yeast extract. The inhibition

effect of elicitors on primary metabolism would on the other hand enhance secondary metabolism, and thus caused high saponin production.

Clearly, biotic elicitors were more effective in inducing saponin biosynthesis than osmotic stress. Moreover, these elicitors had minor inhibition to cell growth. It might be due to that biotic elicitors can directly act on the cell wall components of the cells and induce high profile signal transduction for secondary metabolism. Apart from elicitor dosage, exposure time is also an important factor to determine the effect of elicitor on saponin levels. Thus, further experiment should be carried out to study the dosage and time of exposure.

CHAPTER 8 RESPONSE TO ELICITATION

8.1 Introduction

It is well known that plants have developed an array of defense strategies that allow them to resist microbial infections and other physiological and environmental perturbations. These defense mechanisms include the production of active oxygen species, e.g. superoxide and hydrogen peroxide, the synthesis of antimicrobial phytoalexins, the induction of hydrolytic enzymes and the construction of defensive barriers to fortify the cell wall against pathogen aggression, and the hypersensitive response. Among them, production of antimicrobial phytoalexins (secondary metabolites) received highest attention because these chemicals have great benefit to human beings (Murphy and Huerta, 1990; Cazalé et al., 1998; Sharan et al., 1998; Yuan et al., 2001).

One of the earliest events during the hypersensitive response to elicitation is the production of reactive oxygen intermediates, including the superoxide anion and hydrogen peroxide (H_2O_2) through the oxidative burst (OB). Transient induction of hydrogen peroxide at the cell surface was initiated within 2 to 3 minutes of addition of elicitors to soybean cell suspension culture. It is believed that because of its speed of appearance, the oxidative activity may serve as a first line of defense against the invading pathogen (Apostol et al., 1989). The induction of H_2O_2 production in plant culture system by abiotic elicitation, e.g. change of osmotic pressure and mechanical disturbance (Yahraus et al., 1995) and biotic elicitation, e.g. addition of biotic elicitors to the culture system (Apostol et al., 1989; Levine et al., 1994), has been widely reported.

Wu and Lin (2002) reported that H_2O_2 production was induced and ginsenosdie biosynthesis was enhanced after exposure of *P. ginseng* cell suspension culture to low-energy ultrasound. Yahraus et al. (1995) reported that dilution of the medium with water or resuspension of cells in sucrose solutions of reduced osmolarity caused an oxidative burst similar to those stimulated by chemical elicitors. However, the effect was not observed under hyperosmotic conditions. H_2O_2 production from the oxidative burst after application of biotic elicitors was reported in the plant cell suspension cultures (Apostol et al., 1989; Levine et al., 1994). Indirect evidence to the role of H_2O_2 to secondary metabolites production has been reported by Apostol et al. (1989) which showed that direct application of H_2O_2 to the soybean culture system stimulated a significant production of phytoalexins. Similar result was also reported at fungal elicitor induced oxidative burst and alkaloid biosynthesis in *C. roseus* suspension cultures.

Hydrogen peroxide is known to serve as a second messenger in the stress signal cascades of plant cells to activate a more diverse set of stress responses, e.g. cross-linking of cell wall proteins (Cazalé et al., 1998), the induction of defense-related genes, stimulation of phytoalexin biosynthesis and promotion of the hypersensitive response (Low and Merida, 1996; Fellbrich et al., 2000; Yuan et al., 2001). Thus, there is a close relationship between hydrogen peroxide overproduction and secondary metabolite biosynthesis. Measurement of hydrogen peroxide can be an indication of the defense response of cells under stimulation. The production of H_2O_2 during the elicitation process can be demonstrated by detecting the fading of fluorescence emitted by pyranine owing the bleaching activity (Apostol et al., 1989; Yahraus et al., 1995).

Another effect of elicitors on the suspension cell culture is the induction of enzymatic activities. The phytoalexin defense response involves the rapid transcriptional activation of genes encoding a number of phytoalexin biosynthetic enzymes, including phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS). The elicitor induces the plant cell to begin *de novo* synthesis of mRNA coding for enzymes involved in the formation of these antibiotics (Dixon and Hamson, 1991; Gowri et al., 1991).

In plants, phenylalanine ammonia-lyase (PAL) plays a key role in linking primary metabolism to phenylpropanoid metabolism by converting L-phenylalanine to *trans*-cinnamic acid. This reaction is considered to be a key step in the phenylpropanoid pathway (Hahlbrock and Scheel, 1989) because it provides an entry point for the biosynthesis of a large number of products derived from the phenylpropane skeleton. These products include lignins and isoflavonoid phytoalexins, both of which are involved in plant defense reactions. Thus, PAL is regarded as a plant cell defense marker due to its capacity of being activated by microbial elicitation.

This study would focus on the production of hydrogen peroxide that is believed to have direct antimicrobial effect and act as a secondary messenger in the transcriptional activation of defense genes, and induction of PAL activity after application of elicitors *P. ginseng* cell culture systems.

8.2 Methodology

Details of the methodology about the preparation of *P. ginseng* cell cultures for this

experiment have been described in Chapter 7.

In this work, hydrogen peroxide produced during the oxidative burst by *P. ginseng* cells and released into the culture medium was detected by oxidative quenching of fluorescent pyranine as described by Yahraus et al. (1995).

8.3 Results and discussion

Oxidative burst

The fluorescence intensity of the ginseng cell suspension cultures supplemented with fluorescing pyranine declined to a certain degree within a two hour period (Figure 8-1). The fluorescence intensity also decreased gradually in the control cultures which implicated that oxidative species also generated by the cells in control culture. The mixing of cell suspension with fresh medium during the preparation of the cell culture for measurement of fluorescence and the cultivation of the cells in the shaker created hyperosmotic stress and environmental disturbance to the cells that finally induced OB.

However, the degree of fluorescence quenching activity in the control culture was much lower than that in the cultures fed with the elicitors. Fluorescence intensity decreased rapidly as the culture was fed with nutrients and 0.2 M sorbitol. This result showed that the hypersensitive stress created by metabolic inert carbohydrate, sorbitol, can also induce OB in the *P. ginseng* suspension culture. This result was different from that reported in other plant cell suspension cultures which showed that hyperosmotic stress

did not induce OB (Yahraus et al., 1995) or just induced weak H_2O_2 production (Cazalé et al., 1998).

In the cultures fed with MJ alone, MJ plus nutrients or MJ plus nutrients and sorbitol, the degree of fluorescence quenching was not distinct. Thus, feeding of sorbitol together with MJ did not have additive effect on the oxidative burst. YE (1 g/l) was the most effective elicitors among those under study in inducing OB. Fluorescence intensity nearly dropped to zero during the first half hour. The situation had no difference when YE was fed alone or together with nutrients. Since the extinction of fluorescence intensity was less obvious in control cultures as compared to the other cultures, the OB reaction may be attributed mainly to the cells stimulated by different agents. It can be said that feeding nutrients, osmotica and chemical elicitors could induce OB in suspension of cells.

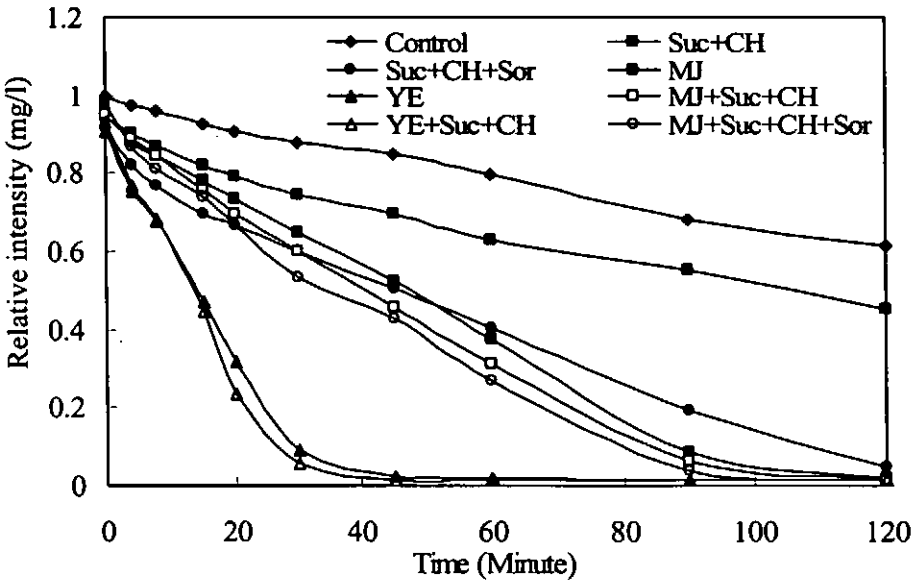

Figure 8-1. The decrease of fluorescence due to oxidative quenching of pyranine by hydrogen peroxide released from *P. ginseng* cells under different culture conditions.

It is noted through this experiment that H_2O_2 overproduction was induced just after addition of elicitors. However, the effect of elicitors on secondary metabolite biosynthesis was seen at several days later, i.e. stationary growth phase. Thus, it may be said that overproduction of H_2O_2 is just an initial response of cascade of induced secondary metabolic biosynthesis. Moreover, high H_2O_2 was not in direct proportion to the high secondary metabolite biosynthesis. As it can be seen that yeast extract (1 g/l) caused the highest H_2O_2 overproduction but not the highest saponin content (Figure 7-2 and Figure 8-2).

Phenylalanine ammonia-lyase (PAL) activity

PAL plays an important role in linking primary metabolism to pheynlpropanoid metabolism by converting L-phenylalanine to *trans*-cinnamic acid. The reaction is considered to be a key step in the phenylpropanoid pathway as it provides an entry point for the biosynthesis of a large number of products derived from the phenylpropane skeleton (Li et al., 2001).

Our investigation showed that the feeding of nutrients, sorbitol, YE and MJ to the ginseng cell cultures induced the intracellular enzymatic activities of PAL (Figure 8-5). PAL activity in the control culture only increased slightly. Much higher PAL activity was observed in the cultures fed with sorbitol. This indicated that the sudden change in osmotic stress could stimulate PAL enzymatic response. For the cultures fed only with elicitors, MJ caused more than one fold increase in PAL activity as compared to the control while YE only caused 40 % increase in the PAL activity. The high PAL activity

was observed in cultures fed with MJ plus nutrients, and MJ plus nutrients and sorbitol. PAL activity in these cultures was enhanced by 3 fold as compared to that of the control. The highest PAL activity was detected at four days after feeding. The increase in PAL activity during the culture processes means that the cell metabolism shifted from primary to secondary metabolism, which contribute to cellular defense against stresses. Previous studies also revealed that treatment of tobacco-suspended cells with MJ or elicitor derived from *F. solani* led to a transient increase in PAL activity (Sharan et al., 1998).

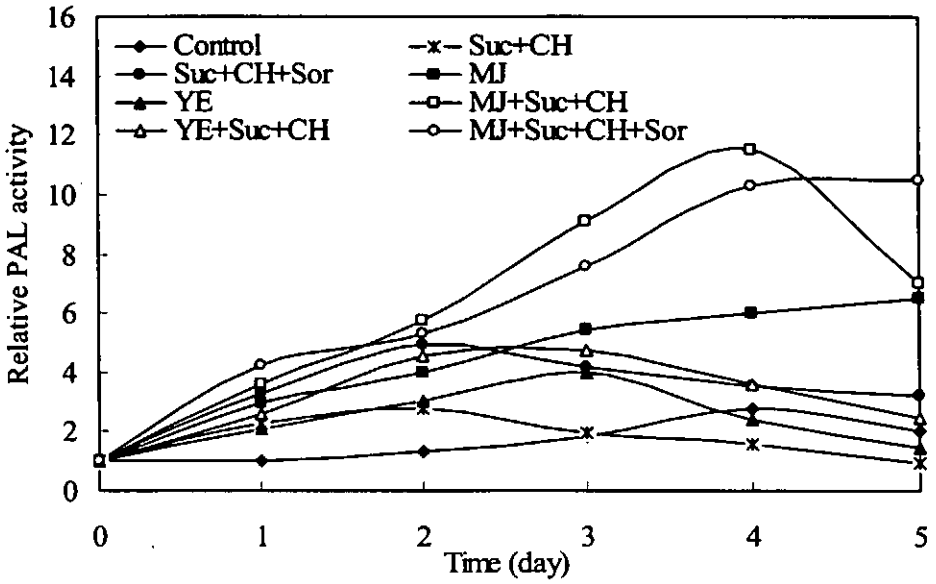

Figure 8-2. The stimulation of PAL activity of cells by nutrients, osmoticum and elicitors.

In conclusion, the study showed that osmotica and chemical elicitors can enhance saponin biosynthesis in *P. ginseng* cell culture. The effect of these stimuli on cell cultures could be reflected by the increased activity of OB and PAL. There was no quantitative

correlation between OB, PAL activity and saponin biosynthesis, although the increase in OB and PAL activity always accompanied with the increase in saponin biosynthesis.

CHAPTER 9 OPTIMIZATION OF FEEDING STRATEGY BY STATISTICALLY-DESIGNED EXPERIMENTS

9.1 Introduction

The traditional approach to the optimization problem is the one-variable-at-a-time method. In this process, all variables but one are held constant and the optimum level of this variable is determined, and the optimum level of each variable is determined step by step. This method works only if there is no interaction between the variables. However, because of the complexity of plant metabolism, interaction between the variables is inevitable, especially when many variables need to be optimized. Thus, use of the one-variable-at-a-time method cannot find the true optimum. For effective and comprehensive evaluation and optimization of process variables, statistical methods for experimental design and analysis are most useful (Box et al., 1978).

Statistical experimental design has been used to optimize the cell culture process, e.g. optimize of medium constitutes and fermentation conditions, and optimize fed-batch productions. Sunitha et al. (1998) applied the central composite experimental design to optimize medium constitutes, such as glucose, urea and biotin, and the conditions of fermentation, e.g. temperature, pH, and the time of fermentation, of the coimmobilized whole cells suspension culture of *Micrococcus glutamicus* and *Pseudomonas reptilivora*. A higher yield of L-glutamic acid was obtained. Toivonen and Rosenqvist (1995) studied the effect of phosphate, ammonia, nitrate and ferric-EDTA concentrations of culture

medium on the growth and phenolics production of the plant hairy root cultures of *Glycyrrhiza glabra*. By employing statistical experiment design and linear regression analysis, an improved B5 medium was developed. The dry root weight obtained from the improved medium was doubled compared to that on the standard B5 medium. The production of total phenolic substances was also higher in the improved medium. Tuominen et al. (1988) used a statistical (Box-Wilson) experimental design to study the effect of different nutrients on the growth, differentiation, and cardenolide production of *Digitalis lanata* tissue culture. It was found that the concentration of maltose and NO_3^- NH_4^+ ratio were significant variables for both growth and cardenolide production.

9.2. Objectives

The objectives of this study are to apply statistically-designed experiments to test the effect of nutrient feeding and osmotic stress on biomass and saponin production in *P. ginseng* cell cultures, and to find the optimal feeding scheme and level of osmotic stress for higher biomass and saponin yields.

9.3. Methodology

Based on our previous studies (Chapters 5-7), sucrose (suc.) and casein hydrolysate (CH) were chosen as the feeding nutrients, and sorbitol as the osmotica (Table 9-1). The concentration of nutrients and sorbitol, and the feeding time were the experimental variables, which were all evaluated at three levels. The levels of these variables were selected based on our previous experiments. In order to test the effects of all the variables

and combinations, a 2^3 factorial design was used to set up the experimental groups (Table 9-2). The culture characteristics to be measured in response to the change of the experimental variables included the biomass productivity (Y_{BP}) and the saponin content of cell (Y_{sp}). The experimental results were fitted to a second order polynomial,

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_2 + b_5X_1X_3 + b_6X_2X_3 + \\ + b_7X_1^2 + b_8X_2^2 + b_9X_3^2 \quad (1)$$

Table 9-1. Variables and their limits used in the experimental plan.

Factor	Nutrient (X_1)	Sorbitol (M) (X_2)	Feeding time (day) (X_3)
Lower level (-1)	Suc. (15 g/l) + CH (250 mg/l)	0.1 M	8
Base level (0)	Suc. (30 g/l) + CH (500 mg/l)	0.2 M	12
High level (1)	Suc. (45 g/l) + CH (750 mg/l)	0.3 M	16

The above equation was solved by using STATISTICA to estimate the response of the dependent variables. The equation was optimized by nonlinear regression analysis to get the optimum value. The contour plot was also drawn by using STATISTICA. An idea of the approximate optimal range of the factors was obtained from the plot.

9.4. Results and discussion

Table 9-2 shows that the biomass productivity and saponin contents of cell measured over a culture period during which the maximum biomass and saponin were observed. To

avoid the bias in taking the maximum cell concentration and saponin content, the values obtained on three different days were averaged, and the average biomass productivity and saponin content were used in fitting the polynomial models.

Table 9-2. The observed and predicted biomass productivity and saponin content obtained from the statistically-designed experiments.

Group	Code level			Average biomass productivity (Y_{BP}) (g/l-d)		Average saponin content (Y_{sap}) (%)	
	X_1	X_2	X_3	Observed value	Predicted value	Observed value	Predicted value
1	-1	-1	-1	1.45 ± 0.49	1.54	2.49 ± 0.43	2.34
2	1	-1	-1	1.64 ± 0.34	1.54	2.17 ± 0.33	2.32
3	-1	1	-1	1.34 ± 0.29	1.29	2.94 ±0.44	2.93
4	1	1	-1	1.25 ± 0.17	1.29	3.71 ± 0.87	3.54
5	-1	-1	1	0.60 ± 0.04	0.67	2.19 ± 0.33	2.34
6	1	-1	1	0.77±0.09	0.67	2.47 ± 0.51	2.32
7	-1	1	1	0.72 ± 0.11	0.74	2.91 ± 0.46	2.93
8	1	1	1	0.78 ± 0.05	0.74	3.36 ± 0.68	3.54
9	0	0	0	1.41 ± 0.10	1.38	2.85 ± 0.28	2.83
10	0	0	0	1.35 ± 0.27	1.38	2.81 ± 0.31	2.83
11	0	0	0	1.39 ± 0.27	1.38	2.83 ± 0.30	2.83

* Note: Average biomass productivity and average saponin content were obtained by taking the average of three days' measurements. The predicted biomass productivity and saponin content were calculated by using equation 2-3 and 3-4 respectively.

9.4.1 Average biomass productivity

The constants in the mathematical models describing the effect of the selected variables X on the average biomass productivity were derived from the experimental results with regression analysis,

$$Y_{BP} = \underset{(b_0)}{0.791} + \underset{(b_1)}{0.048X_1} - \underset{(b_2)}{1.803X_2} + \underset{(b_3)}{0.964X_3} - \underset{(b_4)}{0.0325X_1X_2} + \underset{(b_5)}{0.0003X_1X_3} + \underset{(b_6)}{0.197X_2X_3} -$$

$$\underset{(b_7)}{0.0007X_1^2} - \underset{(b_8)}{1.119X_2^2} - \underset{(b_9)}{0.009654X_3^2} \qquad (2\text{-}1)$$

$R^2 = 0.996$ and confidence level 95%.

The fitting of the model to the data is good ($R^2 = 0.996$). The model equation can be simplified by deleting the insignificant terms. The interaction term between nutrient concentration (X_1) and feeding time (X_3) is very small, $b_5 = 0.0003$. Another negligible term is the second order effect of nutrient concentration (X_1), b_7 (=0.0007). After neglecting these two terms, the regression analysis of the data gives

$$Y_{1pr} = \underset{(b_0)}{198.51} + \underset{(b_1)}{0.00925X_1} + \underset{(b_2)}{1587.1X_2} - \underset{(b_3)}{59.24X_3} - \underset{(b_4)}{0.0325X_1X_2} + \underset{(b_6)}{0.197X_2X_3} - \underset{(b_8)}{3972.37X_2^2}$$

$$+ \underset{(b_9)}{2.46X_3^2} \qquad (2\text{-}2)$$

$R^2 = 0.994$ and confidence level 95%.

Equation 2-2 shows that nutrient concentration (X_1), interaction between nutrient concentration (X_1), and sorbitol concentration (X_2), and square of sorbitol concentration (X_2^2), i.e. b_1, b_4 and b_7, may be considered of less significance. Omitting these terms, regression analysis of the data gives

$$Y_{BP} = \underset{(b_0)}{0.17083} \underset{(b_2)}{-2.825X_2} \underset{(b_3)}{+0.3447X_3} \underset{(b_6)}{+0.1969X_2X_3} \underset{(b_9)}{-0.0197X_3^2} \qquad (2\text{-}3)$$

$R^2 = 0.969$ and confidence level 95%.

The term X_1, nutrient concentration, is absent from equation 2-3. This means that X_1 is not a significant factor affecting the biomass productivity, and even lower level of nutrient feeding is sufficient for the culture.

The predicted average biomass productivity using the above equation is also shown in Table 9-2, along with the experimental data. The predicted and experimental values are also plotted, in order to show the goodness of fit of the model (Figure 9-1). The table and graph both indicate that the predicted values are close to the experimental results.

The closer the value of R (correlation coefficient) to 1, the better is the correlation between the observed and predicted values. Now, R in this case is equal to 0.984, showing a good agreement between the experimental and predicted values of biomass productivity. The coefficient of determination (R^2) is a measure of the goodness of fit of the regression surface. In this case, R^2 is 0.969 which indicates that nearly 97 % of the observed values of biomass productivity can be expressed by the regression model.

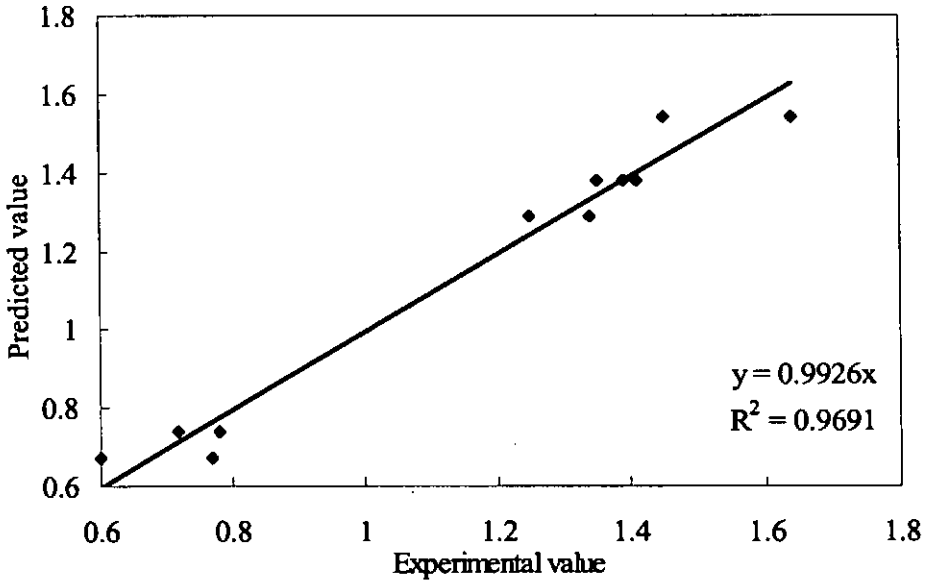

Figure 9-1. A plot of predicted values against experimental values of average biomass productivity (g/l-day).

The effect of sorbitol concentration and feeding time on the biomass productivity is represented by the contour plot in Figure 9-2. The highest average biomass productivity is always reached by feeding 0.1 M or less sorbitol between day 8 to day 10. After conducting the steepest ascent treatment, the global maximum of average biomass productivity is obtained at X_1 = 9.76 days and X_2 = 0 M. The zero value (0 M) of X_2 (sorbitol) means that we should use the least possible amount. It can be seen from the plot that high biomass productivity could be achieved without sorbitol feeding which usually imposes an osmotic stress to the cell growth. Under this circumstance, we have to make a compromise between sorbitol concentration and the feeding time. As for the feeding time, the days after day 12 had insignificant effect on biomass porductivity as the new fed nutrients at that stationary phase only extended the growth time and reduced the biomass productivity. The optimal conditions for maximum average biomass productivity are

sorbitol (X_2) at 0.1 M and feeding time at day 9.76, i.e. day 10. In this case, the biomass productivity is 1.64 g/l-d.

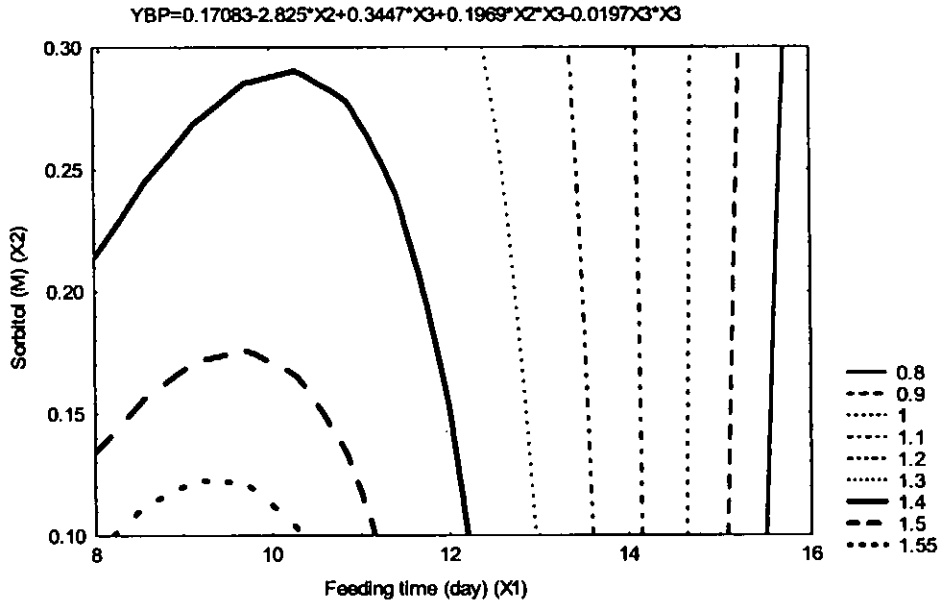

Figure 9-2. A contour plot of average biomass productivity: the effect of feeding time and sorbitol concentrations.

9.4.2 Saponin content

The mathematical models describing the effect of the selected variables X on the average saponin content can be derived from the experimental results (Table 9-2) by regression,

$$Y_{SP} = \underset{(b_0)}{1.174744} \underset{(b_1)}{-0.0532X_1} \underset{(b_2)}{+2.367826X_2} \underset{(b_3)}{+0.281702X_3} \underset{(b_4)}{+0.105X_1X_2} \underset{(b_5)}{+0.000583X_1X_3}$$

$$\underset{(b_6)}{-0.11875X_2X_3} \underset{(b_7)}{-0.000584X_1^2} \underset{(b_8)}{- 1.017853X_2^2} \underset{(b_9)}{- 0.01197X_3^2} \qquad (3\text{-}1)$$

$R^2 = 0.950$ and confidence level 95%.

The fitting of the model is quite good ($R^2 = 0.950$). The coefficients, b_5, b_7 and b_9, are negligibly small, so that the interaction between the nutrient concentration (X_1) and feeding time (X_3), the second order effect of nutrient concentrations (X_1^2), and the second order effect of feeding time (X_3^2) has insignificant effect on saponin biosynthesis. Now, the equation is reduced to,

$$\underset{(b_0)}{Y_{SP} = 1.9225} \underset{(b_1)}{-0.01117X_1} \underset{(b_2)}{+4.775X_2} \underset{(b_3)}{+0.011875X_3} \underset{(b_4)}{+0.105X_1X_2} \underset{(b_6)}{-0.11875X_2X_3} \underset{(b_8)}{-5X_2^2} \quad (3\text{-}2)$$

$R^2 = 0.946$ and confidence level 95%.

This polynomial equation 3-2 can give satisfactory prediction. However, in order to make the model simpler, the term X_3 can also be neglected without losing much of accuracy. With the elimination of X_3, the regression analysis gives,

$$\underset{(b_0)}{Y_2 = 2.065} \underset{(b_1)}{-0.01117X_1} \underset{(b_2)}{+4.205X_2} \underset{(b_4)}{+0.105X_1X_2} \underset{(b_6)}{-0.07125X_2X_3} \underset{(b_8)}{-5X_2^2} \quad (3\text{-}3)$$

$R^2 = 0.944$ and confidence level 95%.

After this analysis, it is found the term b_6 is also a less significant one. The equation after regression analysis is,

$$\underset{(b_0)}{Y_{SP} = 2.065} \underset{(b_1)}{-0.01117X_1} \underset{(b_2)}{+3.35X_2} \underset{(b_4)}{+0.105X_1X_2} \underset{(b_8)}{-5X_2^2} \quad (3\text{-}4)$$

$R^2 = 0.929$ and confidence level is 95% showed

Therefore, the variables X_1 and X_2 are the only variables in the model equation. This means that the saponin content is independent of the feeding time (X_3) for nutrient and sorbitol. Table 9-2 and Figure 9-3 show the saponin contents measured and predicted (use equation 3-4) at various experiment conditions. It can be seen that equation 3-4 can give a close prediction of the experimental results.

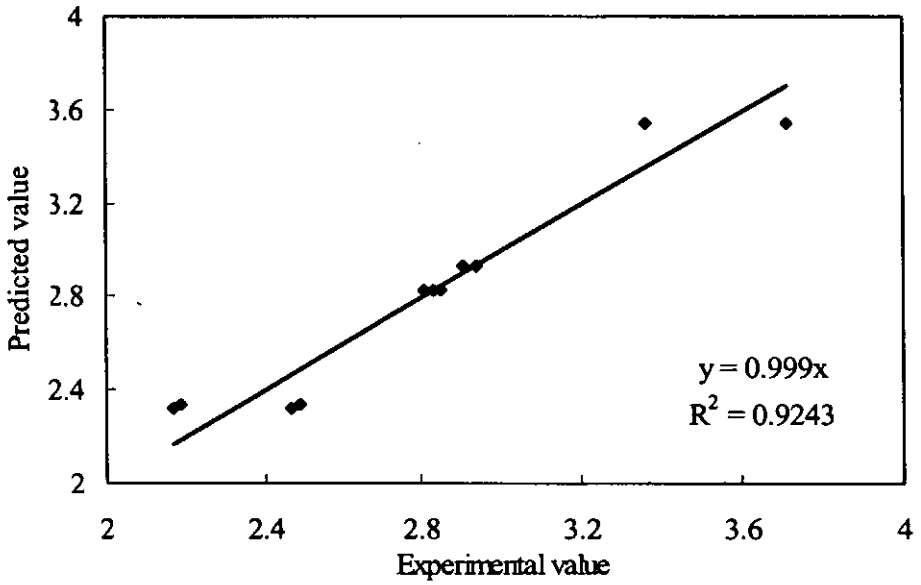

Figure 9-3. A plot of predicted values against experimental values of the saponin content (%).

The effect of nutrient and sorbitol concentrations on saponin is represented by the contour plot at Figure 9-4. The result shows that we can increase the saponin content by augmenting the values of both the nutrients and sorbitol. There is, theoretically, also no upper limit for both factors. Thus, no optimal values of these two factors could be found from this plot. This plot matches with previous study that saponin biosynthesis was enhanced at high osmotic stress. The higher the concentration of sorbitol and nutrients fed, the more the saponins obtained. Under this experimental condition, the optimal

conditions for maximum saponin content were (X_1) sucrose at 45 g/l and CH at 1.5 g/l, and sorbitol at 0.3 M. The saponin content obtained is 3.53 %.

The R value of this equation is 0.964 which indicates a good agreement between the experimental and predicted values of saponin content. R^2 is equal to 0.929 indicating that nearly 93 % of the observed saponin content value is explained by the regression model.

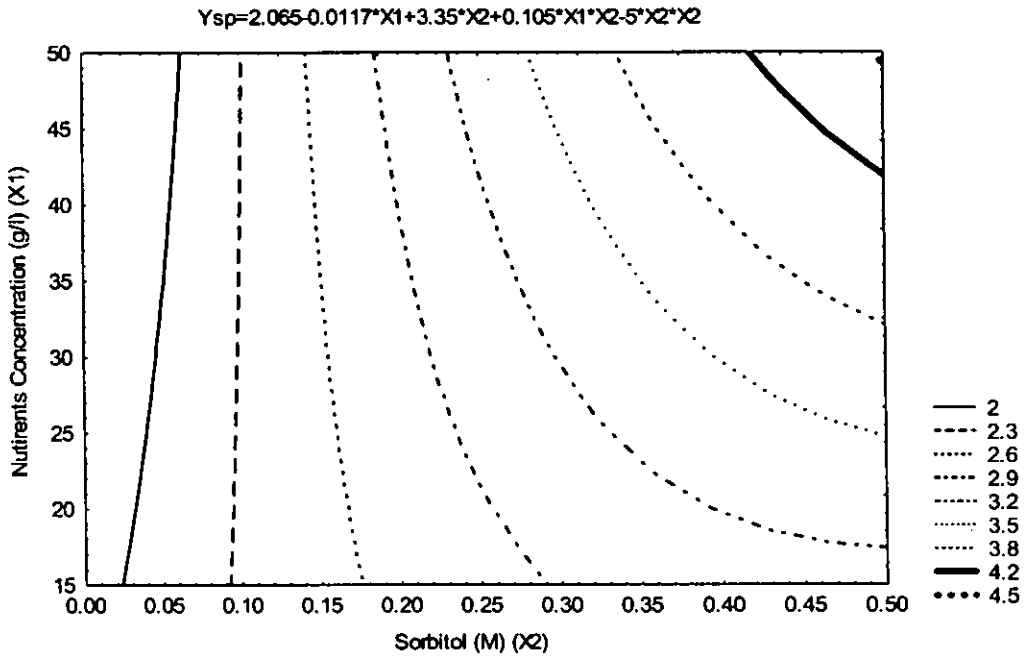

Figure 9-4. A contour plot of saponin content : the effects of nutrient concentration and sorbitol concentration.

9.5. Conclusion of statistical analysis

Both models have quite satisfactory fitting (R^2 = 0.969 for biomass productivity and 0.929 for saponin content), which means that the phenomenon can be well described by a quadratic model. The fitting coefficient can even take higher values, but that leads to very wide confidence intervals. If we want to make them smaller, we have to lower the

significance level of the coefficients (less than 90%), or cut out some terms, making the model easier but losing some fitting.

Nutrients feeding (X_1) had the least effect on the biomass productivity. However, it is very essential factors for the saponin content. The results – contours plot for saponin content – show that there is no upper limit for the nutrient feeding. This upper limit is going to be specified by the physical properties of the system.

The effect of sorbitol (X_2) is more complicated because it affects both the biomass productivity and saponin content. Highest sorbitol concentration gave high saponin content but biomass productivity was inhibited. It can be seen from the optimization of each model that saponin biosynthesis and cell growth prefer contradicting sorbitol conditions. When increasing the sorbitol, more saponin was obtained, but biomass growth was inhibited.

Feeding time (X_3) seems only affects the biomass productivity. It is a very convenient variable, because it takes only integer values (8, 9, 10 ...) so it is easy to manipulate and optimize.

The experimental result obtained in the enhancement of biomass productivity and saponin biosynthesis was in contradictory. The approach of the experimental data is mathematical only and does not concern the physical parameters of the problem. Thus, application of the results from this approach should also consider the agreement with the physical factors and reality of the cell culture. We have to come to a compromise

between the biomass productivity and saponin biosynthesis when these three factors are applied in order to obtain a high saponin yield.

9.6 Testing of optimum condition

Study showed that optimum culture strategy for biomass productivity could be achieved by feeding nutrients at day 10 without feeding osmotica, and that for saponin biosynthesis was feeding unlimited nutrients, and sorbitol. However, this combination could only fit the statistical parameter but not the physical parameters of the culture system. Sorbitol had to be added in order to promote saponin biosynthesis, and too high sorbitol and nutrient concentrations would lead to growth inhibition. In fact, previous study has showed that sorbitol at 0.3 M or above and sucrose above 50 g/l would cause depression to cell growth. Thus, a set of experiment as listed at table 9-3 was designed to test the optimum culture strategy.

Table 9-3. Combinations of the nutrients fed into the culture system at day 10.

Group	Nutrient combination
Control	None
1	Suc. (45 g/l) + CH (1.5 g/l)
2	Suc. (45 g/l) + CH (1.5 g/l) + sorbitol (0.1 M)
3	Suc. (45 g/l) + CH (1.5 g/l) + sorbitol (0.2 M)
4	Sorbitol (0.2 M) + CH (1.5 g/l)

The result of cell growth after feeding was shown at figure 9-5. The dry cell weight of those groups with nutrients feeding at day 10 increased rapidly, especially group 1 without osmotica. However, dry cell weight at the groups fed with osmotica was similar to group 1 at day 24. The dry cell weight at group 2, fed with nutrients and sorbitol at 0.1 M, was higher than that obtained at group 3, fed with nutrients and 0.2 M sorbitol. This indicated that the increase in osmotic stress would repress cell growth. However, the cells would finally adapt to the stress if it was not high enough to cause cell death.

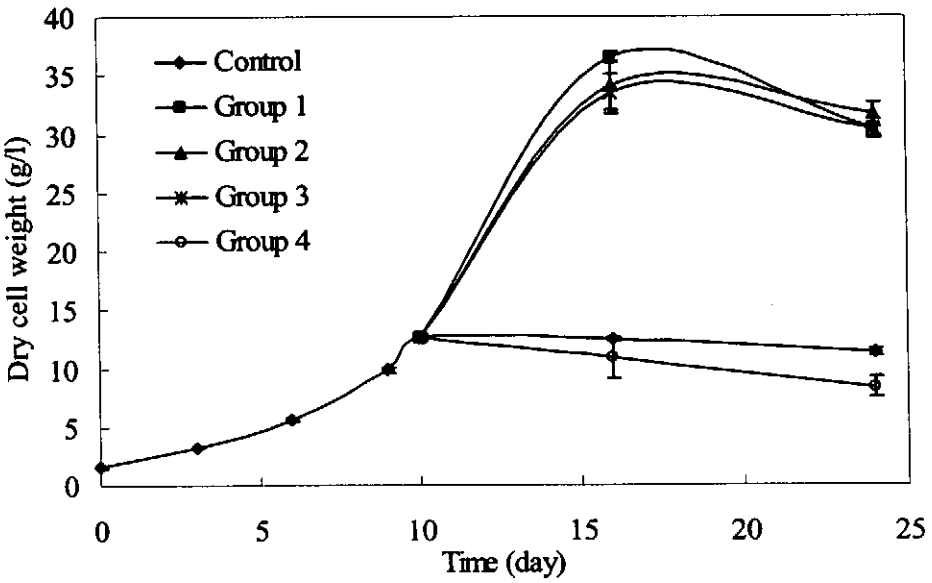

Figure 9-5. Time profile of the growth of *P.ginseng* cells under different nutrient and osmotic conditions.

In the culture without sucrose feeding, i.e. the control set and the group fed only with sorbitol and casein hydrolysate, the highest dry cell weight obtained is 12.5 g/l and 10.98 g/l respectively, that were much lower than those in nutrients feeding groups, up to 36.44 g/l. On the whole, by the nutrient feeding process at the early stationary growth phase, cell growth was much enhanced.

Saponin content was enhanced in the cells with nutrient and osmotica feeding or osmotica feeding only as that induced high osmotic stress to the cells (Figure 9-6). High sorbitol concentration would induce more saponin biosynthesis. 0.2 M sorbitol could not only promote saponin biosynthesis but caused less inhibition to cell growth. As the enhanced saponin biosynthesis owing to increased osmotic stress could counterbalance its inhibition effect on cell growth, high total saponin content could finally be achieved (Figure 9-7).

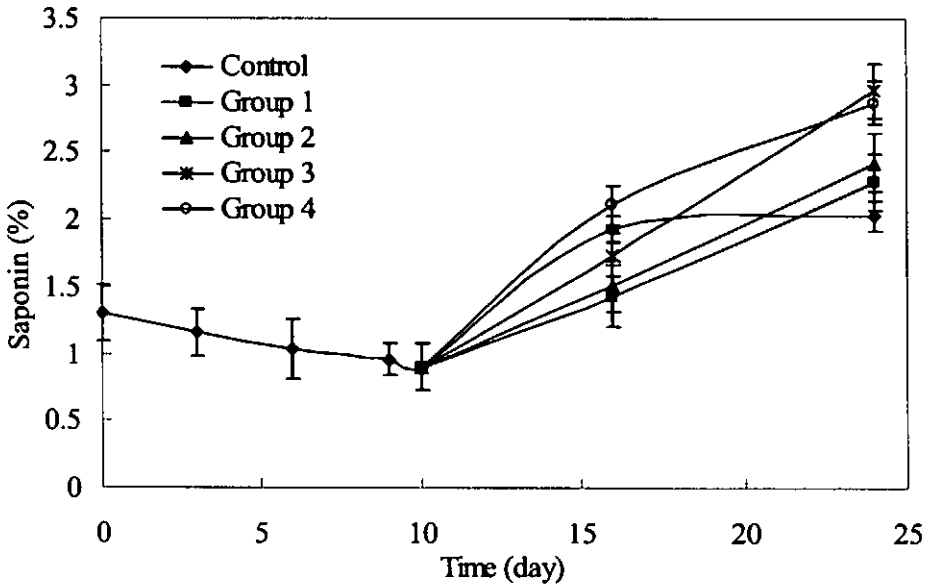

Figure 9-6. Time profile of saponin content of cells with and without nutrients and sorbitol feeding.

The experiment confirmed that biomass growth was affected by the sorbitol concentration. Less sorbitol concentration contributed to more biomass growth. Highest biomass growth was obtained as there were nutrients feeding only. Both nutrients and sorbitol feeding can promote saponin biosynthesis.

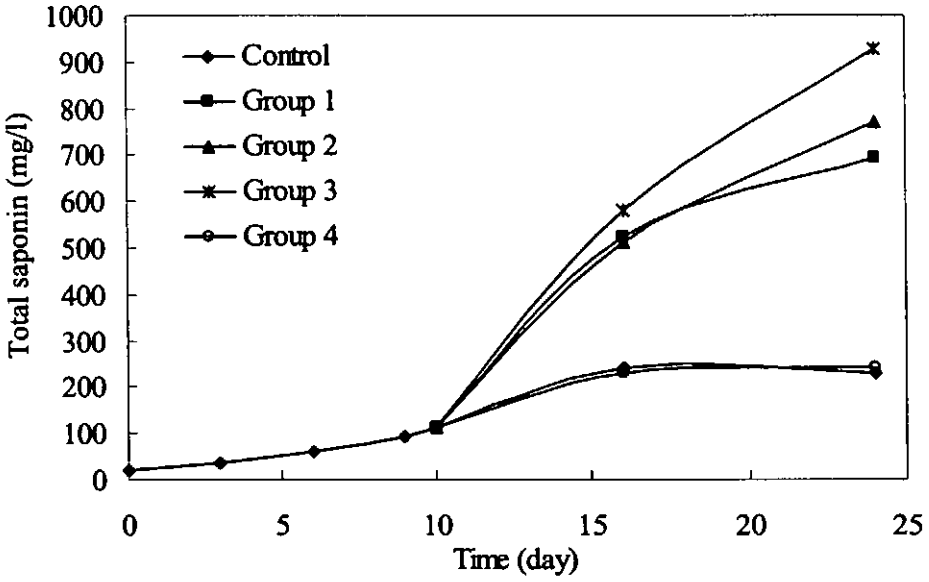

Figure 9-7. Time profile of saponin yield in the *P.ginseng* cells with and without nutrients and sorbitol feeding.

CHAPTER 10 OVERALL SUMMARY

10.1 Conclusion

In this thesis, some development in plant cell culture technology has been reviewed. It has also demonstrated that *P. ginseng* cell suspension culture shared the similar culture pattern as other plant species. The pattern of cell growth consisted of lag growth phase, exponential growth phase, stationary phase and death phase. Saponin content decreased during the exponential growth phase, and increased gradually as the cell growth attained steady stage.

A relatively high initial sucrose concentration would only inhibit cell growth. However, feeding of sucrose and nutrients at the late exponential growth phase could not only extend the growth period but could increase the biomass productivity. These results are useful in understanding the sucrose effect in *P. ginseng* cell culture.

Osmotica could, on the one hand, enhance saponin biosynthesis, but, on the other hand, inhibit cell growth. Regulation of osmotic stress by feeding of osmotica together with growth-limiting growth nutrients could improve saponin biosynthesis without significant cell growth depression. The control of osmotica application was effective to enhance saponin production in *P. ginseng* suspension cell culture. High osmotic stress generated by metabolically inert carbohydrate (sorbitol) was better than that by NaCl which imposed toxicity on the cell culture.

Biotic elicitors were more effective than abiotic elicitors (osmotica) in stimulation of saponin production. Elicitation is a key step to stimulate saponin biosynthesis in *P. ginseng* and has minor inhibition on cell growth. Exposure of cells to elicitors at correct time can be used to probe the complex pathways of secondary metabolite biosynthesis pathway. Following elicitor perception, a number of rapid reactions are detectable in *P. ginseng* cells, including enhanced production of active oxygen species, H_2O_2, and stimulated enzymatic activity, e.g. PAL. Biotic elicitors seemed to be more effective in inducing these activities than abiotic elicitors.

Statistical experimental design showed that nutrient feeding was the major factor that affects biomass productivity while osmotica feeding affected both biomass productivity and saponin contents. The most suitable feeding time was day 10 post inoculation.

10.2 Future study

This investigation revealed that saponin yield could be increased by separation of the culture profile into two stages. Thus, effort in future should be directed to promote high biomass growth at first stage; then, their secondary metabolite biosynthesis was enhanced by feeding of elicitors at late exponential growth phase.

Elicitors have been demonstrated to enhance saponin biosynthesis. However, their effect on different groups of ginsenosides, e.g. Rb1 group (protopanaxadiols) and Rg1 group

(the protopanaxatriols), has not yet studied. Thus, future study should analyze these specific ginsenosides by using more advanced analytical technique such as HPLC.

Now, signal reception of elicitation, the transfer of this stimulation, and the biosynthesis of the ginsenosides are still not fully understood. It is the breakthrough in plant cell culture technology if the mechanism behind is realized. Thus, further study can also be directed at this filed by using precursor feeding and radioactive labeling technology.

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APPENDICES

Appendix 1

Components of the Murishige and Skoog (1962) salt and vitamin mixture (Life Technologies, Grand Island, NY, USA, Cat. No. 10632).

Inorganic salts:	Powder (mg/l)
NH_4NO_3	16500
H_3BO_3	6.20
$CaCl_2$	332.20
$CoCl_2$	0.025
$CuSO_4 \cdot 6H_2O$	0.025
Na_2EDTA	37.26
$FeSO_4 \cdot 7H_2O$	27.80
$MgSO_4$	180.70
$MnSO_4 \cdot H_2O$	16.390
$Na_2MoO_4 \cdot 2H_2O$	0.25
KI	0.83
KNO_3	1900.00
KH_2PO_4	170.00
$ZnSO_4 \cdot H_2O$	8.60
Vitamins	
Myo-Inositol	100.00
Nicotine Acid	0.50
Pyridoxine·HCl	0.50
Thiamine·HCl	0.10
Other components	
Glycine (Free Base)	2.00

Appendix 2

Procedures for determining glucose, fructose and sucrose.

(a) Determination of glucose

Materials

1. o-toluidine reagent (sigma catalog No. 635-6).
2. o-toluidine, 6% (v/v), in glacial acetic acid with thiourea added as stabilizer.
3. Glucose standard solution (Sigma Catalog No. 635-100).
4. Standardized at 100 mg/l (5.55mmol/l) with benzoic acid, 0.1% (w/v), added as preservative.

Methods

1. Label test tubes or cuvette BLANK, STANDARD, TEST. To BLANK add 100 µl water. To STANDARD add 100 µl Glucose standard solution (Catalog No. 635-100). To TEST add 100 µl test sample.
2. To each tube adds 5.0 ml o-Toluidine Reagent. Mix by lateral shaking.
3. Put all tubes into a vigorously boiling water bath for exactly 10 minutes.
4. Quickly remove all tubes and cool to room temperature by placing in water for approximately 3 minutes.
5. Transfer contents of tubes to cuvette and read absorbance at 620 nm.
6. Calculate the content by the following formula:

$$\text{Glucose(mg/dl)} = A_{TEXT}/A_{STANDARD} \times 100$$

Note: If the reading of TEST indicates a glucose concentration greater than 250 mg/l, dilute the TEST with an equal volume of o-toluidine Reagent. Read absorbance of the diluted TEST and multiply result by 2.

(b) Determination of fructose

Materials

1. 70% (v/v) H_2SO_4.
2. Anthrone reagent (prepared by dissolving 150 mg anthrone in 100 ml 70% H_2SO_4. It is imperative that the final color of this solution be yellow, not green. This reagent is stable for 2 weeks at 4 °C.
3. Standard fructose solution (1 mg/ml). This solution should be made fresh each time.

Methods

Preparation of calibration curve

1. Pipe 20,40,60,80, and 100 µl aliquots of standard fructose solution into duplicate sets of tubes, and make up the volume in each tube to 100 µl with distilled water.
2. At timed intervals, add 3 ml of anthrone reagent, and place in a 40 °C water bath for 20 min. Read the absorbance at 620 nm.
3. Construct a standard curve of fructose (mg/ml) vs. absorbance reading at 620 nm. The absorbance at 620 nm for the tube containing 100 µl standard fructose solution should be approximately 1.8±0.2.

Analysis of medium samples

1. Pipe appropriate amounts of samples and makes dilution (usually 30 times) which will give an absorbance reading within the standard range.
2. Measurement of fructose concentration by repeating No. 2 in "preparation of calibration curve". Fructose concentration can be obtained by referring to the standard curve constructed.

Note

The procedure described above determines both fructose and sucrose amounts in the given sample. It is therefore necessary to run sucrose assays on medium samples as described above.

(c) Determination of sucrose

Materials

1. 30% (w/v) KOH solution.
2. 70% (v/v) H_2SO_4.
3. Anthrone reagent (prepared by dissolving 150 mg anthrone in 100 ml 70% H_2SO_4. It is imperative that the final color of this solution be yellow, not green. This reagent is stable for 2 weeks at 40 °C.
4. Standard sucrose solution (1mg/ml). This reagent should be made fresh each time.

Methods

Preparation of calibration curve

1. Pipe 20, 40, 60, 80, 100 µl standard sucrose solution into duplicate sets of test tubes. Make up all volumes to 100 µl with distilled water and mix well.
2. Add 100 µl of 30% KOH to each tube and mix well.
3. Cover the tubes and place them in a boiling water bath for 10 minutes. Remove and leave to cool.
4. Add 3 ml of anthrone reagent to the first tube and mix well. Place in a 40 °C water bath, and start the timer. After exact 2 minutes, repeat with the second tube. Follow this pattern with the remaining tubes.
5. After exactly 20 minutes from the addition to the first tube, remove this at 2 minutes intervals with the remaining tubes. The absorbance at 620 nm should be approximately 1.1±0.1.
6. Construct a calibration curve of sucrose concentrations (mg/ml) against absorbance readings.

Analysis of medium samples

1. Pipe appropriate amounts of samples and make dilution (usually 30 times) which will give an absorbance reading within the standard range.
2. Measurement of sucrose concentration by repeating No. 2-5 in "preparation of calibration curve". Sucrose concentration can be obtained by referring to the standard curve constructed.

Appendix 3

Procedures for determining total sugar by phenol-sulfuric assay

The general phenol-sulfuric acid assay for carbohydrate:

1. Prepare the reagent: Dissolving phenol in water (5% w/v). This reagent is stable indefinitely.
2. Prepare the standard: Dissolving 100mg sucrose in 20 ml distilled water. Pipe 200 μl and dilute to 50 ml, 25 ml, 17 ml, 13 ml, and 10 ml to make the concentration as 4 μg, 8 μg, 11.76 μg, 15.38 μg, and 20 μg per 200 μl.
3. Prepare the sample: Pipe 100 μl supernatant of culture medium and dilute to 40 ml solution.
4. Pipe 400 μl samples, standard, and blank to the test tubes and mix with 400 μl of phenol reagent.
5. Add 2 ml of concentrated sulfuric acid rapidly and directly to the solution surface without allowing it touch the sides of the tube. (The reproducibility of this assay is strongly dependent on the manner of the addition of the sulfuric acid.)
6. Leave the solutions undisturbed for 10 minutes before shaking vigorously.
7. Determine the absorbance at 490 nm after a further 30min.

Note:

1. This assay is very sensitive. All things have to be kept clean.
2. Absorbance is varied with temperature and operation time. Standard curve must be constructed each time.

Appendix 4

Procedures for determination of nitrate

Materials

1. 3.94 mM CuSO4.5H2O (A)
2. 0.1 N NaOH (B)
3. 0.21% w/v hydrazine sulphate (C).
4. 10 % v/v acetone (D).
5. Diazo reagent: 10% v/v ortho-H3PO4, 4% w/v sulphanilamide, 0.2% w/v N-naphthyl-(1)-ethylene-diammonium dichloride (E).
6. Nitrate standard: 3.03g (30 mM) KNO_3/l.

Methods

1. Mix 4 ml nitrate standard/ diluted samples with 0.2 ml A, 1.0 ml A and 0.2 ml C.
2. Incubate in 65 °C water bath for 30 minutes.
3. Place samples into ice for 5 minutes.
4. Add 0.4 ml D, 1.2 ml E and 3.0 ml dd water. Stand in room temperature for 15 minutes.
5. Measure absorbance at 540 nm wavelength.

Appendix 5

Procedures for determining of ammonium

Materials

1. Ammonium standard: 44.4 mg NH_4NO_3 in 1000 ml distilled water (10 μg/ml; OD 0.95).
2. Phenol reagent: 25 g phenol; 312 ml 1 N NaOH, and add distilled water until 1000 ml.
3. Sodium nitroprusside reagent: 1 g nitroprusside sodium, 100 ml distilled water (stable for one month at 4 °C).
4. Sodium hypochlorite solution: 22 ml NaOCl solution (appropriate 15 % active chlorine), 478 ml distilled water. If the NaOCl is not fresh, the volume added should be increased.

Methods

1. Dilute the sodium nitroprusside solution 1:100 in distilled water. Prepare ammonium standards by setting up a series of dilutions of the ammonium nitrate solution in the range 1-5 ml made up to 10 ml in distilled water.
2. Prepare 1 ml sample (water for reference), 1 ml deionized and distilled water, 2 ml phenol reagent, 3 ml sodium nitroprusside solution and 3 ml sodium hypochlorite solution.
3. Shake well and incubate for 30 min in the dark at room temperature. Determine OD_{630} (1 ml cuvette).

Appendix 6

Procedures for determination of phosphate

Materials

1. Standard phosphate solution: 0.2 g KH_2PO_4 in 1000 ml distilled water.
2. Ammonium molybdate: 5 g ammonium molybsate in 40 ml dH2O (A); 17 ml concentrated sulphuric acid diluted with 40 ml distilled water (B). Immediately prior to use, the solutions, (A) and (B), are mixed and brought up to 100 ml with distilled water.
3. Hydroquinone/ sodium bisulphate: 0.5 g hydroquinone in 40 ml distilled water (A); 20 g sodium bisulphite in 40 ml distilled water (B). Immediately prior to use, the solutions, (A) and (B), are mixed and made up to 100 ml with distilled water.

Methods

1. Prepare 0.1 ml sample (or water for control).
2. Add 2.0 ml ammonium molybdate solution to the sample.
3. 1 ml hydroquinone / sodium bisulphite solution was added 1 minute later.
4. Then, 2.5 ml distilled water was added.
5. The sample was left to stand at room temperature for 30 minute before determining OD_{720} (1 ml curvet). For the calibration curve the phosphate standard solution should be diluted at ratio from 1:1 to 1:8.

Appendix 7

Procedures for determination of ginseng saponin by thin layer chromatography (TLC)

Colorimetric method

The higher concentration of acid favors dehydration of cyclohexanol or oxidation by the excess of aldehyde. This colour reaction is applied to simple cyclohexane derivatives. The principles are also applied in the steroid and terpenoid field. Perchloric acid-Vanillin reagent can give the best result (simple, sensitive and relatively precise) in analysis of ginseng saponins among the various colorimetric reagents.

Materials

1. Vinillin: 99 % (GCT) ALDRICH.
2. Perchloric Acid: (WAKO Pure Chemical Industries. LTD).
3. Acetic acid: 99.8%, Riedel-de Haen.
4. Water-bath: Grant JB4.
5. Spectrometer: Spectronic Genesys 5 spectrophotometer.
6. TLC glass plates: Layer thickness: 0.25 mm; Format: 20 × 20 cm.

Methods

(a) Extraction of saponin

1. Dry ginseng cells were ground into powder as small as possible.

2. 100 mg ginseng cell powder was soaked in 5 ml saturated butanol for five hours and then stored in freezer until frozen.
3. Then the samples were ultra-sonicate for 3 hours.
4. The samples were then centrifuged until the cell powder and the solvent were separated into two layers (usually 3000 rpm for 15 minutes).
5. 2 ml supernatant was extracted into test tube and then stored in a vacuum oven below 40 °C and below 26 Hg vacuum until dry.

(b) Preparation of TLC silicate plate

6. A G-silica plate was placed into a glass chamber with Ethanol-Acetone solvent in order to remove impurities that may affect the accurate of the experiment to the top edge. The top edge of the plate would be in yellow.
7. The plate was dried inside oven.
8. Then, it was cut into 4 equal parts (5 cm width for each). The one with yellow edge should be disposed. The other three parts were further cut into small plates each with 3 cm width.
9. The small plates should be stored in desiccator in order to avoid from the moisture if the plate was not used immediately.
10. A line was drawn near the bottle of the plate by pencil. The sample number was marked below.

(c) Running TLC Plate

11. The test tubes were taken from the vacuum oven after drying. 1 ml methanol was added into the test tubes for dissolving the samples.

12. Take 20 μl sample and injected the sample along the line drawn on the small plate evenly. Electric dryer should be used during the process in order to evaporate the solvent from the plate quickly. A blank was prepared by using pure methanol to replace the sample. An extract plate was prepared for locating the positions of the chemicals on the plate later.
13. Afterwards, place the small plates with samples into a glass chamber with the mobile phase solvent (Chloroform: Methanol: Water in 75: 60: 10 i.e. 75 lm: 60 ml: 10 ml). The loading line should not be immersed below the solvent in order to avoid the samples from dissolving in it.
14. When the solvent running to the top of the plate, the plate was taken out and dried under air.
15. 5% sulfuric acid was sprayed on the extract plate. Location of the chemical constitutes in the sample would be seen upon heating.
16. Then, the powder on the plate was scrapped from the located position and stored in centrifuging tube.

(d) Preparation of standard samples

17. The standard solutions for saponin were prepared by using oleanolic acid, panaxatriol and ginsenoside Rb1. The volume added is 75 μl, 150 μl, 225 μl and 300 μl.
18. The tubes with standard solution were also dried in oven. Since it is solution, time of drying is very short.

(e) Determination of saponin

19. 0.2 ml 5 % vanillin acetic acid solution (solid vanillin added to acetic acid) and then 0.8 ml perchloric acid were added into the centrifuging tubes with sample powder. The mixture was warmed in water bath at 60-70 °C for 15 minutes. The procedures were also performed to the standard solution.
20. Then, the tubes were cooled with water. 5 ml acetic acid is added to each sample.
21. The tubes were centrifuged at 3000 rpm for about 5 minutes until the powder are separated from the solution. Since the standard solution is in liquid state, centrifugation is not necessary.
22. The absorbance of the samples is determined at 560 nm wavelength.

Appendix 8

Procedures for determination of phenylalanine ammonia-lyase (PAL)

Materials

1. Boric acid
2. pH meter.
3. Centrifuging machine.
4. L-phenylalanine
5. Incubator

Methods

1. 1 ml of 0.5 M borax-borate buffer at pH 8.8 was added to 0.5 g dried ginseng cells. Then, the cells were homogenized below 5 °C in order to extract the enzymes.
2. The cell homogenate was centrifuged at 13000 rpm for 3 minutes.
3. The supernatant was filtered and used in PAL assay.
4. 500 µl of the extract was mixed with 1900 µl of borax-borate buffer and 600 µl of 0.1 M L-phenylalanine solution and then incubated at 37 °C.
5. The reaction was allowed for 30 minutes. Then, tetraacetic acid was added to cease the reaction.
6. Absorbance at 290 nm was then measured.

Appendix 9

Procedures for determination of fluorescence

Materials

1. Pyramine.
2. Luminescence spectrometer

Methods

1. Prepare a flask containing 25 ml fresh medium.
2. Add the freshly-prepared medium to 25 ml samples.
3. Allow the samples to settle down for several hours.
4. Add 100 µl pyramine (1 mg/ml) to the samples to make final concentration to 2 µg/ml.
5. The flask containing the samples was placed back to the shaker in order to make pyramine evenly distributed in the sample.
6. 3 ml cells were extracted from the sample to act as control before elicitor, if any, was added.
7. The cells were then filtered through a cotton-filled pipette tube, and supernatant was collected for analysis.
8. Then, elicitor, if any, was added to the sample.
9. 3 ml cells were extracted from the samples at different time interval for analysis at the luminescence spectrometer.
10. The excitation wavelength was set to 405 nm, and the emission wavelength was set to 512 nm.