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Ginsenoside content in suspension cultures of *Panax quinquefolium* L. cultivated in shake flasks and stirred-tank bioreactor

SUMMARY

Plant suspension cultures are described as a source for the acquisition of medicinal secondary metabolites which in the future may become an alternative to traditional raw materials. This study demonstrates that the cell cultures of one of the ginseng species – *Panax quinquefolium* L. synthesize ginsenosides, which are triterpene saponins having a multidirectional pharmacological effects. Tested suspension cultures were run on a small scale in the shake flasks and in scale up of the process in a 10-liter stirred tank. In the shake flasks, the highest biomass yield (2.28 gl^{-1} for dry and 33.99 gl^{-1} for fresh weight) was reached on day 30 of culture, and the highest content of saponins (2.66 mg g^{-1} dw) was determined on day 28 of culture. In the bioreactor, nearly 2.67 and 3-fold increase of respectively dry and fresh biomass was recorded in relation to the inoculum. Large-scale cultures synthesized protopanaxatriol derivatives such as Rg1 and Re ginsenosides, however, no saponins belonging to the protopanaxadiol derivatives were reported.

Keywords: *Panax quinquefolium* L. suspension culture, ginsenosides, bioreactor

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INTRODUCTION

Plant cultures *in vitro* are seen as an alternative source of natural substances normally acquired from ground plants, that are used as medicines in the prevention of selected diseases or being components of a diet because of their specific pro-health properties (7, 28). Long-term research indicates that even undifferentiated cells such as callus or cell suspensions produce the same secondary metabolites as the ground plants. For example, callus cultures of *Citrus* L. *spp.* and *Coffea arabica* L. accumulate narigin, limonin and caffeine respectively and the suspension cultures of *Cassia acutifolia* Mill. and *Catharanthus roseus* (L.) G. Don synthesise anthraquinone and indole alkaloids as traditionally cultivated plants (10). However, for most of cell cultures no high yield of secondary metabolites production has been obtained that would allow to start the production in scale up. There are several fundamental reasons for this situation. Firstly, the content of secondary metabolites in the culture is much lower than in the field crop material. Secondly, the change in the profile of synthesized compounds is much lower compared to the field plants. The increase of biosynthesis level of selected secondary metabolites can be achieved by selection of appropriate cell lines, optimization of culture conditions or genetic modification. The result can be a much more efficient production of secondary metabolites by *in vitro* cell cultures than the field crops.

The illustration of highly productive cell lines can be suspension cultures of *Coleus blumei* Benth. and *Lithospermum erythrorhizon* Siebold & Zucc. that produce 9- and 13.5-fold more rosmarinic acid and shikonin respectively than parent plants (23).

Finally, the strategy of increasing the production of a given substance using *in vitro* biomass can be implemented through large-scale cultures of highly-productive biomasses carried out in bioreactors (16).

From the technological point of view, just suspension cultures seem to be the most favorable for the industrial production of secondary metabolites. They enable the use of classic or slightly modified bioreactor constructions with mixed (stirred-tank) or pneumatic mixing (balloon, column and air-lift reactors) used for years in microbiological research. The advantage of suspension cultures is also their relatively simple adaptation to industrial installations. The homogeneity of the suspension combined with good mass and gas transfer makes it possible to obtain high-density cultures. Good contact between cells and growth medium also increases the efficiency of elicitation techniques such as permeabilization or feeding aimed at stimulating the production of secondary metabolites. The disadvantage of suspension cultures is their relatively high susceptibility to mechanical stress limiting the ability to conduct them using classic stirred-tank bioreactors. The limitation for the industrial use of the described biomass is also the relatively slow growth of plant cells, as well as the high somaclonal variation, which can generate strong variations in productivity and growth parameters of culture (16).

Mentioned restrictions of suspension cultures, although important from a technological point of view, are not the most significant factors inhibiting the development of industrial biotechnology based on *in vitro* cultures of higher plants. In practice, the most successful projects using plant biomasses are based on suspension cultures. An example of this is the industrial production of paclitaxel in *Taxus* L. spp. cultures. In a German company Phyton Gesellschaft für Biotechnik mbH, tests were carried out in bioreactors with a capacity of 75 m^3 (6). Meanwhile, for several years the Korean company SamYang Genex Corp. offers as a commercial product Genexol containing paclitaxel from bioreactor suspension cultures Taxus L. spp. (7). Another example can be the Japanese company Nitto Denko Corporation which attempts to obtain ginsenosides from *Panax ginseng* C.A. since the end of the 1980s. Meyer suspension cultures led to develop the technology implemented in bioreactors with a capacity of 2 m^3 and then 20 m^3 (26, 29). Despite obtaining biomass of ginseng from the field crops and the suspension cultures of *P. ginseng* C.A., the demand for ginseng saponins is still increasing. Ginseng saponins known as ginsenosides are biologically active metabolites responsible

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for most of the multidirectional pharmacological effects of ginseng. They are oligosaccharide glycosides of either dammarane or oleane type triterpenoids. Based on the structure differentiation of the sapogenins, the ginsenosides can be reasonably classified into different subtypes: protopanaxadiol type (PPD), protopanaxatriol type (PPT) and octillol type. The main of them are PPD and PPT ginsenosides. PPD ginsenosides possess a 20(s)-20(R)-protopanaxadiol sapogenin conjoined with one to six monosaccharides. Metabolites Rb_1, Rb_2, Rc and Rd belong to this group. PPT ginsenosides possess a 20(s) or 20(R)-protopanaxatriol sapogenin and one to four sugar units. They include such metabolites as Rg_1 and Re (21). Considering the fact that the demand for ginseng saponins is still increasing, our study was focused to obtain suspension culture of another species of ginseng – *Panax quinquefolium* L. Moreover, the kinetics of growth and dynamics of ginsenoside biosynthesis in this culture were investigated.

In the study, we also attempted to enlarge the scale of the process in a 10-liter stirred-tank bioreactor.

MATERIALS AND METHODS

PLANT MATERIALS

The leaf blades of a 4-year-old ground plant of *Panax quinquefolium* were obtained from the Garden of Medicinal Plant of the Medical University in Łódź. They were sterilized with 2% sodium hypochlorite solution for two minutes and after rinsing with sterile distilled water, the leaf blades were cut into 0.5–1 cm fragments and placed on solid agar (0.7%) woody-plant (WP) (14) medium with 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4 D) (Sigma-Aldrich), 1 mgl^{-1} (alpha)-naphtalene acetic acid (NAA) (Sigma-Aldrich) and 0.5 mgl^{-1} 6-benzylamino purine (BAP) (Sigma-Aldrich) in dark at 26ºC. After two weeks the callus tissue appeared on the edges of the explantant. After six weeks the callus tissue was transferred on new portion of the medium. The callus tissues were subcultured every five weeks.

ESTABLISMENT OF SUSPENSION CULTURE AND GROWTH CONDITION IN THE SHAKE FLASKS

Suspension culture of *P. quinquefolium* L. was initiated from callus tissue after XXIII passage and was conducted in Erlenmeyer flasks (300 ml) containing 50 ml of liquid Murashige and Skoog (MS) medium (15) with 2.4 D 1 mg l^{-1}, kinetin (kin) 0.1 mg $l^{-1,}$ pH 5.6-5.8. The medium was sterilized in the temperature of 123°C and 1 ATM pressure for 21 min. The flasks were placed on rotary shaker (100 rpm), at 26±2°C temperature, with 90% humidity, in dark for 40 days. Average fresh biomass of inoculum was 3.33 gl^{-1}, and average dry weight was 0.262 gl^{-1}. Biomass from day 5, 10, 15, 20, 25, 28, 30, 32, 35, 40 of cultivation was separated through filtration (using vacuum pomp) and fresh weight and was weighed. The dry weight was also measured. The cells separated from the medium were dried at 100°C for 1 hour first, then at 80°C temperature for 24 hours. The fresh and dry biomass was showed in g l^{-1}. The treatment was repeated three times.

SUSPENSION CULTURE GROWTH CONDITION IN BIOREACTOR

With the purpose to enlarge the culture scale, the incubation of suspension culture in stirred-tank bioreactor of 10 dm^3 (Fig.1) was carried out. The initial volume of the medium (MS with 2.4D 1 mg l-1, kin 0.1 mg l-1) was 3 l. The constant temperature of bioreactor was controlled by a Thermomix MM thermostat (B Brown Biotech. International). The other process parameters were: rotations 50 min-1, aeration 100 vvm. The bioreactor was inoculated with cell suspension from shake flask cultures after 14 days of cultivation (approx. 16.6 gl^{-1} fresh and 1.41 gl^{-1} dry weight). The incubation was carried out at 26°C in natural photoperiod, for 30 days. Fresh cell suspension was separated through filtration (using vacuum pomp), weighted and dried at the room temperature.

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Fig. 1. Callus tissue (after passage XXIII) and suspension culture of *P. quinquefolium* L. in shake flask and stirred-tank bioreactor. The suspension cultures are after 4 weeks of cultivation. Scale bar means 1 cm

MEDIUM COMPONENTS

After separating the biomass from the medium during 40 days of cultivation, the consumption of the main medium components saccharose, phosphate and ammonium ions was indicated.

Determination of sacharose content

The changes of saccharose content in the medium was determined using an antron method according to references (19). The method involves the action of sulfuric acid on the hexose or pentose molecule contained in tested samples. After separation of the water molecule hydroxymethylfurfural or furfural is produced, that gives a blue-green complex with the anthrone. The intensity of the color obtained is directly proportional to the amount of sugar in the tested sample and is measured spectrophotometrically at 620 nm.

Determination of phosphate ions

The content of phosphate ions in the culture medium was determined according to the Polish standard PN 89-C 04537/02 (17). The method involves the reaction of orthophosphates with ammonium molybdate in an acidic medium and the formation of yellow phosphomolybdic acid, which is then reduced to blue phosphoromolybdate. The intensity of the resulting blue color is proportional to the content of orthophosphate and is measured spectrophotometrically at 690 nm.

Determination of ammonium ions

The determination was made by the direct Nesslerization method in accordance with the Polish PN-C-04576-4 standard (18). To determine the ammonium content of the tested medium sample, the

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reaction of the $NH4^+$ ion with the Nessler reagent, resulting in a yellow-brown complex compound was used. The color intensity of the sample increases in proportion to the ammonium content. The content of ammonium ion was determined by photocolorimetry at 420 nm.

EXTRACTION PROCEDURES

After separating cells from the medium, the plant material was dried in room temperature and then it was used for ginsenoside extraction. The samples of 1±0.1 g of dry raw material were placed in 250 ml flasks. They were extracted three times with 50 ml of 80% methanol for 30 min at solvent boiling temperature under a reflux condenser. The combined methanol extracts were evaporated to dryness in a vacuum evaporator under lowered pressure at 60°C. The flask with dried residues was placed in a desiccator filled with drying agent. The dried methanolic extract was weighed.

GINSENOSIDE ANALYSIS USING HPLC METHOD

Dried extracts were dissolved in 1 ml of methanol (HPLC-grade, J.T.Baker, Netherland) and filtered through a 0.2 µm pore diameter Millex®-FG Hydrophobic Fluoropore filters (PTFE) (Sigma-Aldrich). The volume of 20 µl was introduced to liquid chromatography system (LiChroART® 250-4, Waters 600 Controlled pump, UV-Vis 996 detector combined with Pentium 60PC hardware equipped with Millenium software). The mixture of acetonitrile (J.T. Baker, Netherland) with water (J.T.Baker, Netherland) was used as eluent. Acetonitrile to water ratio 30:70 was used for determination of saponis Rb_1, Rb_2, Rc and Rd, analysis time was 45 min and flow rate was 2 ml min^{-1}. The eluent for determination of metabolites Rg_1 and Re was acetonitrile to water ratio 18:82, flow rate was 3 ml min^{-1}, analysis time was 40 min. Ginsenoside detection was made at 203 nm wavelength. All standards of tested ginsenosides were purchased from C. Roth GmbH+Co (Karlsruhe, Germany). Ginsenosides were quantified (mg g^{-1} d.w.) by comparing retention time and peak areas between standards and samples.

STATISTICAL ANALYSIS

All treatments were performed in triplicate. Data were analysed using the Kruskal-Wallis test with STATISTICA (StatSoft, Inc. 2011, version 10, www.statsoft.com) and $p<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

The dynamic of growth of *P. quinquefolium* L. was studied in MS medium with 2.4D 1 mg l^{-1}, kin 0.1 mg l^{-1} and 30 g l^{-1} saccharose. During 40 days of cultivation, every 2–3 samples were taken between the 25^{th}–32^{nd} day of culture or every five days for other days of culture to estimate the fresh and dry suspension biomass. The dynamic of ginsenoside biosynthesis was also investigated (Fig. 2). The culture growth curves in relation to both fresh and dry biomass were similar and had sigmoid character. The maximum biomass yield was 2.28 g l^{-1} for dry and 33.99 g l^{-1} for fresh weight after 30 days of culture and it was 8.7- and 10.2-fold increase in relation to inoculum respectively. These results indicate that the increase of *P. quinquefolium* L. suspension biomass was significantly higher than that in suspension culture of other species of ginseng such as *P. ginseng* C.A. Meyer (13), *P. japonicus* (T. Ness) C.A. Mey (12) or *P. vietnamensis* Ha et

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Grushv. (25). These different findings may be a result of using different lines of suspensions and different growth condition such as for instance the set of plant growth regulators in the medium.

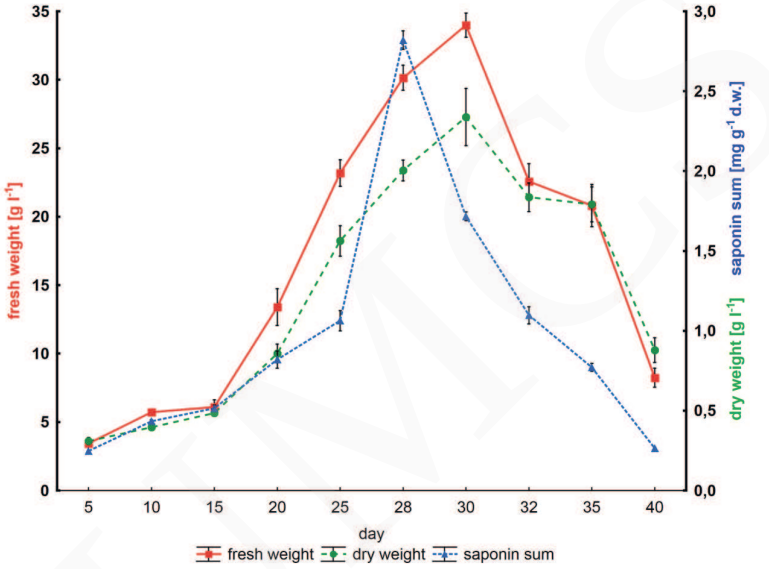

Fig. 2. Changes of biomass and ginsenoside biosynthesis during 40-days suspension culture of *P. quinquefolium* L.

The study of kinetics of *P. quinquefolium* L. cell growth expressed as cell dry and fresh biomass indicated the following pattern: dry and fresh weight increase was not considerable up to 15th day of culture. A gradual increase of biomass was observed between 15th and 30th days of cultivation and then, dry and fresh weight increased 4.6- and 5.58-fold respectively. Typical stationary stage was not observed and after 30 days culture gradual decrease of biomass took place (Fig. 2). The lack of stationary phase is sometimes observed in plant cell cultures (2, 22) and it may indicate a rapid cell degeneration process resulting from the lack of nutrients in the medium. The confirmation of this thesis seems to be the study of the changes of concentrations of saccharose, phosphate and ammonium ions in the medium which were also studied during culture growth.

The analysis of the biomass increase, saponin biosynthesis and the changes of the concentrations of studied medium components indicate the reverse dependence between these parameters. During the first five days of suspension culture, the concentrations of saccharose, phosphate and ammonium ions changed slightly. The most intensive of their consumption was observed between the 10th and 30th day of cultivation, when the suspension cultures were showing the most intensive

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increase of both biomass and ginsenoside accumulation. The phosphate ions assimilation was the fastest from all the studied components (Fig. 3). Its complete utilization was observed in the 20th day of culture time. The intensive cell growth stage, when the rich energy substances synthesis takes place, was characterized by complete phosphate ion depletion. Also, the hydrocarbon, i.e. saccharose assimilation depended on ATP accessibility, which in turn was the result of phosphate ion accessibility. The study of *Catharanthus roseus* (L.) G. Don cultured cells revealed that phosphate ions were rapidly absorbed during initial stage of batch cultivation, and during subsequent growth they were consumed gradually (20).

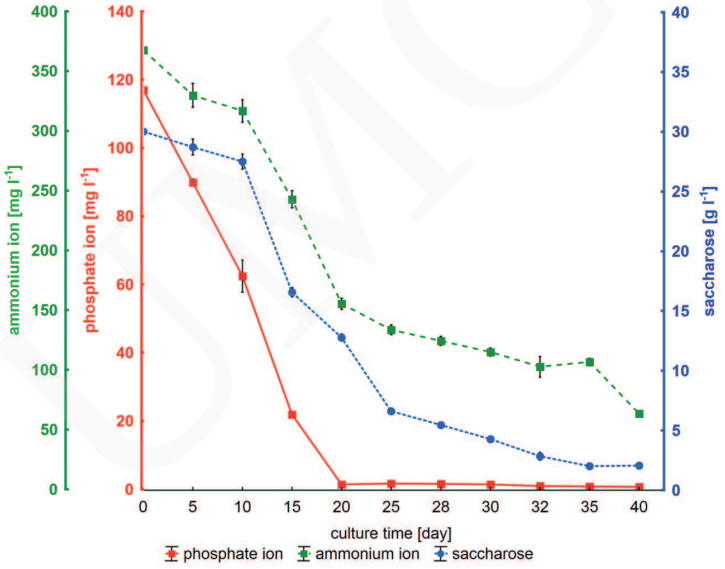

Fig. 3. Consumption of phosphate, ammonium ions, and saccharose in the medium during 40 days suspension culture of *P. quinquefolium* L.

Our results demonstrated that the assimilation of studied medium ingredients was in a direct relationship to the dynamic of cell growth and metabolism. These results are consistent with those obtained for *P. quinquefolium* L. and *P. notoginseng* C.A. Meyer suspension cultures by the other authors (32, 33).

The presence of six ginsenosides, i.e. Rb_1, Rb_2, Rc, Rd, Re and Rg_1 in biomass of suspension culture of *P. quinquefolium* L. was also determined (Fig. 4). The highest content of examined saponins in 1 g of dry biomass was determined on day 28 of culture and it was 2.66 mg g^{-1} dw. Lower level (1.82 mg g^{-1} dw) of sum of eight ginsenosides was detected in suspension cultures of *P. sikkimensis* R.N. Banerejee (3), however another line of *P. quinquefolium* L. suspension accumulated nearly 30 mgg^{-1} dw. saponins (11).

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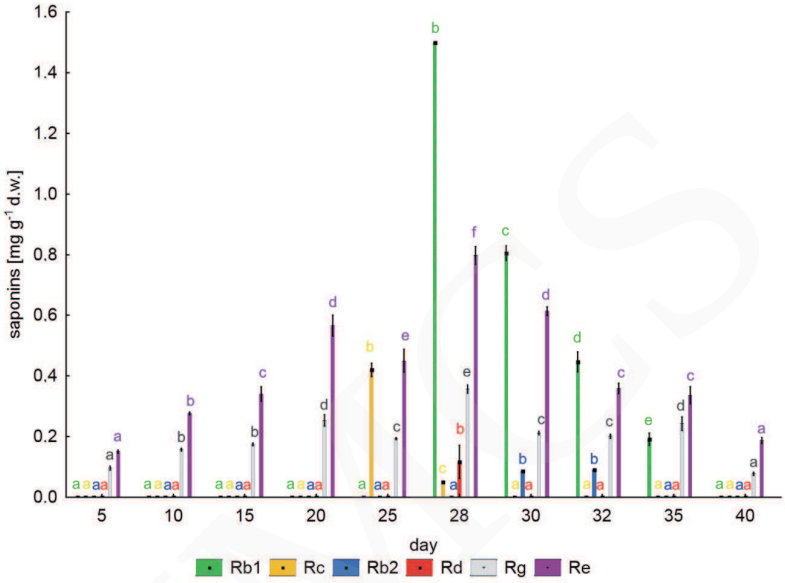

Fig. 4. The content of individual ginsenosides during 40-days suspension culture of *P. quinquefolium* L. Each value represents the mean of three replicates ± SE. The various letters for the same parameter means statistically significant differences at $p \leq 0.05$ (Kruskal-Wallis test)

The results described in this study demonstrated that two metabolites: Rg_1 and Re (both belong to protopanaxatriol derivatives) were always detected during 40-days culture time. Moreover, it was stated that the change of dynamic of their production was similar to the dynamic of change of biomass. In contrast with our results, Kochkin et al. (12) found that Rg1 metabolite was accumulated in greater amount in the first 10 days of cultivation of *P. japonicus* (T. Ness) C. A. Mey suspension, and after that Rg1 level decreased below its content detected in the day of inoculation of *P. quinquefolium* L. suspension culture described in this paper. Other observation was noted in relation to ginsenosides belonging to protopanaxadiol derivatives. For example, ginsenoside Rc appeared in day 25 of cultivation and the metabolites: Rb_1, and Rd were detected for the first time three days later. In day 28 of culture, the level of ginsenoside Rb_1 achieved its maximum level. Then, its amount gradually decreased while Rc and Rd saponins were not detected.

The obtained outcomes suggest that the change in the content of individual ginsenosides may be a result of the changes of the activity of key enzymes involved in biosynthetic pathway of ginseng saponin. It seems that glycosyltransferases, responsible for the last stage of ginseng saponin biosynthesis, leading to the formation of particular metabolites, may be particularly sensitive to these changes.

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BIOREACTOR CULTURE

The growth and ginsenoside production of *P. quinquefolium* L. suspension culture were investigated in the stirred-tank bioreactor after 30 days of cultivation. Our previous research (data not published) indicated that then the biomass and ginsenoside content achieve the maximum yield. The obtained results showed that fresh and dry weight increased 3- and 2.48-fold in relation to inoculum respectively. Threefold increase of biomass was also noted for batch bioreactor culture of oil palm (8). The results described in this paper indicated that bioreactor culture of *P. quinquefolium* L. achieved lower biomass increase than that in cultures growing in the shake flasks. The same observation was noticed for suspension of *Thevetia peruviana (Pers.) K. Schum.* also cultivated in the stirred tank bioreactor (1). Using the other type of bioreactor such as an airlift bioreactor and balloon-type bubble bioreactor system allowed to obtain a significantly higher biomass yield for cultures of *P. japonicus* (T. Nees) C.A.Mey and *P. ginseng* C.A. Meyer (5, 24). The differences in biomass growth may result from different aeration conditions in different types of bioreactors and the size of the inoculum (4, 5).

Our findings indicated that suspension culture growing in larger scale accumulated only protopanaxatriol derivatives – metabolites Re and Rg_1. The level of Re was 3-fold higher than the level of Rg_1 (Table 1). The content of these ginsenosides in the shake flasks was higher by about 13% and 39% for Re and Rg1 respectively.

Table 1. Biomass yield and ginsenoside production in bioreactor cultures of P. *quinquefolium* L. suspension. The various letters in the upper index means statistically significant differences at $p \leq 0.05$ (Kruskal-Wallis test)

Yield biomass [g l^{-1}] ± SE		Ginsenoside content [mg g^{-1} dw] ± SE		
fresh	dry	Rg1	Re	total
50.43 ± 4.32^a	3.49 ± 0.78^b	0.21 ± 0.01^a	0.62 ± 0.05^b	0.83 ± 0.04^c

This result is not surprising because it is often observed that transferring the plant cell cultures from the shake flask to the bioreactor cause a decrease in secondary metabolite content, and different cultivation environment between the shake-flask and the bioreactor could affect the cell physiology as well as the secondary metabolite biosynthesis (31). The literature data also reported that other bioreactor culture of ginseng suspensions can synthesize both protopanaxatriol and protopanaxadiol derivatives, however PPT saponins dominate quantitatively (5, 27, 30). The differences in the findings obtained for bioreactor cultures described in this paper and presented by the other researchers may be a result of different ginseng species used in the studies and culture conditions in different

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bioreactor systems. The selection of a suitable bioreactor can be crucial for a successful scaling up process. Indeed, it is also important to properly optimize various parameters that affect growth and production of secondary metabolites in the bioreactor culture. The obtained results in this study demonstrated that further researches are needed to focus on the optimization of the process of biomass yield and ginsenoside biosynthesis in the bioreactor cultures.

CONCLUSIONS

The maximum yield of biomass was achieved after 30 days of culture and the highest ginsenoside level was detected in 28-days culture of *P. quinquefolium* L. suspension in the shake flask. Saccharose, phosphate and ammonium assimilation were in a direct relationship to the dynamic of cell growth and metabolism.

The cultures carried out in the stirred-tank bioreactor demonstrated 2.48- and 3-fold increase of dry and fresh biomass respectively. The cultures produced only protopanaxatriol derivatives. The next stage of the study should be performed to optimize the process of biomass growth and the enhancement of saponin production in the stirred-tank bioreactor.

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