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STUDIES OF THE CULTURE CONDITIONS FOR *PANAX GINSENG* CELLS IN JAR FERMENTORS[1]

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ABSTRACT.—Newly isolated callus of ginseng showing a low level of differentiation, coded Pg-3 B2K, was similar to Pg-1 IBA1 callus in growth and saponin content but proved to be more amenable to mass culture. When it was cultured as a suspension, the effect of carbon and nitrogen sources on cell growth was examined. The culture conditions for maximum increase of fresh and dry weight were defined as follows: the callus is cultured on Murashige and Skoog's agar medium minus NH_4NO_3 and plus 0.5% glucose and 2% sucrose for an initial period of 2 weeks, and after adding 2% sucrose for an additional 2 weeks. A fermentor culture was performed in a 30-liter vessel, and it was demonstrated that the mode of agitation (turbine types and turning speeds) has an important effect on the growth and saponin content of ginseng cells.

In our previous papers (1-4), it has been reported that ginseng (*Panax ginseng* C.A. Meyer) callus, coded Pg-1, produces almost the same pharmacologically active saponins, ginsenosides, as that of cultivated ginseng root.

Although IBA1 callus in Pg-1, which was mainly redifferentiated roots, was the most excellent in saponin production (1), it was thought that large lumps formed during suspension culture might be a problem for mass cultivation, in particular for the transfer from suspension culture to suspension or jar fermentor.

Newly isolated ginseng callus, coded Pg-3 and of a lower degree of differentiation, was examined with respect to culture conditions for growth and saponin production when cultured as a large-scale suspension. Results indicate that Pg-3 callus is a strain of higher saponin productivity than Pg-1 callus.

MATERIALS AND METHODS

INDUCTION OF PG-3 DK CALLUS AND B2K CALLUS.—A 5-year-old ginseng root (*P. ginseng*) cultivated in Korea was surface-sterilized with 70% EtOH and a saturated aqueous solution of commercial bleaching powder and then rinsed with sterile H_2O, in December 1978. After cylinders (diam. 1 cm) were removed with a cork borer, 1-2 mm slices were cut. The slices were placed on Murashige and Skoog's (1962) agar medium (MS) supplemented with 2,4-dichlorophenoxyacetic acid (D) 1 ppm and kinetin (K) 0.1 ppm. The developing calli (Pg-3) were transplanted onto the same medium, maintained at 25° in the dark, and subcultured at intervals of 4 weeks (Pg-3 DK callus). After a third subculture, the calli were transferred onto MS medium containing indole-3-butyric acid (IBA) 2 ppm and K 0.1 ppm, which had been the best condition for the saponin production in Pg-1 IBA1 callus as described in a previous paper (1). The calli were kept at 25° in the dark and subcultured at intervals of 4 weeks for about a year until, eventually, vigorous growth was achieved (Pg-3 B2K callus).

PG-1 IBA1 CALLUS.—Selection of Pg-1 IBA1 callus from D1 callus has been reported previously (1).

DETERMINATION OF GROWTH RATIO AND SAPONIN CONTENT IN AGAR CULTURES.—Callus (*ca.* 3 g) was transferred onto the test medium in a 100-ml Erlenmeyer flask. After 4 weeks of culture at 25° in the dark, the growth ratio and saponin content were determined. We used six to ten flasks in each experiment.

DETERMINATION OF GROWTH RATIO OF SUSPENSION CULTURES.—Test medium (250 ml) in a 1000-ml Erlenmeyer flask was inoculated with callus of agar cultures and cultured on a reciprocal shaker (80 strokes/min, each stroke 8 cm in length) at 25° in the dark. After 4 weeks of culture, the callus was harvested, and the growth ratio was determined. Two flasks were used in each experiment.

EXAMINATION OF CARBON AND NITROGEN SOURCES.—MS media supplemented with 2 ppm IBA and 0.1 ppm K were used. A combination of glucose and sucrose was examined as carbon source.

[1]This is part 40 in this series "Studies on Plant Tissue Cultures," for part 39 see reference (1).

Examination of the nitrogen source consisted of removal of NH_4NO_3 and addition of KNO_3. Each experiment was performed twice, if necessary.

JAR FERMENTOR CULTURE.—Callus cultured on agar-media was transferred to a 5-liter jar fermentor[2] containing 4 liters of test medium. The aeration ratio was 1 volume (aeration) per volume (medium) per minute (VVM) and the turning speed was 100 rpm.

Callus grown on agar-medium also was transferred to 500 ml medium in a 1000-ml Erlenmeyer flask and cultured on a reciprocal shaker for 4 weeks. Cells cultured in this manner were used as inoculum for a 30-liter jar fermentor[3] containing 25 liters of test medium. The inoculum size was 24 g/liter or 48 g/liter. The aeration ratio was 0.25 VVM. Three turbine types (disc, angled disc, and anchor type) shown in Figure 1 and two turning speeds (100 and 150 rpm) were examined.

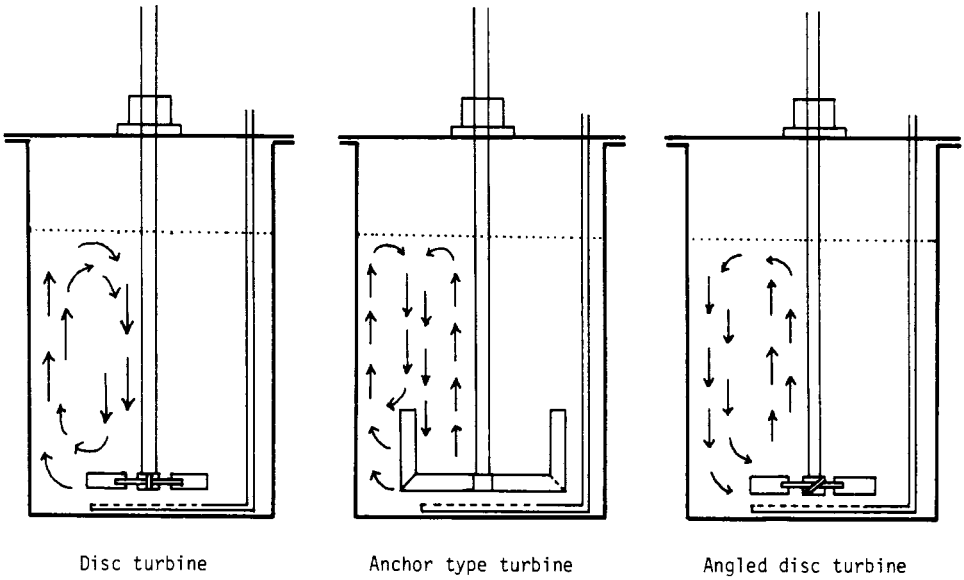

FIGURE 1. Three turbine types used in 30-liter jar fermentor culture.

EXTRACTION AND DETERMINATION OF SAPONIN.—The methods of extraction (4) and determination (1) of saponin have been described previously. The summary of the determination method of the total saponin was as follows: The MeOH extract from 50 g of fresh weight of callus was suspended in H_2O and extracted with Et_2O. The H_2O layer was extracted with *n*-BuOH, and the *n*-BuOH layer was evaporated under reduced pressure (crude saponin). Silica gel tlc plates quantitatively spotted with this crude saponin were developed in *n*-BuOH-EtOAc-H_2O (4:1:5), upper layer, and colored by spraying 10% H_2SO_4 and heating. Then each spot density of ginsenoside was measured using a dual wave length tlc scanner (λ_{obs}=530 nm, λ_{ref}=700 nm) and total saponin was calculated.

RESULTS AND DISCUSSION

The newly isolated Pg-3 DK callus and B2K callus (derived from DK callus) were cultured on several agar media, and the growth ratio and saponin content were determined. According to Table 1, Pg-3 B2K callus showed a higher growth ratio and saponin content than DK callus. This result was similar to Pg-1 D1 and IBA1 callus (1). The culture on MS medium supplemented with 2 ppm IBA and 0.1 ppm K showed a high growth ratio and the highest saponin content.

Pg-1 IBA1 callus when cultured as a 250-ml suspension with MS medium containing IBA 2 ppm and K 0.1 ppm yielded the highest saponin production (1). Therefore, Pg-1 IBA1 and Pg-3 B2K callus were suspended and cultured in the same medium (IBA 2 ppm and K 0.1 ppm) in a 5-liter jar fermentor, and subsequently the growth ratio and saponin content were compared. The results are presented in Table 2. Pg-3

[2]Model MD-75 or MD-300, Marubishi Laboratory Equipment Co., Ltd.

[3]Model SKJ-3-30, Suzuki Seiko Co., Ltd.

TABLE 1. Growth and Saponin Content of Pg-3 Callus in Static Culture

Cell line	Medium[a]	Growth ratio	Total saponin content (mg/100 g fresh wt)
Pg-3 DK	MS D 1 K 0.1	2.46	9.7
	MS D 1 K 0.1 CM 7%	3.10	15.0
	RT N 1 K 0.1 C 0.1%	3.26	18.1
Pg-3 B2K	MS IBA 2 K 0.1	3.30	55.6
	MS IBA 5 P 0.1	3.37	38.9

[a]Plant growth regulator concentration=ppm.

MS=Murashige and Skoog's medium (1962); RT=Khanna and Staba's revised tobacco medium (1968); D=2,4-dichlorophenoxyacetic acid; K=kinetin; IBA=indole-3-butyric acid; P=*N*-phenyl-*N'*-(4-pyridyl)urea; CM=coconut milk; C=casamino acid (casein hydrolyzate).

TABLE 2. Growth and Saponin Content of Ginseng Callus Cultured in a 5-Liter Jar Fermentor

Cell line	Growth ratio	Dry weight (g)	Total Saponin (mg)	
		100 g (fresh wt)	100 g (fresh wt)	1 g (dry wt)
Pg-1 IBA1	4.02	3.63	38.5	10.6
	4.41	4.88	52.4	10.7
Pg-3 B2K	2.63	4.43	44.7	10.1
	3.82	3.70	40.5	10.9

B2K callus yielded almost the same dry weight per 100 g fresh weight and saponin content as Pg-1 IBA1 callus, but showed a lower growth ratio.

The effect of carbon and nitrogen sources was examined with callus suspended in liquid MS medium with 2 ppm IBA and 0.1 ppm K. In suspension culture containing 3% sucrose, the growth of Pg-3 B2K callus was slow for the first 2 weeks. This result was probably caused by low availability of sucrose at the initial stage of the culture. The callus was transferred to medium containing 1% sucrose and various amounts of glucose and cultured for 2 weeks. After an initial 2 weeks of culture, the growth ratio reached a maximum at 0.5-0.6% glucose concentration. Therefore, 0.5% glucose and various concentrations of sucrose were tested. Sucrose 2% was shown to produce maximum growth during the initial 2 weeks. These results indicated that a medium with 0.5% glucose and 2% sucrose is best for growth during the initial 2 weeks.

For further increase of growth, the callus was transferred to media containing 0.5% glucose and 2% sucrose and cultured for 2 weeks. Then, various amounts of sucrose (0-3%) were added to the medium, and the callus was cultured for an additional 2 weeks. The control experiment was performed with medium containing 3% sucrose for 4 weeks. The callus was harvested, and the growth ratio and dry weight were measured. The results are shown in Figure 2.

MS medium with 0.5% glucose and 2% sucrose showed a higher growth ratio than with 3% sucrose. Except for the addition of 3% sucrose, the growth ratio had increased by the addition of sucrose after 2 weeks of culture. Dry weight per 100 g fresh weight also increased by the addition of sucrose after 2 weeks' culture. Based on these results, an addition of 2% sucrose was thought best to achieve a high growth ratio.

Furthermore, it appeared that a high concentration of mineral salts or NH_4^+ ions might prevent the callus from growing rapidly. So, the MS medium was modified by removing NH_4NO_3 and adding KNO_3. It was found that MS medium minus NH_4NO_3 and with added KNO_3 gives a higher growth ratio and dry weight per 100 g fresh weight than regular MS medium or MS medium minus KNO_3. The growth ratio

TABLE 3. Effect of Medium Conditions and Turbine Types in 30-Liter Jar Fermentor Culture of Pg-3 B2K Callus

Medium	Turbine (rpm)	Inoculum size (g/liter)	Growth ratio	Dry weight		Total saponin content		
				g/100 g (fresh wt)	g/liter	mg/100 g (fresh wt)	mg/100 g (dry wt)	mg/liter
MS Sucrose 3%	Disc (100)	24	4.45	5.71	6.1	41.1	720	43.9
	Disc[a] (50)	48	4.33	5.64	11.7	23.1	410	48.0
MS-NH_4NO_3 Sucrose 3%	Disc (100)	24	5.00	8.86	10.6	41.3	466	49.4
		48	4.10	5.56	10.9	18.6	335	36.5
MS-NH_4NO_3 Sucrose 2% + glucose 0.5%	Disc (100)	24	4.33	7.54	7.8	55.3	733	57.2
		48	6.12	3.17	9.3	18.4	580	53.9
MS-NH_4NO_3 Sucrose 2% + glucose 0.5% add sucrose 2% after 2 weeks	Disc (100)	48	5.04	5.95	14.4	20.7	348	50.1
	Anchor (100)	48	4.66	6.86	15.3	19.5	284	43.5
	Angled (100)	48	6.45	5.48	17.0	9.7	177	30.1
	Disc (150)	48	3.86	5.82	10.8	26.7	459	49.6
	Angled (150)	48	3.81	7.74	14.2	24.2	313	44.4

[a]0.5 VVM, 2 disc turbines.

Aeration ratio=0.25 VVM; culture period=28 days; disc=disc turbine; anchor=type turbine; angled=angled disc turbine.

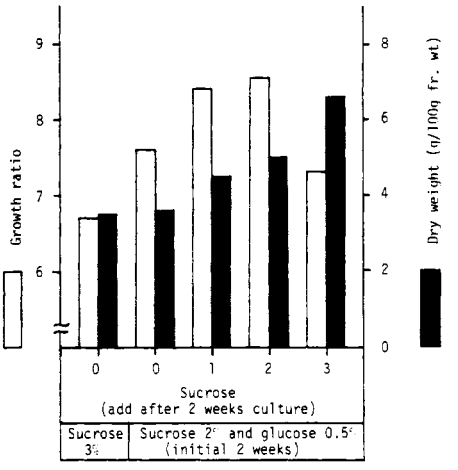

FIGURE 2. Effect of sucrose addition on the growth after 2 weeks' culture of Pg-3 B2K callus with 2% sucrose and 0.5% glucose.

obtained with such medium is higher, and the dry weight per 100 g fresh weight is similar when compared with Gamborg's B5 medium (5). Although the medium contained various amounts of KNO_3, the growth ratio was similar over the range of KNO_3 concentration tested (500-6000 mg/liter). Therefore, a medium containing only 1900 mg/liter of KNO_3 (*i.e.*, MS medium minus NH_4NO_3) as the nitrogen source was used in the following 30-liter jar fermentor cultures.

Based on the above results that the medium conditions (carbon and nitrogen sources) had an effect on the cell growth in shake flask suspension culture, suspension culture of Pg-3 B2K callus was performed in a 30-liter jar fermentor with several media; growth ratio, dry weight, and total saponin content were determined. The results are summarized in Table 3. MS medium minus NH_4NO_3 was similar to regular MS medium for growth and saponin content. Using MS medium minus NH_4NO_3 and plus 0.5% glucose and 2% sucrose, and 2% sucrose added after 2 weeks of culture resulted in a higher growth ratio and higher dry weight (g/liter) than using regular MS medium containing 3% sucrose. Also, the relative amounts of saponins (mg/liter) decreased only slightly.

Among three turbine types (Figure 1), the angled disc turbine provides for the best growth ratio and dry weight increase (g/liter) but the lowest saponin content. When the agitation was accelerated to 150 rpm, the growth ratio and dry weight decreased, but the saponin content increased, giving a saponin production (mg/liter) similar to that of 100 rpm.

In a 30-liter jar fermentor culture, the increase of the growth ratio and dry weight were not accompanied by an increase of the saponin content. This observation indicated that the saponin production per culture was about equal. A jar fermentor culture is not comparable to a shake flask suspension culture. Therefore, it is necessary to examine further the culture conditions for cells in jar fermentors.

Pg-3 callus has a capacity for producing a high yield of saponins and is more suitable for the large-scale culture than Pg-1, differentiated callus, because it is possible to transfer the callus through the pipes and valves connecting jar fermentor and tanks. Repeated selection of cell lines from Pg-3 callus and the engineering of a new device for mass culture are in progress.

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