BIOTECHNOLOGY LETTERS
Volume 16 No.9 (Sept.1994) p.943-948
Received as revised 2nd August

PRODUCTION OF POLYSACCHARIDES IN LIQUID CULTURES OF *POLIANTHES TUBEROSA* CELLS

Kazuya Otsuji [1]*, Yasuki Honda [2], Yukio Sugimura [2], and Akira Takei [1]

[1] Kashima Research Laboratories, Kao Corporation, Kashima, Ibaraki 314-02, Japan
[2] Tochigi Research Laboratories, Kao Corporation, Haga, Tochigi 321-34, Japan

SUMMARY

The effects of components of the medium on the production of extracellular polysaccharide (EPS) by cultured cells of *Polianthes tuberosa* (tuberose) were studied. Optimization of media components culturing in flask resulted in increasing EPS production from 1.4 to 4.1 g/l. In particular, relatively high concentration (10^{-5}M) of 2,4-dichlorophenoxyacetic acid (2,4-D) markedly stimulated the production of EPS. Based on these results, EPS production by a 30-l jar fermentor was attempted and the final rate of production was 4.6 g/l at 30th day of culture. The EPS consisted mainly of acidic polysaccharides with glucuronic acid, mannose, arabinose, galactose, glucose and xylose.

INTRODUCTION

Cells derived from higher plants are potentially important alternative systems for production of useful plant metabolites *in vitro*, and many trials have been carried out to date (Fujita et al., 1981; Parr, 1989; Yamada and Endo, 1984; Furuya et al., 1983; Smith et al., 1987). However, low yields of metabolites and the genetic instability of cell lines are major obstacles to the successful commercialization of cell culture processes. On the other hand, it is well known that in liquid culture polysaccharides are secreted from cells(Becker et al., 1964; Goto, 1986; Hale et al., 1987; Mante and Boll, 1975; Takeuchi and Komamine, 1978; Aspinall et al., 1969; Akiyama et al., 1984). Production of polysaccharides by plant cell culture is generally more stable than that of secondary metabolites but their satisfactory utilization has not been found.

In this paper, we found that callus derived from petals of *Polianthes tuberosa* (tuberose) secreted large amount of polysaccharides into medium. Therefore , to enhance production of extracellular polysaccharides (EPS) in the liquid culture, we examined the effects of major constituents of the medium. Based on these results, we tried to scale up production using a 30-L jar fermentor. The sugar analysis of the ESP were also carried out.

MATERIALS AND METHODS

Plant materials and cell culture: The cells used in the various experiments were

derived from petals of tuberose flowers on Linsmaier and Skoog (LS) medium (Linsmaier and Skoog, 1965) solidified with 0.8% agar and supplemented with 10^{-5} M 1-naphthaleneacetic acid (NAA), 10^{-6} M 6-benzyl aminopurine (BA) and 3% sucrose. The cells were transplanted to LS liquid medium supplemented with plant growth regulators (Sigma Chemical Co.,USA) at the same respective concentration and sucrose. Cultures were incubated with shaking at 120 rpm in darkness at 26°C, and cells were subsequently subcultured at 30-day intervals. One gram fresh weight of cells was inoculated into a 100-ml volumetric Erlenmeyer flask that contained 30 ml of culture medium, which included various components, such as basal elements, plant growth regulators, and carbon sources, and then cells were cultivated for 30 days as described above. In the scaled-up procedure, 1,400 g of fresh weight of cells were inoculated into a 30-l volumetric jar fermentor that contained 20 l of LS medium supplemented with 5% sucrose and combinations of 10^{-5} M NAA and 10^{-6} M BA or 10^{-5} M 2,4-D. The jar fermentor was operated with stirring at 80 rpm and aeration (10 l/min) in darkness at 26°C for 30 days. All chemicals used for the basal medium were reagents of analytical grade (Wako Pure Chemical Industries, Ltd., Japan).

Preparation of EPS and cultured cells: Culture medium containing EPS and cultured cells was filtered through two layers of gauze and the filtrate was centrifuged at 10,000 x g for 30 min to remove any remaining cell debris. Three volumes of ethanol were added to the supernatant, with thorough mixing by inversion. After storage at 4 °C overnight, the precipitate was pelleted by centrifugation at 800 x g for 10 min, then it was lyophilized and weighed. The cultured cells that had been recovered by filtration and centrifugation were washed twice with distilled water, then lyophilized and weighed.

Chemical analysis of sugars:Total neutral sugars were quantified by the phenol-sulfuric acid method (Hodge and Hofreiter, 1962) with glucose as the standard. Uronic acids were quantified by the carbazole-sulfuric acid method (Bitter and Muir, 1962) with glucuronic acid as the standard. For analysis of the monosaccharide composition of EPS, samples of EPS were hydrolyzed by incubation with 2 M trifluoroacetic acid in sealed tubes at 120°C for 1 h. Each hydrolysate was vacuum-dried at 40°C and dissolved in 1 ml of water. Monosaccharides in the hydrolysate were converted to the corresponding alditol trifluoroacetates by the method of Albersheim et al. (1967) and analyzed by gas chromatography (GC). GC was carried out with a hydrogen flame ionization detector on a capillary column (25 m x 0.2 mm i. d.). The column oven temperature was linearly raised from 120 to 175°C at a rate of 4°C/min.

RESULTS AND DISCUSSION

Effects of standard media

Three standard media, White (White, 1963), Gamborg (Gamborg et al., 1968) and LS were selected for their different nutrient compositions. Each medium contained 10^{-5} M NAA, 10^{-6} M BA and 3% sucrose. LS medium gave the best results for both EPS production (1.4 g/l) and cell growth (18.3 g/l). EPS productions in other standard media were 1.1 g/l and cell growths in White and Gamborg media were 5.9 and 14.6 g/l, respectively. It should be noted that LS medium has the highest nutrient concentrations among the three tested media, in particular in terms of total nitrogen, phosphate and potassium. Since EPS production and cell growth were best in LS medium, this medium was used in subsequent attempts to optimize the levels of the major constituents of the medium for elevated production of EPS.

Effects of plant growth regulators

As shown in Table 1, a higher concentration of NAA, irrespective of the concentration of BA, favored EPS production only slightly, but it simultaneously lowered cell growth.

Especially, see that a combination of 10^{-5} M NAA and 10^{-6} M BA gave 15.9 g/l. By contrast, a lower concentration of NAA (10^{-6} M) lowered EPS production considerably. Since enhancement of EPS production was not expected with combinations of NAA and BA, we investigated the use of 2,4-D as an auxin. Addition of 10^{-5} M 2,4-D was the most effective method to enhance EPS production (3.3 g/l) and, at either higher or lower concentrations of 2,4-D, EPS production decreased. Note that a lower concentration (10^{-6} M) also resulted in a considerable increase in cell growth. Addition of 10^{-4} - 10^{-6} M BA to medium that contained 10^{-5} M 2,4-D did not favor EPS production, but significantly enhanced cell growth, giving dry weight of cells of 15.2 g/l. Thus, maximum production of EPS was obtained at 10^{-5} M 2,4-D, while cell growth was maximal with a combination of 10^{-5} M NAA and 10^{-6} M BA. These observations suggest that nature and concentration of plant growth regulators must be changed for seed culture and production, for example, 10^{-5} M NAA and 10^{-6} M BA for culture of plant seeds and 10^{-5} M 2,4-D for production of EPS. There are numerous reports on the secretion of EPS, but hardly found are publications that studied correlation between plant growth regulators and secretion of EPS. Previous workers simply advocated the use of 2,4-D and its initial concentrations such as 5×10^{-6} M 2,4-D for *Zea mays*, (Bacic et al., 1987), 4.5×10^{-6} M 2,4-D for *Phleum pratense*, (Hale et al., 1987) and 4.5×10^{-5} M 2,4-D for *Arabidopsis thaliana*. (Goto, 1986). Concentrations from 4.5×10^{-6} to 4.5×10^{-5} M 2,4-D have frequently been used in examinations of polysaccharide production. These suggest that relatively high concentration of auxin, especially, 2,4-D is required for the secretion of polysaccharides from liquid cultured cells.

Effects of sugars

Table 2 shows EPS production was from 2.8 to 3.4 g/l by glucose, fructose, sucrose and mannose. Xylose and galactose were not effective for EPS production, while arabinose and galacturonic acid caused cell necrosis. The effect of sugar species on cell growth was similar to that of sugars on EPS production: sucrose, mannose, fructose, and glucose favored cell growth, (5.8 to 6.5 g/l). Numerous examples of hydrolysis of sucrose are reported in a wide range of cell culture systems. Thus, an addition of sucrose to a cell culture yields glucose and fructose by hydrolysis (Fowler and Stepan-Sarkissian, 1985).Therefore, it seems highly probable that glucose and fructose, as well as sucrose, are utilized by cultured cells. We selected sucrose as a cheap carbon source for culture of tuberose cells.

The effects of the initial concentration of sucrose on EPS production and cell growth were examined. Sucrose concentrations from 1 to 5% increased, in proportion to it, both EPS production and cell growth, giving maximum values of 3.7 and 7.5 g/l, respectively. But, when 6% of sucrose was used, both values leveled off. Generally, use of higher concentrations of sucrose would give rise to osmotic shock or unfavorable effect on biosynthetic pathways. Fujita et al. (1981) reported that, in the synthesis of shikonin by cultures of *L. erythrorhizon* cells,

concentrations of sucrose up to 4% increased the rate of synthesis. For production of polysaccharides, a concentration of 3% of sucrose is often adopted, but the optimum concentration must be determined in conjunction with size of the inoculum.

Table 1. Effects of plant growth regulators on EPS production and cell growth in liquid cultures of tuberose cells in LS medium that contained various plant growth regulators, 3% sucrose, and 3.3% inoculum. Measurements were made after 30 days in culture.

Plant growth regulators (M)			EPS (g/l)	Dry weight of cells (g/l)
2,4-D	NAA	BA		
10^{-4}			2.4	6.6
10^{-5}			3.3	9.6
10^{-6}			2.1	13.2
10^{-4}		10^{-5}	1.9	5.9
10^{-5}		10^{-4}	1.5	14.5
10^{-5}		10^{-5}	1.7	15.2
10^{-5}		10^{-6}	1.6	15.2
10^{-6}		10^{-5}	1.1	11.9
	10^{-4}	10^{-5}	1.9	10.1
	10^{-4}	10^{-6}	1.9	9.2
	10^{-4}	10^{-7}	2.1	12.9
	10^{-5}	10^{-5}	1.5	13.2
	10^{-5}	10^{-6}	1.8	15.9
	10^{-5}	10^{-7}	1.6	14.2
	10^{-6}	10^{-5}	0.3	12.9
	10^{-6}	10^{-7}	0.3	10.9

Table 2. Effects of various sugars on EPS production and cell growth in liquid cultures of tuberose cells in LS medium that contained 10^{-5} M 2,4-D, 3% sugar, and 3.3% inoculum. Measurements were made after 30 days in culture.

Sugar	EPS (g/l)	Dry weight of cells (g/l)
Arabinose	-	-
Galactose	1.7	4.6
Galacturonic acid	-	-
Glucose	3.4	5.8
Fructose	3.3	6.0
Mannose	2.8	6.5
Sucrose	3.1	6.5
Xylose	2.1	4.7

Effects of the size of inoculum

EPS production was also affected by the size of inoculum (3.3 to 10.0 %); the effect was

from 3.6 to 4.1 g/l. Maximum production occurred with an inoculum of about 7 to 8%. While cell dry weight decreased with increased size of inoculum (data not shown), the dry weight of cells harvested after a 30-day cultivation was fairly constant (4.8 to 5.9 g/l) despite different size of inoculum (data also not shown). Although many studies considered effects of inoculum size on cell cultures (Panda et al., 1992), no consistent conclusion has been reached yet about correlation among parameters such as inoculum size, cell growth and metabolite production. This negligence is surprising whenever the overall performance of a given culture system is to be assessed.

Production of EPS in a 30-l jar fermentor

As described above, EPS production was markedly enhanced by optimization of constituents of the medium. However, commercial production of EPS will not be performed in flasks but in fermentors. Therefore, EPS production in a 30-l jar fermentor was done using the medium whose constituents were optimized from flask tests. Results from the flask tests that were described above recommend us different combinations and concentrations of plant growth regulators; 10^{-5} M NAA and 10^{-6} M BA for cultures of seeds and 10^{-5} M 2,4-D for production of EPS. Fig. 1 shows the time-course of EPS production and cell growth for seed culture (A). After 30 days of culture, dry weight of cells reached 390 g/l and 1.8 g/l of EPS was obtained. On the other hand, for the production of EPS (B), 4.6 g/l of EPS was produced and dry weight of cells was 165 g/l on the final day of cultivation. Production of EPS in the jar fermentor may have been superior to that in flasks because aeration was more advisable than in flasks. This productivity 4.6 g/l during one-month culture is thought to be of the highest level ever reported on useful metabolite production by plant cell culture.

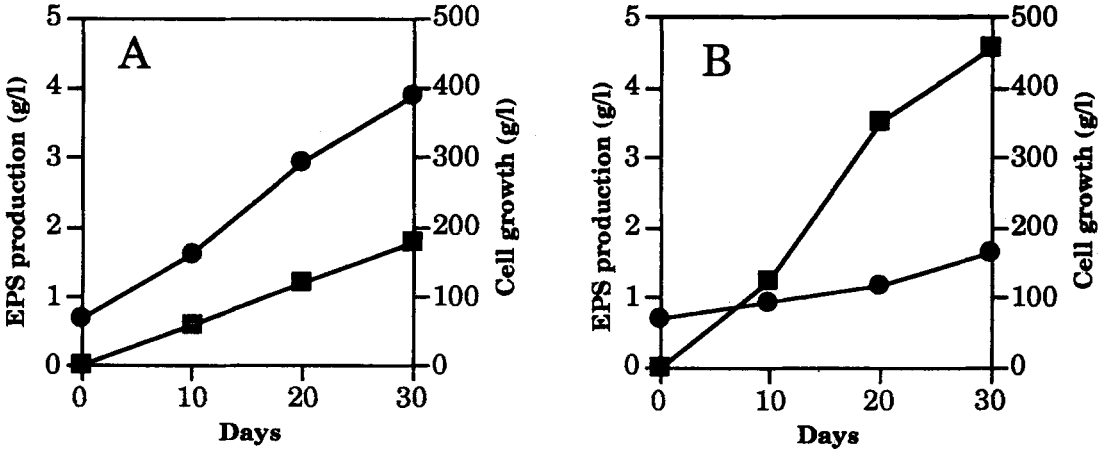

Fig. 1. EPS production(■) and cell growth(●) by tuberose cells in a 30-l jar fermentor using the optimized medium for culture of seeds (A) and production of EPS (B).

Analysis of sugars in EPS

Neutral sugar in EPS, recovered as an ethanol precipitate, accounted for about 70% and the remaining 30% was uronic acid. The neutral sugar consisted of five monosaccharides, namely arabinose (39%), galactose(24%), glucose(6%), mannose(24%) and xylose(7%). Further details of chemical structures will be reported elsewhere together with results on separation and purification of EPS.

In conclusion, callus derived from the petals of tuberose flowers secreted polysaccharides into medium. To enhance EPS production in liquid culture, constituents of the medium were studied and optimized to increase the production of EPS from 1.4 to 4.1 g/l. In a 30-l jar fermentor, the rate of production of EPS was 4.6 g/l during one-month culture. These EPS are being commercially produced and utilized as ingredients of cosmetics.

ACKNOWLEDGEMENTS

The authors are very grateful to Mr. Motoki Nakajima for providing the tuberose bulbs as a gift. The authors also gratefully acknowledge the valuable suggestions and valuable encouragement from Drs. Susumu Ito, Naotake Takaishi, and Kikuhiko Okamoto.

REFERENCES

Akiyama Y., Eda S., Mori M., Kato K. (1984) Agric. Biol. Chem. 48: 403-407.
Albersheim P., Nevins D.J., English P.D. Karr A. (1967) Carbohydr. Res. 5: 340-345.
Aspinall G.O., Molloy J.A., Craig J.W.T. (1969) Can. J.Biochem. 47: 1065-1070.
Bacic A., Moody S.F., McComb J.A., Hinch J.M., Clarke A.E. (1987) Aust. J. Plant Physiol. 14: 633-641.
Becker G.E., Hui P.A., Albersheim P. (1964) Plant Physiol. 39: 913-920.
Bitter T., Muir H.M. (1962) Anal. Biochem. 4: 330-334.
Gamborg O.L., Miller R.A., Ojima K. (1968) Exp. Cell Res. 50: 151-158.
Goto N. (1986) Physiol. Plant. 66: 49-52.
Fowler M.W., Stepan-Sarkissian G. (1985) Primary and Secondary Metabolism of Plant CellCultures, pp.66-73 Springer-Verlag, Berlin,Heidelberg.
Fujita Y., Hara Y., Ogino T., Suga C. (1981) Plant Cell Reports 1: 59-60.
Furuya T., Yoshikawa T., Orihara Y., Oda H. (1983)Planta Med. 48: 83-87.
Hale A.D., Pollock C.J., Dalton S.J. (1987) Plant Cell Reports 6: 435-438.
Hodge J.E., Hofreiter B.T. (1962) Methods inCarbohydrate Chemistry, Vol. 1, pp. 380-394 Academic Press, New York.
Linsmaier E.M., Skoog F. (1965) Physiol. Plant. 18: 100-127.
Mante S., Boll W.G. (1975) Can. J. Bot. 53: 1542-1548.
Panda A.K., Mishra S., Bisaria V.S. (1992) Biotechnol. Bioeng. 39: 1043-1051.
Parr A.J. (1989) J. Biotechnol. 10: 1-26.
Yamada Y., Endo T. (1984) Plant Cell Reports 3: 186-188.
Smith J.L., Smart N.J., Kurz W.G.W., Misawa M. (1987)Planta Med. 53: 470-474 .
Takeuchi Y., Komamine A. (1978) Physiol. Plant. 42:21-28.
White PR (1963) The Cultivation of Animal and Plant Cells, 2nd ed. pp. 59 Ponald PressCo., New York.