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Production of Polysaccharides by Plant Cell Culture and Their Applications to Cosmetics

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Introduction

Production of useful metabolites by plant cell culture is expected in various fields such as pharmaceutical, cosmetic and food industries. So many trials have been attempted to date[1-5], however, low yields of metabolites and genetic instability of cell lines are major obstacles to the successful commercialization of plant cell culture. Various approaches, such as selection of high producing cell line, control of culture conditions and release of veneficial metabolites into culture medium, have been carried out to overcome low yields of metabolites. It has been known that some of the cultured cells secreted macromolecules into the medium[6-10], but satisfactory productivity for industrial production has not been realized, in addition, their useful utilizations have not been found so far. In spite of such situations of the production and uses of the metabolites by plant cell culture, we found that cells induced from petals of tuberose(*Polianthes tuberosa*) is capable of secreting large amount of polysaccharides(EPS) into the medium. Based on this finding, we investigated the possibility of mass production of EPS and searched their utilizations.

In this review, we present the media constituents and culture conditions for high production of EPS and application as a ingredient of cosmetic.

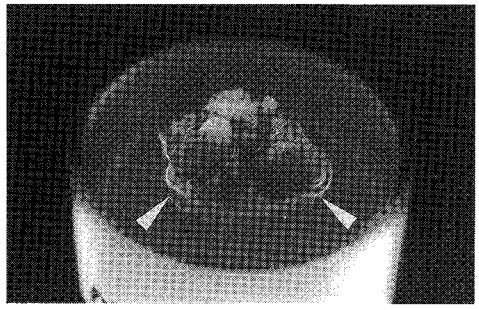

Fig. 1 Secretion of EPS(arrows) from the callus of tuberose.

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Effects of media constituents[11]

1. *Callus induction and selection*

Sterilized young petals of tuberose were bedded on the Linsmaier and Skoog (LS) medium[12] solidified with 0.8% agar and supplemented with 10^{-5}M α-naphthaleneacetic acid (NAA) and 10^{-6} M 6-benzylaminopurine (BA) and 3% of sucrose. Derived callus were subcultured at same respective conditions. During subculturing of the callus, quantity of the secretion of EPS was monitored with the naked eye and callus of higher secretion were selected. **Fig. 1** shows the selected callus and the manner of secreting EPS from the callus.

2. *Selection of standard medium*

Three standard media, White[13], Gamborg[14] and LS were employed to investigate the effect of production of EPS and growth of cells. Three grams of fresh weight cells were inoculated into a 200 m*l* volumetric Erlenmeyer flask containing 80 m*l* of a culture medium comprised of various components including basal elements, plant growth regulators, and carbon sources, then cultivated for 30 days with shaking at 120 rpm in darkness at 26°C. The culture medium containing EPS and cells were filtered through two-fold gauze and the filtrate was centrifuged at 10,000×g for 30 min. to remove any remaining cell debris. Three volumes of ethanol were added to the supernatant and mixed thoroughly by inversion. After storage at 4°C for overnight, ethanol-precipitate was pelleted by centrifugation at 800×g for 10 min. then lyophilized and weighed. The cultured cells recovered by filtration and centrifugation were combined and washed twice by distilled water, then lyophilized and weighed. Other investigations on the media constituents were basically carried out by the methods as shown above.

LS medium contained 10^{-5} M NAA, 10^{-6} M BA and 3% sucrose gave the best results in both production of EPS (1.4 g/*l*) and growth of cells (18.3 g/*l*). Production of EPS in other standard media were 1.1 g/*l* and growth of cells 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

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

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

three tested media, in particular in terms of total nitrogen, phosphate and potassium. Since production of EPS and growth of cells were best in LS medium, this medium was used in subsequent attempts to optimize the levels of the major constituents in the medium to elevate the production of EPS.

3. *Effects of plant growth regulators*

In this studies, NAA, BA and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were examined. As shown in **Table 1**, a higher concentration of NAA, irrespective of the concentration of BA, favored the production of EPS only slightly, but it simultaneously lowered growth of cells. Especially, 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 production of EPS considerably. Since enhancement of the production of EPS 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 the production of EPS (3.3 g/*l*) and, at either higher or lower concentrations of 10^{-5} M 2, 4-D, production of EPS decreased. Note that a lower concentration (10^{-6} M) also resulted in a considerable increase in growth of cells. Addition of 10^{-4} -10^{-6} M BA to medium that contained 10^{-5} M 2, 4-D did not favor the production of EPS, but significantly enhanced growth of cells, 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 growth of cells 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 in the culture of seed and production, for example, combination of 10^{-5} M NAA and 10^{-6} M BA for culture of seeds and 10^{-5} M 2, 4-D for production of EPS. There are numerous reports on the secretion of polysaccharides but no evidence on their mechanisms. Previous studies suggested that relatively high concentration of auxin, especially, 2, 4-D is required for the secretion of polysaccharides from liquid cultured cells[15-17].

4. *Effects of carbon sources*

Both production of EPS and growth of cells were markedly enhanced by glucose, fructose, sucrose and mannose while slightly increased by xylose and galactose. Arabinose and galacturonic acid, however, caused cell necrosis. 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[18]. 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 the production of EPS and growth of cells were examined. Sucrose concentrations from 1 to 5% increased in both production of EPS and growth of cells. But, when 6% of sucrose was used, both values leveled off. Generally, use of higher concentrations of sucrose would give rise to osmotic pressure of medium or unfavorable effect on biosynthetic pathways. Fujita *et al.*[16] 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.

As described above, by the optimization of media constituents, the production of EPS has been enhanced from 1.4 to 3.7 g/*l*. Plant growth regulators, especially, played an important role to increase the production of EPS.

Effects of lowering viscosity of culture medium

Increasing the production of EPS resulted in increasing viscosity of the culture medium. The viscosity of the culture medium is the most significant property with respect to the flow behaviour of the fluid[19]. Such behaviour of the medium seems to affect mass transfer, heat transfer and aeration[20-24]. Therefore, a decrease in the viscosity of the culture medium might be expected to enhance the production of EPS.

1. *Molecular weight of EPS*

High correlation between molecular weight of EPS and viscosity of the solution has generally been recognized. To measure the molecular weight of EPS, gel-permeation chromatography (GPC) was carried out on a high-performance liquid chromatograph. **Fig. 2** shows the GPC elution profile of EPS. It reveals that EPS consists of at least two polysaccharides with different molecular weight. The high molecular weight one was estimated to be 2.5×10^6 D or more, while the low molecular weight one was around 1.5×10^5 D. This suggests that the increase of viscosity in the culture medium is caused by high molecular weight of EPS.

2. *Lowering viscosity of EPS solution by addition of mineral salts*

Lowering viscosity of culture medium is expected to result in increase of the production of EPS.

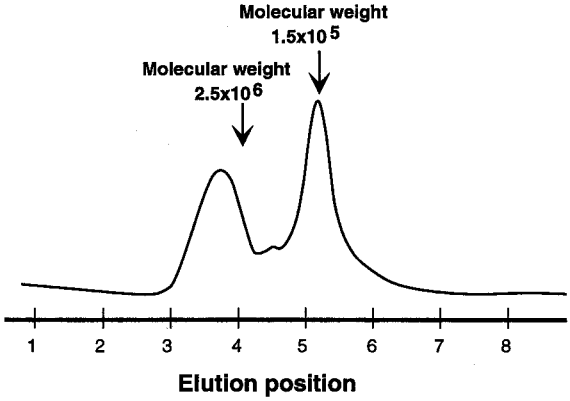

Fig. 2 Elution profile of EPS: column, TSK-Gel 6000 PW; detector, RI; eluant, 0.2 M acetate buffer.
Arrows show the molecular weight markers as indicators.

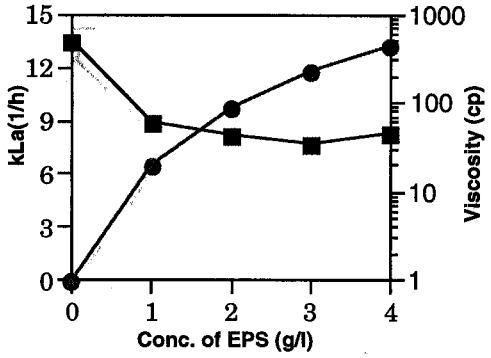

Fig. 3 Effects of the concentration of EPS on the viscosity (●) and k_La (■).

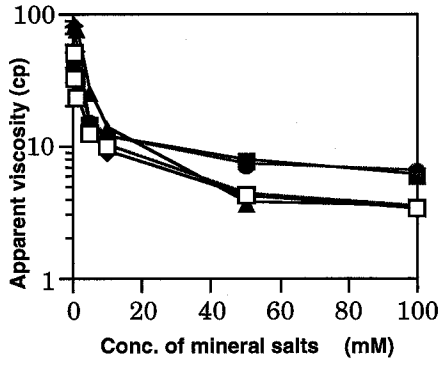

Fig. 4 Effects of the addition of various mineral salts on the viscosity of a solution of EPS. Symbols: ■, NaCl; ●, KCl; ▲, $CaCl_2$; ◆, $BaCl_2$; □, $MgCl_2$.

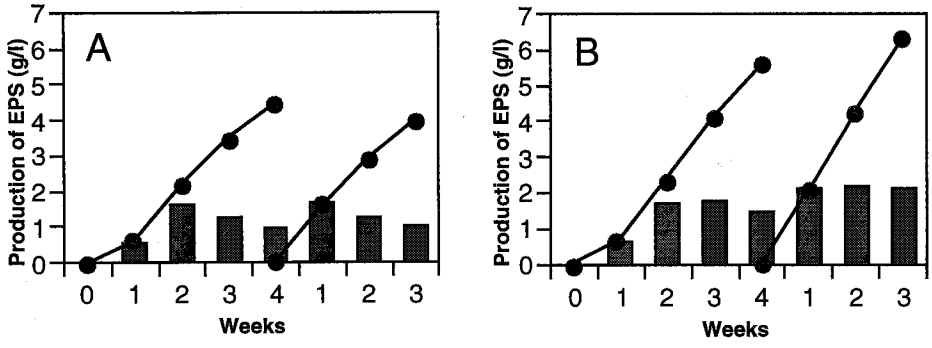

Fig. 5 Time course of the production (●, cumulative production; ■, weekly production) of EPS in LS medium without addition of mineral salts (A) and addition of 30 mM $CaCl_2$ (B).

As shown in **Fig. 3**, the viscosity of an aqueous solution of EPS increased with increasing the concentrations of EPS, for example, a solution of EPS at 4.0 g/*l* had a viscosity of 550 cp. Moreover, k_La decreased from 13 to 9.5/h with the increasing concentrations of EPS and remained constant. **Fig. 3** suggested that the high viscosity of the culture medium can be assumed to be due to EPS accumulated in the culture medium. Therefore, we investigated methods for reducing the viscosity of the culture medium.

Many investigators have reported that the addition of certain mineral salts effectively reduced the viscosity of the solutions of polysaccharides[24,25]. We examined the effects of the addition of mineral salts to viscous solutions of EPS. As shown in **Fig. 4**, all mineral salts tested (NaCl, KCl, $CaCl_2$, $BaCl_2$ and $MgCl_2$) had a mitigating effect on the viscosity. In particular, salts of bivalent cations, such as $CaCl_2$, $BaCl_2$, and $MgCl_2$, were more effective than salts of monovalent cations. The viscosity at 30 mM $CaCl_2$ or $MgCl_2$ was one tenth of the control value. Although some mineral salts are also present in LS medium, these levels are too low to reduce the viscosity of the medium, moreover, they are utilized by cells as nutrients. Based on the results described above, cultures were maintained in LS medium supplemented with 30 mM $CaCl_2$. As show in **Fig. 5-B**, the production of EPS increased markedly as compared to that without the addition of $CaCl_2$ (**Fig. 5-A**). In particular, high production after the second week was maintained.

In investigations of culture conditions, addition of mineral salt such as KCl or $CaCl_2$ was effective in the reduction of the viscosity of culture medium. Resultant production of EPS was 6.5 g/*l*. These results could be reproduced at industrial production by using big vessels.

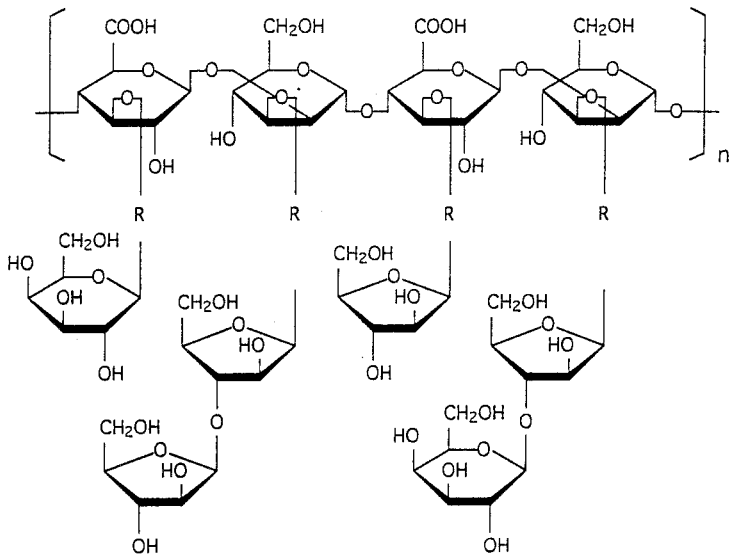

Fig. 6 A tentative structure of the acidic flaction of EPS.

Chemical structure of EPS

It was assumed that EPS recovered from culture medium were consisted of plural macromolecules. Results in colorimetric determinations of sugars by carbazolesulfuric acid method[25] suggested that acidic sugars were contained in EPS. Therefore the EPS were fractionated by ion-exchange chromatography on a column of DEAE Sephadex A-25. From the elution profiles of EPS, acidic and neutral polysaccharides were present at concentrations of about 90% and 10%, respectively. The acidic polysaccharide, a major compornent, was separated and used for stractural analysis. Methylation and GC-MS analysis revealed the presence of 1, 2, 3-mannosyl, 1-arabinosyl, 1, 3-arabinosyl, 1-galactosyl and 1, 3, 4-glucronosyl residues in the molar ratio of 1.00 : 1.08 : 0.85 : 0.75 : 1.08. Additional analysis of its mild acid hydrolysate indicated a tentative structure of the acidic polysaccharide as shown in **Fig. 6**. It possessed a backbone structure composed of a repeating unit of -2)-α-D-mannose(1-4)-β-D-glucronic acid(1-. The side chain units, -1)-α-L-arabinose, -1)-β-D-galactose, -1)-α-L-arabinose-(3-1)-arabinose and -1)-α-L-arabinose-(3-1)-galactose, were connected randomly at c-3 position of the backbone sugars. About 45% of glucronic acids was present as methyl esters.

Application of EPS as ingredients of cosmetic

Topical application of EPS on the skin dose not made tight feeling but smoothing the skin

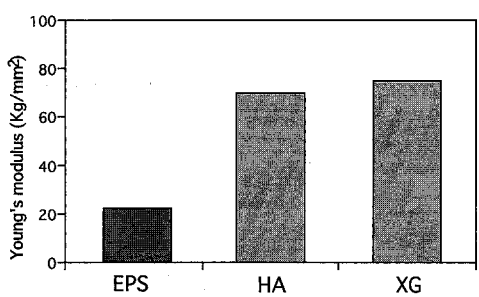

Fig. 7 Young's modulus of various polysaccharides.
HA, hyaluronic acid; XG, xanthan gum.

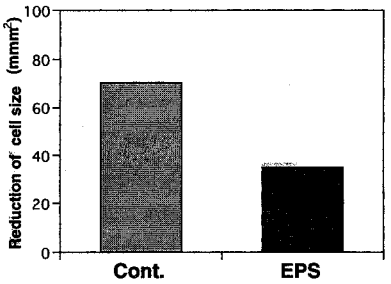

Fig. 8 Effect of long term application of EPS on the chapped skin.

surface. The film of EPS showed lower value of young's modulus than that of other polysaccharides such as xanthan gum and hyaluronic acid used widely as ingredients of cosmetics, especially, at low(40%)relative humidity. The value of young's modulus of EPS was similar to that of epidermis, furthermore, small changes of young's modulus recognized with the changes in relative humidities(**Fig. 7**). It can therefore be presumed that the feeling described above is brought on by such characteristics of EPS. The topical application of EPS also improved chapped skin occurring in chilly and dry conditions. Long term applications of EPS on the skin resulted in the reduction of frictional scaling. It has been known that a part of the skin troubles are caused by external stimuli such as low temperature, dry condition and mechanical irritation. Turnover of epidermal cells was accelerated by such external stimulus, and smaller and nucleated cells are frequently observed consequently[26]. **Fig. 8** shows the effect of long term application of EPS, four months from November to March, on the chapped skin. By long term application of EPS, decrease of the cell size was mitigated compared with control. Application of EPS on the skin might be expected to accelerate normalization of the turnover of epidermis.

In conclusion, the investigations of media constituents and culture conditions brought on the high yield of EPS in the liquid cultures of tuberose cells. Especially, higher concentration of 2, 4-D and addition of mineral salt to reduce the viscosity of culture medium played an important role to increase the production. Produced EPS containing acidic polysaccharide was effective in the chapped skin occurring in chilly and dry conditions. On the bases of this finding, EPS has been commercialized as a ingredient of cosmetics.

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