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Cultivation of *Digitalis lanata* Cell Suspension in an Aqueous Two-Phase System

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Abstract Suspension cultures of *Digitalis lanata* were successfully performed in an aqueous two-phase system (ATPS) of 4.5% polyethylene glycol (PEG) 20,000 and 2.8% crude dextran. Cell growth in the medium containing an individual ATPS-forming polymer was inhibited due to the toxicity of PEG and a high viscosity of dextran. Formation of ATPS supported cell growth by showing a considerably decrease in viscosity and partitioning of cells into a PEG-lean dextran phase. It was found that an aqueous two-phase cultivation of plant cells in a stirred tank bioreactor could be successfully applied.

Key words: Aqueous two-phase system, plant cell culture, *Digitalis lanata*

Plant cells in a suspension culture used as a tool for the production of helpful secondary metabolites have been recognized for many years. Attempts to produce taxol by plant cell cultures instead of extraction from intact plants are a good example for an industrial application [2]. Recently, plant cells have also been considered to be an alternative host for the production of recombinant proteins, because they are able to glycosylate foreign proteins with biological activity [9]. Compared to animal cell cultures, it is easy to cultivate plant cells in a large scale using a simpler and inexpensive medium.

Aqueous two-phase systems (ATPSs) that are composed of two hydrophilic polymers have been used to separate and purify biomolecules and cells [8]. Even though their main application is in bioseparation, increasing attention has been concentrated on its use in extractive bioconversion or *in situ* extraction with a cell cultivation. Extractive bioconversion using ATPSs is suitable for integrating cell cultures and downstream processing for the enhanced production of an extracellular product. For an efficient *in situ* extraction, good cell growth in ATPS is necessary. Growth of microbial cells in ATPS has been performed for an extractive bioconversion [7]. In addition, animal cells such as hybridomas and Chinese hamster ovary cells were cultured in these systems as well [11, 12]. Cultivation of plant cells in an aqueous two-phase polymer system was first reported by Hooker and Lee [4]. They showed that a two-phase system provided a viable medium for *Nicotiana tabacum* suspension culture. The productivity of secondary product was enhanced by immobilizing cells in one phase while collecting and withdrawing products in the other phase. Successful growth of plant cells in two-phase mixtures was also carried out by other researchers [5, 6]. Along with suspension cells, hairy roots of *Tagetes patula* were grown in ATPSs to produce thiophene [1]. However, detailed analysis regarding plant cell culture in ATPSs has been limited today.

In this study, cultivation of *Digitalis lanata* plant cell suspension cultures was looked into by using ATPS composed of polyethylene glycol (PEG) and dextran. Growth characteristics in the individual ATPS-forming polymer system were compared and physical properties were closely examined in order to identify the key factors promoting cell growth in two-phase media. Furthermore, to confirm the scale-up possibility of the plant cell growth in ATPS, cultivation in a stirred tank bioreactor was attempted.

Materials and Methods

Plant Cell Culture and Media

The *Digitalis lanata* cell line of K3OHD was kindly provided by Dr. Wolfgang Kreis (Friedrich-Alexander-Universtät Erlangen-Nürnberg, Germany) and had been maintained in a modified Murashige and Skoog (MS) medium containing 33 g/l of glucose, 4 mg/l glycine, and 170 mg/l of KH_2PO_4. Growth regulator was omitted and pH was adjusted to 5.5. Suspension cells were grown in

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125-ml Erlenmeyer flasks with 50 ml of medium on a rotary shaker at 120 rpm under a dark condition. Culture temperature was 25±1°C. Cells were subcultured every 10 days. The cells in the late exponential growth phase have been used as an inoculum. To investigate the effect of phase-forming polymers on plant cell growth, solutions of 15% (w/w) PEG 20,000 and 4% (w/w) crude dextran were made separately by using a modified MS medium. For the formulation of aqueous two-phase media, each solution was mixed with 1:2.33 mass ratio to make sure that it secures enough bottom phase volume for the cell growth. As a result, the composition of ATPS became 4.5% PEG 20,000 and 2.8% crude dextran. Aqueous two-phase cultivation in a stirred tank bioreactor was carried out in a 5-l plant cell bioreactor (Korea Fermentor Co., Inchon, Korea). All the chemicals used were purchased from Sigma Chemical Co. (St. Louis, U.S.A.) and the cultures were performed in triplicate.

Dry Cell Weight Determination

For dry cell weight measurements, cell suspension was filtered through Whatman No. 1 filter paper and washed with hot distilled water to remove polymers. The cells were dried at 60°C to constant weight.

Viscosity and Osmolality

Viscosity of each polymer solution and an aqueous two-phase system in cp (centipoise) was measured using a viscometer Model RI:1:L (Rheology International Shannon Ltd., Co. Clare, Ireland). Osmolality was measured with an OSMETTE A osmometer (Model No. 5002, Precision Systems Inc., Natick, U.S.A.) and expressed in units of milliOsmole per kg of water (mOsm/kg).

Results and Discussion

Plant Cell Growth in Aqueous Two-Phase System

Aqueous two-phase systems can be applied to plant cell suspension cultures as one of the *in situ* extraction methods to enhance production of hydrophobic secondary metabolites or extracellular proteins such as enzyme. Selective removal of the product during bioconversion using plant cells may also be possible in ATPS. Prior to obtaining phytochemicals with high yields using ATPS, however, reliable cultivation of plant cells in ATPS should be proved.

In preliminary experiments, a 4.5% PEG 20,000 and 2.8% crude dextran system was made by mixing 15% of PEG 20,000 and 4% of crude dextran solution in the mass ratio of 1:2.33, that was selected for *Digitalis lanata* cell cultures based on the distribution characteristics of cells and product. In this system, cells were totally partitioned in the bottom phase. The time course of growth pattern was investigated in shake flasks containing the selected aqueous two-phase polymer system, and the results are shown in Fig. 1. For comparison, control culture in the normal MS medium was performed with respective cultivation in a medium containing 15% PEG 20,000 or 4% crude dextran to clarify the influence of individual ATPS-forming polymer. Cell growth was significantly inhibited by the addition of 15% PEG 20,000 only. The length of the lag period was extended up to 4 days and a slight extent of the growth was observed. In the presence of 4% crude dextran, it also lengthened the lag period, but growth rate after the lag was much higher than that with PEG. However, in ATPS with both polymers, the time course of cell growth showed a similar pattern to that of the control culture. A toxic effect of PEG at high concentrations was also observed in animal cell cultures [11], even though low concentrations of PEG were reported to stimulate cell growth [10]. The toxicity at high concentrations may be due to changes in physical properties of the culture medium. PEG may contain some inhibitory compounds which can exert a toxic influence on the cells [7]. Since PEG is a well-known fusogen for various types of cells, direct interaction of PEG with the cell surface may also affect cell growth. The molecular weight of PEG can be an another factor to be considered for the plant cell growth. It was shown that growth rate and stationary phase cell concentration decreased with the lower molecular weight of PEG in an aqueous two-phase cultivation of *Nicotiana tabacum* [4]. Although dextran was much less inhibitory once cells started to grow, the concentration and molecular weight of dextran could also affect cell growth. The reason why it took a considerably long time to enter the exponential growth phase in ATPS and individual ATPS-forming polymer was possibly due to the fact that time was required for plant cells to adjust to a

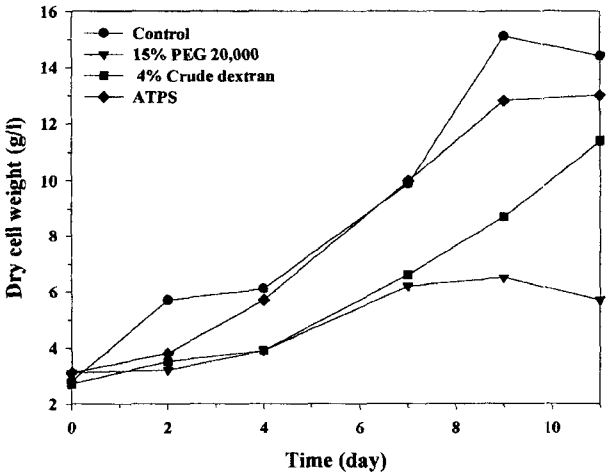

Fig. 1. Comparison of cell growth in an individual polymer solution and ATPS using flask cultures.
The composition of ATPS was 4.5% PEG 20,000 and 2.8% crude dextran which was a mixture of 15% PEG 20,000 and 2.8% crude dextran solution in the mass ratio of 1:2.33.

new environment. Ileva *et al.* [5] suggested that the slow growth in an aqueous two-phase cultivation system might be due to the mass transfer limitation of the culture medium components.

Viscosity and Osmolality of Aqueous Two-Phase System

It was of interest to observe that plant cells could grow successfully in ATPS despite the fact that their growth was inhibited in an individual ATPS-forming polymer. Therefore, effects of ATPS and ATPS-forming polymers on the physical properties such as viscosity and osmolality of the culture medium were examined. At first, the viscosity of solutions without polymers, with both PEG and dextran alone, and ATPS was measured based on water, growth medium (modified MS medium), and medium used for bioconversion (8% glucose solution in plain water without nutrients). As shown in Fig. 2, the viscosity of the 4% crude dextran solution was found to be very high, but that of the 15% PEG was much lower in all three solutions. Viscosity of any solution without polymers was about 1-2 cp. In a growth medium with dextran alone, it reached up to 350 cp. At this high viscosity, the adequate mixing required for cell culture could not be expected. This may be the reason for the growth inhibition shown in Fig. 1. Interestingly enough, when both polymer solutions were mixed to form ATPS, viscosity became considerably lowered because of the formation of emulsion. Reduction of viscosity probably enhanced the oxygen transfer that is required for cell growth. Reasonable cell growth in ATPS could be explained by this reduction of viscosity of the medium. Hooker and Lee [4] pointed out the importance of miscibility of the two polymer phases. Higher phase miscibility may cause less resistance to the nutrient mass transfer including oxygen, thus facilitating growth. As the cells were completely partitioned in a dextran phase, direct contact with PEG could be prevented. In combination, cell

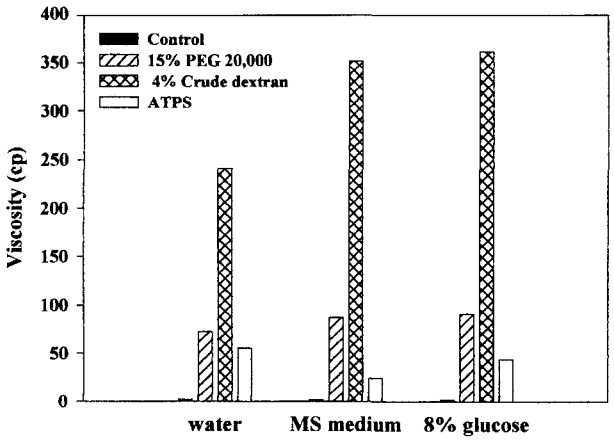

Fig. 2. Viscosity of each polymer solution and ATPS based on water, MS Medium, and 8% glucose solution.
The composition of ATPS was 4.5% PEG 20,000/2.8% crude dextran.

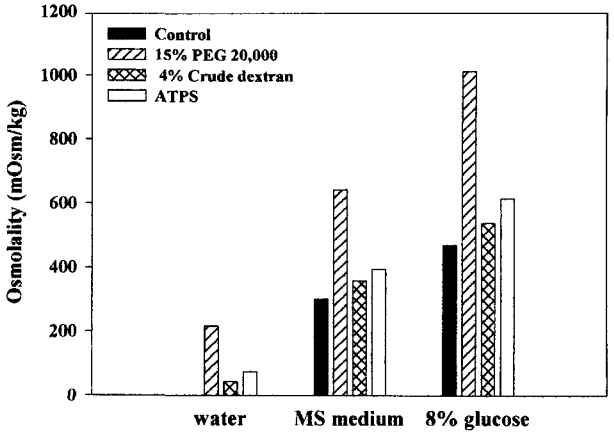

Fig. 3. Osmolality of each polymer solution and ATPS based on water, MS Medium, and 8% glucose solution.
The composition of ATPS was 4.5% PEG 20,000/2.8% crude dextran.

growth in ATPS seemed to be much better than that in each phase-forming polymer.

Dextran and especially PEG were found to have strong concentration-dependent effects on osmolality of the animal cell culture medium [11]. Therefore, an effect of ATPS-forming polymers on osmotic pressure in a plant cell culture medium was tested (Fig. 3). Addition of 15% PEG into the MS medium gave an osmotic pressure of over 640 mOsm/kg while 4% crude dextran gave 358 mOsm/kg. In ATPS, 395 mOsm/kg was observed (301 mOsm/kg in normal MS medium). Use of 8% glucose solution showed much higher osmotic pressure due to the presence of a high concentration of sugar. Osmolality is an important variable in an animal cell culture, but this is not the case in a plant cell suspension culture. Since plant cells have cell wall, this range of osmolality is tolerable. High osmolality usually reduced plant cell size without having an apparent effect on the growth rate.

Aqueous Two-Phase Cultivation of Plant Cells in a Bioreactor

Finally, in order to confirm the scale-up possibility of plant cell growth in ATPS, cultivation in a bioreactor was performed. Cell growth in 4.5% PEG 20,000/2.8% crude dextran ATPS was monitored in a 5-liter stirred tank bioreactor with a 2-liter working volume. The results are shown in Fig. 4. Growth pattern was similar to that in the shake flasks shown in Fig. 1. Aqueous two-phase cultivation of *D. lanata* suspension cells was possible in bioreactors and final cell mass was slightly lower than that of the control culture. During the cultivation in ATPS, the cell size index (fresh cell weight/dry cell weight) was maintained at a low value compared to the control and this was caused by the high osmolality of the two-phase medium (data not shown). Viscosity of an aqueous two-phase culture was sustained between 25 and 35 cp.

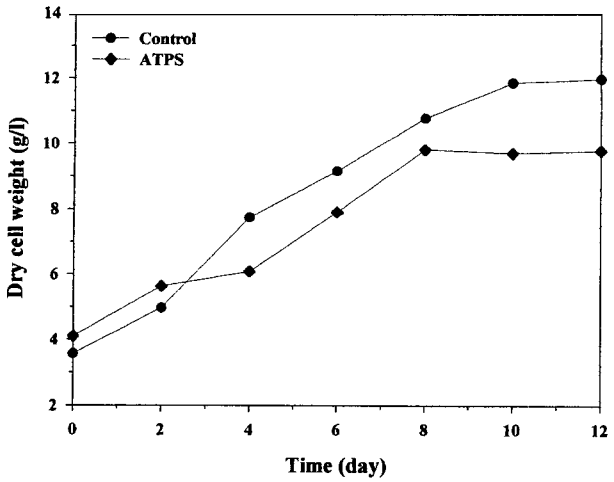

Fig. 4. Aqueous two-phase cultivation in plant cell bioreactors.

However, in the control culture, it kept increasing from 6 to 25 cp until the end of cultivation.

In conclusion, it was observed that plant cell suspension cultures in an aqueous two-phase polymer system was feasible in shake flasks as well as in bioreactors for the production of various phytochemicals. This was made possible by lowering the viscosity with the formation of ATPS, which could promote the oxygen transfer. However, the cell growth in ATPS fell somewhat short of reaching full growth of the control culture. Therefore, factors enhancing cell growth in ATPS up to or more than the control level should be found to make this system more valuable. In addition to the cell growth, *in situ* production of phytochemicals will be studied. Since the production of digoxin by digitoxin biotranformation was enhanced by *in situ* adsorption in *D. lanata* cell cultures [3], it is expected that an application of ATPS with an adequate integrated bioprocess should possess a potential enhancement of digoxin production. We strongly expect that this system can also be used for the production of secretory enzyme or recombinant protein in plant cell cultures.

Acknowledgments

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