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Effects of Pluronic F-68 on Cell Growth of *Digitalis lanata* in Aqueous Two-Phase Systems

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Abstract The effects of Pluronic F-68, a non-ionic surfactant, on the growth and physical characteristics of *Digitalis lanata* suspension cultures were investigated in aqueous two-phase systems (ATPSs) composed of 4.5% polyethylene glycol (PEG) 20,000 and 2.8% crude dextran. In the range of 0.1–10.0 g l^{-1}, Pluronic F-68 enhanced the maximum cell density in a medium with ATPSs, even though Pluronic F-68 did not affect cell growth in a normal growth medium. In terms of physical properties of ATPSs with cell suspension cultures, 0.2 g l^{-1} of Pluronic F-68 reduced viscosity by up to 40%, while 0.1 g l^{-1} of Pluronic F-68 significantly enhanced the oxygen transfer rate. In addition, we successfully performed aqueous two-phase cultivation in a 5-l stirred tank bioreactor with 0.5 g l^{-1} of Pluronic F-68, and discovered that cell growth in ATPSs was similar to that in normal growth medium.

Key words: *Digitalis lanata*, aqueous two-phase system, Pluronic F-68, bioreactor, oxygen transfer rate

Aqueous two-phase systems (ATPSs) are composed of either a hydrophilic polymer and a concentrated salt solution, or two hydrophilic polymers, and are designed to separate or purify biomolecules and biomass [18]. Recently, more attention has been focused on the use of ATPSs to integrate cell cultures with downstream processing, because ATPSs could be more advantageous due to their high water content, low interfacial tension, which facilitates mass transfer, and more biocompatible characteristics, compared to the conventional extraction system using an organic solvent [1]. An example of this is the extractive bioconversion or *in situ* extraction of secreted protein with fermentation [12]. For efficient integrated bioprocessing, ATPSs must support cell growth. In addition to microbial fermentation, ATPSs could be a valuable medium for animal cell growth [25].

Plant cell suspension cultures have been recognized as an alternative route for producing useful metabolites [4, 23]. Furthermore, plant cells could be a cost-effective expression system for producing recombinant proteins as biopharmaceuticals with appropriate post-translational modifications [13, 14, 16]. Thus, the application of ATPSs into plant cell suspension cultures may be valuable for the enhanced production of foreign proteins. However, optimization of ATPS composition is required for the proper partition of cells and products, securing enough volume in the cell-rich phase and adequate cell growth. An attempt to optimize the composition of ATPSs for plant cell growth was first reported by Hooker and Lee [8]. They found that *Nicotiana tabacum* could grow in optimized aqueous two-phase formulas with proper partitioning characteristics of cells and nutrients. Other researchers have also reported that the growth of plant cell suspensions could be maintained in ATPSs, and that the hairy roots of *Tagetes patula* could be grown in ATPSs with enhanced secretion of thiophenes [3]. Unfortunately, the growth in ATPSs was lower compared to a control culture, using normal growth medium. Therefore, in order to maximize the performance of integrated bioprocesses for the enhanced recovery of secreted products with appropriate partitioning, cell growth in ATPSs should be improved to a level similar to the control culture.

Pluronic F-68, a copolymer of ethylene oxide and propylene oxide, has been widely used to protect animal or insect cells from hydrodynamic stress in bioreactors [21, 24]. The mechanism of shear protection by Pluronic F-68 has generally been regarded as its interaction with cell surface, which reduces the adsorption between the cells and gas bubbles in a sparged environment, and the cells could consequently be protected from bubble-rupture damage [20]. In addition to shear protection, positive effects of Pluronic F-68 on cell growth in serum-free media were also reported. According to Palomares *et al.* [22], Pluronic

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F-68 could strongly bind to the cell surface and favorably affect cell physiology.

In our previous study, it was shown that the cultivation of *Digitalis lanata* suspension cell cultures in ATPSs was possible [5]. However, it was observed that a gap between cell growth in ATPSs and full growth in the control culture still existed both in a flask and a 5-l stirred tank bioreactor. Therefore, the effects of Pluronic F-68 on cell growth and physical properties in ATPSs were investigated in the present study to overcome the cell growth limitation in ATPSs. Additionally, to confirm the possibility of scale-up and reproducibility of growth promotion by Pluronic F-68, we studied the cultivation of suspension cells, using ATPSs in a 5-l stirred tank bioreactor.

MATERIALS AND METHODS

Plant Cell Line, Medium, and Culture

The *Digitalis lanata* K3OHD was kindly provided by Dr. Wolfgang Kreis (Friedrich-Alexander-Universtät Erlangen-Nürnberg, Germany) and has been maintained in a modified Murashige and Skoog (MS) medium, containing 33 g l^{-1} of glucose and 0.17 g l^{-1} of KH_2PO_4 without growth regulators. The pH of the medium was adjusted to 5.5 prior to autoclaving. Cell suspensions were maintained in 500-ml Erlenmyer flasks with 180 ml of fresh medium on a gyratory shaking incubator, operating at 120 rpm under a dark condition. Culture temperature was kept at 25°C. Every 10 days, 70 ml of cell suspensions were transferred to a fresh medium.

Preparation of an Aqueous Two-Phase System (APTS)

Each polymer solution, 15% (w/w) PEG 20,000 and 4% (w/w) crude dextran, was made separately by using a modified MS medium, then mixed with 1:2.33 mass ratio to form ATPS. The resulting composition of ATPS was 4.5% PEG 20,000 and 2.8% crude dextran.

Bioreactor Operation

A 5-l stirred tank bioreactor (Kobiotech Co., Incheon, Korea) with a working volume of 2 l was used to cultivate plant cells. The agitation rate and aeration rate were 80 rpm and 0.1 vvm, respectively. Suspension cells, cultured in flasks for 10 days, were used as an inoculum at 10% (v/v). The temperature was kept constant at 25°C, and agitation was achieved with a 4-bladed hollowed paddle impeller.

Cell Mass Measurement

For the measurement of dry cell weight, cell suspensions were filtered through Whatman No. 1 filter paper in a vacuum and washed three times with hot distilled water to remove polymers. Then, the cells were transferred to pre-weighed dishes and dried at 60°C.

Viscosity and Oxygen Transfer Rate

The viscosity of the ATPSs and MS medium was measured, using a viscometer Model RI:1:L (Rheology International Shannon Ltd., Co. Clare, Ireland) at room temperature. The oxygen transfer rate of the bioreactor was measured by the dynamic gassing-out method, at 25°C, using a YSI model 50B (YSI Inc., Yellow Springs, Ohio, U.S.A.) dissolved oxygen meter.

RESULTS AND DISCUSSION

Effect of Pluronic F-68 on *Digitalis lanata* Cell Growth in Control Culture and ATPS

In this study, the effect of Pluronic F-68 concentration on the growth of *D. lanata* suspension cells in a modified MS medium was investigated, and the results are shown in Fig. 1. No growth promotion of Pluronic F-68 (0.1- 10.0 g l^{-1}) was observed at any of the concentrations in a normal growth medium. Cell growth decreased by 11%, compared to the control culture, at 0.1 g l^{-1} of Pluronic F-68. In contrast, as shown in Fig. 2, the presence of Pluronic F-68 in ATPS promoted cell growth, yielding maximum cell growth at 0.5 g l^{-1} of Pluronic F-68. This result shows that the stimulatory effect of Pluronic F-68 on cell growth in ATPS most likely originates from physical, rather than biological, mechanisms.

There are many substances known to promote plant growth [9, 17]. Pluronic F-68 has been known to stimulate the growth of plant cells in suspension cultures [11]. The stimulating effects varied with the culture type, which include suspension cell cultures, root cultures, and protoplast cultures. Even though the growth of *Solanum dulcamara* suspension cells was not altered by the addition of 0.01-1.0% (w/w) Pluronic F-68, the growth of the transformed roots of *S. dulcamara* was enhanced by the same range of

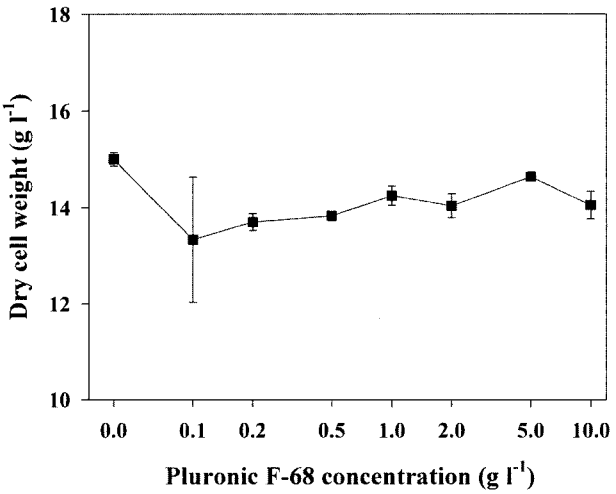

Fig. 1. Effects of Pluronic F-68 on *Digitalis lanata* cell growth in a modified MS medium.

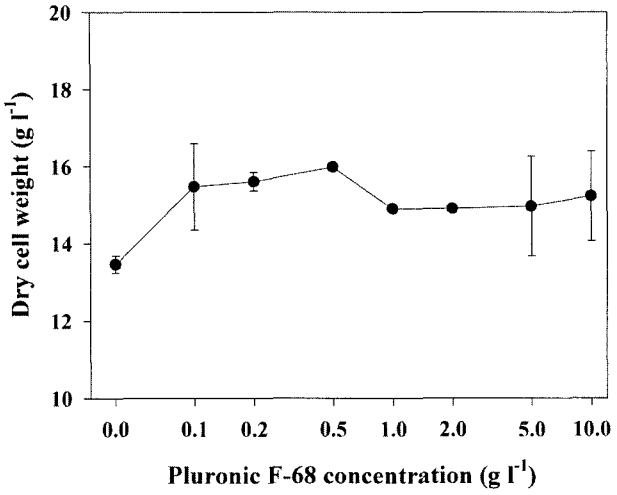

Fig. 2. Effects of Pluronic F-68 on *Digitalis lanata* cell growth in ATPS.

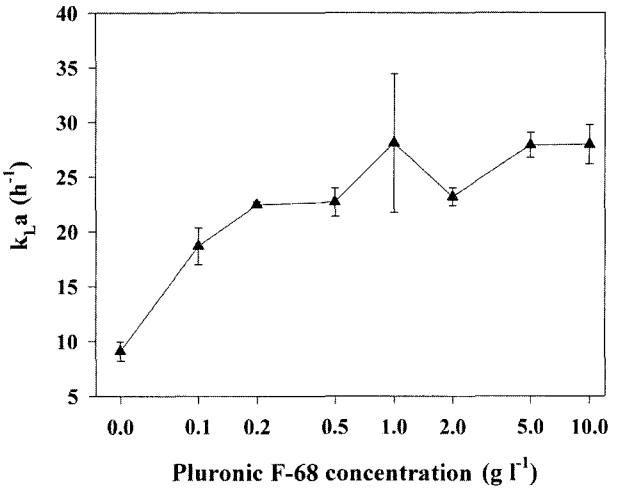

Fig. 4. Changes of the oxygen transfer coefficient in ATPS at various concentrations of Pluronic F-68.

Pluronic F-68 [10]. Furthermore, it was found that Pluronic F-68 enhanced post-thaw growth of cryopreserved *Oryza sativa* Taipei 309 cells [2]. It was suggested that the adsorption of Pluronic F-68 onto cell surfaces exerted growth-stimulating effects on the cryopreserved cells by overcoming certain perturbations, such as respiratory impairment or cellular damage during rehydration. Regardless of whether Pluronic F-68 changes cell physiology or not, it is apparent that Pluronic F-68 can interact directly with cell surfaces, despite the presence of cell walls. Consequently, it is possible that Pluronic F-68 can affect cell growth and their metabolism by either biological or physical mechanisms.

Effect of Pluronic F-68 on Physical Properties of ATPS

In order to confirm the effect of Pluronic F-68 on the physical properties of ATPS, the viscosity of ATPS was measured at various concentrations of Pluronic F-68. As shown in Fig. 3, the viscosity of ATPS containing 0.2 g l^{-1} of Pluronic F-68 was the lowest. The reduction of viscosity was about 40%, compared to that of ATPS without Pluronic F-68. Several researchers have reported reasonable plant cell growth in ATPSs [5, 8], and Choi *et al.* [5] suggested that the formation of ATPS decreased the viscosity of each ATPS-forming polymer. Unfortunately, the viscosity of ATPS was still higher than that of the normal growth medium, therefore, cell growth in ATPS could be inhibited.

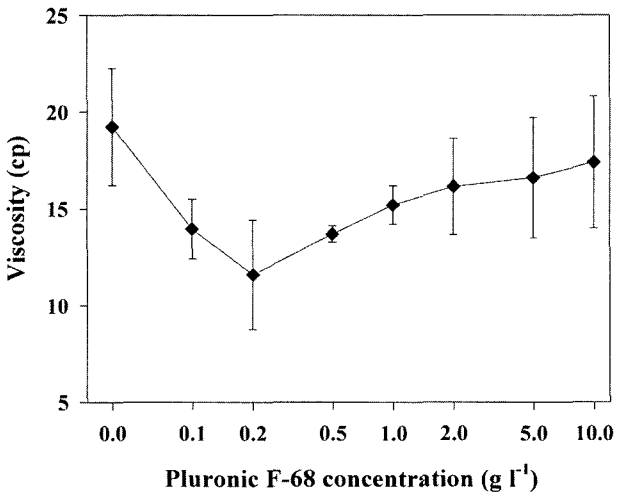

Fig. 3. Changes of the viscosity in ATPS at various concentrations of Pluronic F-68.

The oxygen transfer coefficient in ATPS was measured at various concentrations of Pluronic F-68, since viscosity was most likely an important factor to influence mass transfer. The results are shown in Fig. 4. The oxygen transfer coefficient significantly increased from 9.1 h^{-1} to 28.1 h^{-1} in the presence of 1.0 g l^{-1} of Pluronic F-68. The observation that the addition of Pluronic F-68 into ATPS could enhance the oxygen transfer rate, despite recognition of Pluronic F-68 as an inhibitor of gas-liquid mass transfer, was notable [6]. Morão *et al.* [19] also reported that antifoam reduced mass transfer in a fermenter, but that the reduction of oxygen transfer rate was diminished at high viscosity. In the present experiment, it was found that Pluronic F-68 could enhance the oxygen transfer in ATPS by lowering viscosity. Thus, the adverse effect of Pluronic F-68 as a surfactant in mass transfer could be avoided or compensated by relatively high ATPS viscosity levels.

Cultivation of *D. lanata* Suspension Cells in a Bioreactor

In addition, *D. lanata* suspension cells were cultivated in a 5-l stirred tank bioreactor to confirm the possibility of scale-up by using ATPS and the reproducibility of Pluronic F-68 action in a bioreactor. As shown in Fig. 5, the cell

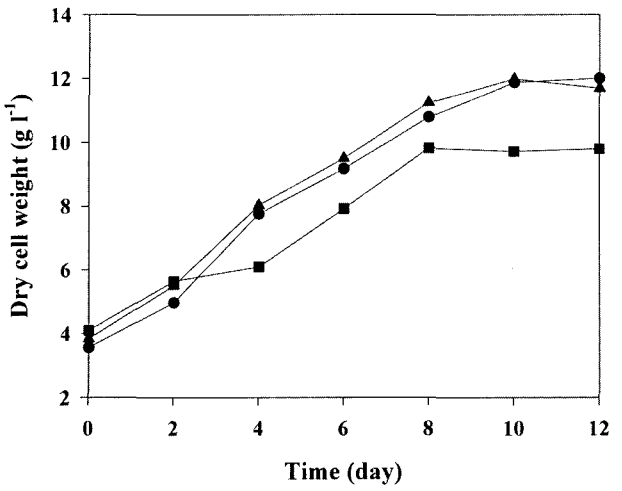

Fig. 5. Time course changes of *Digitalis lanata* cell growth in a 5-l stirred tank bioreactor using a modified MS medium (●) as a control culture and ATPS with (▲) or without (■) 0.5 g l^{-1} of Pluronic F-68.

growth in the bioreactor was similar to the result in flasks as described in our previous report [5]. As mentioned above, maximum cell density in aqueous two-phase cultivation was slightly lower than that of the control culture with a modified MS medium. However, when 0.5 g l^{-1} of Pluronic F-68 was added to the bioreactor, the final dry cell weight in ATPS went up to 11.7 g l^{-1}, which was almost equivalent to the level of the control culture (12.0 g l^{-1}). Consequently, cell growth in ATPS was enhanced to control level by the help of Pluronic F-68 in a stirred tank bioreactor. In conclusion, the decreased cell growth in ATPS was overcome by the addition of Pluronic F-68, which enhanced oxygen transfer in ATPS by lowering viscosity.

Hooker and Lee [8] pointed out the importance of oxygen transfer and its limitations in ATPS. In addition, Zijlstra *et al.* [25] also explained that the poor growth of animal cells in ATPS was due to the changes in the physical properties of the culture medium, such as the reduced oxygen transfer or reduced NaCl levels. Oxygen transfer is also important in microbial cultures producing high molecular weight polymer due to high viscosity [15]. The use of Pluronic F-68 as a medium additive could solve this problem in ATPS, and the increased mass transfer could successfully support cell growth. This result made ATPS a more valuable medium for the integrated bioprocesses with plant suspension cells. Since the bioconversion of digoxin from digitoxin by *D. lanata* was enhanced by *in situ* removal [7], this makes ATPS an alternative choice for *in situ* extraction of natural products or recombinant proteins produced by transgenic plant cell cultures. In that case, combination of ATPS with Pluronic F-68 would enable good plant cell growth as well as efficient production and integrated recovery.

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