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Perfusion Cultivation of Transgenic *Nicotiana tabacum* Suspensions in Bioreactor for Recombinant Protein Production

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Abstract A perfusion culture of transgenic *Nicotiana tabacum* cell suspensions, transformed to express recombinant β-glucuronidase (GUS), was successfully performed in a 5-l stirred tank bioreactor. With 0.1 day^{-1} of perfusion rate, the maximum dry cell weight (DCW) reached to 29.5 g/l in 16 days, which was 2.1-fold higher than the obtained in batch culture (14.3 g/l). In terms of the production of GUS, the volumetric activity could be increased up to 12.8 U/ml by using perfusion, compared with 4.9 U/ml in batch culture. The specific GUS activities in both perfusion and batch cultures were maintained at similar levels, 200–400 U/gDCW. Consequently, a perfusion culture could be a good strategy for the enhanced production of recombinant proteins in a plant cell culture system.

Key words: Bioreactor, β-glucuronidase, *Nicotiana tabacum*, perfusion culture, transgenic plant cell culture

Transgenic plants or plant cell cultures have been recognized as an alternative host for the cost-effective production of valuable foreign proteins [7, 8]. Recent related studies have indicated the feasibility of using plant cell culture systems for the production of pharmaceutically useful proteins such as human interleukin-2, interleukin-4, human granulocyte-macrophage colony-stimulating factor, and α_1-antitrypsin [4, 6, 11, 18]. It is possible for plant cells to grow in simple, cheap, and protein-free media, so that the cost for cell cultivations and downstream processing steps can significantly be reduced, compared with animal cell culture systems. In addition, the expression of foreign proteins in plant cells is more advantageous than that of microbial systems, because plant cells have a post-translational modification machinery, which can play a crucial role in proper protein-folding and in the determination of biological activity of the proteins produced [2, 5]. Moreover, recent advances in plant molecular engineering techniques as well as the growing attention on plant genomes and proteomes are expected to solve several problems related to the use of plant cells in commercial application, such as the low productivity and slow growth rate [9].

High-density cultures are favorable processes for increasing the volumetric productivity with a higher cell density and a prolonged productive period. A high cell density can be achieved by feeding the fortified media or by perfusion [16, 20]. Even though fed-batch cultures have widely been adopted in practical processes, due to their simple operation and ease of control, perfusion cultures have more advantages. For example, because perfusion cultures can be carried out in a constant volume, the need for additional utilities or extra inoculum can be avoided [19]. Additionally, the removal of spent medium by media replacement can promote cell growth by reducing the inhibitory secondary metabolites produced during the culture period [3]. Especially for the operation of two-stage cultures, the perfusion processes make it easy to convert the growth phase to the production phase, simply by exchanging media in the reactor [15]. Optimization of media composition could also offer significant improvements in the production of foreign proteins in transgenic plant cell suspension cultures [14].

In this study, we have demonstrated the potentials of high-density transgenic plant cell suspension cultures using a perfusion strategy for the enhanced production of the recombinant protein, β-glucuronidase, as a model system. Even though there have been several reports regarding the use of perfusion cultures with plant cells for the production of secondary metabolites, to the best of our knowledge, this is the first study presenting the enhanced production of recombinant protein by a perfusion process in a bioreactor.

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MATERIALS AND METHODS

Plant Cell Culture and Media

Transgenic *Nicotiana tabacum* suspension cells were maintained in a modified Murashige and Skoog (MS) medium containing 30 g/l of sucrose, 0.1 g/l of myo-inositol, 2 mg/l of 2,4-dichlorophenoxyacetic acid, and 0.02 mg/l of kinetin. For the continuous selection of transgenic plant cells, filter-sterilized kanamycin (100 mg/l) was added following autoclaving. The pH of the medium was adjusted to 5.9 prior to autoclaving. The cell suspension was maintained in a 500-ml Erlenmyer flask with 180 ml of fresh medium on a gyratory shaking incubator at 120 rpm in the dark. Culture temperature was 25°C. Every 7 days, 70 ml of the cell suspensions was transferred to fresh medium.

Bioreactor Operation and Perfusion Culture

A 5-l plant cell bioreactor (Kobiotech Co., Incheon, Korea) with a 2-l working volume was used for the batch and perfusion cultures of transgenic plant cell suspensions [10]. The initial agitation and aeration rates were 80 rpm and 0.2 vvm, respectively. The agitation rate was gradually increased to 350 rpm as the cell concentration increased. The suspension cells cultivated in the flasks for 7 days were used for the inoculation into the bioreactor. Inoculum size was 20% (v/v). The temperature was kept at 25°C, and the agitation was achieved with a 4-bladed hollowed-paddle impeller. For continuous separation of the media and cells, a mesh filter was built in the inner part of the bioreactor as a perfusion device. The removal of cell-free spent media and the addition of perfused media were carried out using a peristaltic pump. Every day, 10% of the total volume of spent medium was replaced with the same volume of fresh media with 2-fold concentrations of sucrose and basal salt mixtures with cell retention.

Quantitative Analysis of Cell Mass

To measure fresh cell weight (FCW), the cell suspension was filtered through a Whatman No. 1 filter paper under vacuum and washed three times with distilled water to remove the residual sugar from the cell surface. The cells were then transferred to a preweighted dish, and the mass was measured. The dry cell weight (DCW) was estimated after drying for 2 days at 60°C.

Measurement of GUS Activity

The measurement of recombiant β-glucuronidase (GUS) activity in transgenic plant cell suspension was carried out using a fluorometric method employing 4-methylumbelliferone (4MU) as a standard. GUS is originally produced by intestinal bacteria [12]. A fixed amount of fresh cells (0.4 g) was mixed with 0.8 ml of an extraction buffer composed of 50 mM sodium phosphate (pH 7.0), 10 mM β-mercaptoethanol, 10 mM sodium ethylenediaminetetraactetic acid (Na_2EDTA), 0.1% N-lauroylsarcosine, and 0.1% Triton X-100. The cell suspensions were sonicated for 90 sec, an 8 sec on with 2 sec off pulse, at 4°C and they were then centrifuged for 15 min at 12,000 ×*g*. The supernatant was added to the same amount of a reaction buffer containing 1 mM 4-methylumbelliferyl β-D-glucuronide as a substrate in the extraction buffer. The reaction was performed at 37°C for 30 min and stopped by the addition of 0.2 M sodium carbonate. After the reaction, the emitted fluorescence of 4MU produced by GUS was measured with a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc., San Francisco, U.S.A.).

RESULTS AND DISCUSSION

Preliminary experiments have been performed to investigate the effect of the perfusion start time, and exchanging the medium during the late lag phase was found to extend the lag period. The reasons for this inhibition of the cell growth might be the high osmolality resulting from an excess influx of fresh medium, or the loss of unknown factors that could affect the cellular adaptation by the efflux of conditioned medium. In addition, the start of perfusion following the exponential phase could replenish the culture broth and re-boost the cell growth; however, the cell growth might be slightly limited owing to the lowered fraction of viable cells. From the above results, the optimum perfusion start time was selected as the 6th day, at the middle of the exponential phase. Figure 1 shows the cell growth during the perfusion culture at the selected optimum perfusion start time. Compared with a control culture, the replacement with fresh medium by perfusion could extend

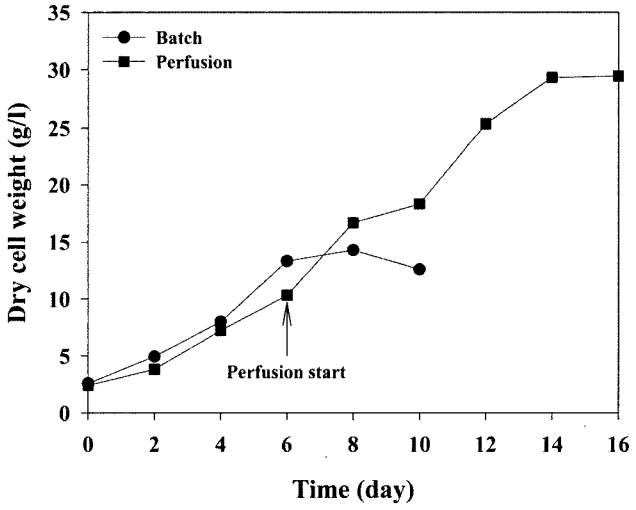

Fig. 1. Time profile of dry cell weight during batch culture and perfusion culture.

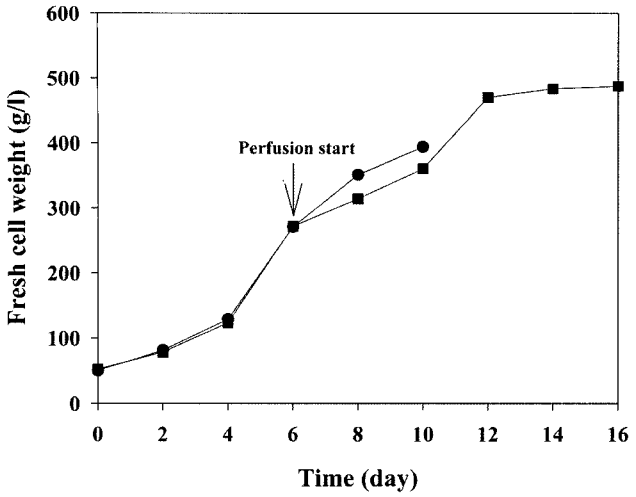

Fig. 2. Time profile of fresh cell weight during batch culture and perfusion culture.

the exponential phase, since the cell density was drastically increased without nutrient depletion. As a result, a maximum cell density of 29.5 gDCW/l could be achieved in 16 days, which was 2.1 times higher than the 14.3 gDCW/l obtained in a batch culture. During the perfusion period, the sugar concentration was maintained in the range of 30–40 g/l, and the conductivity that represents the consumption of minerals or salts in media did not drop below 1000 S (data not shown). However, further cell growth above 30 gDCW/l was not observed despite the presence of sufficient nutrient, and the overproduction of cells made it very difficult to maintain homogeneity in the bioreactor. These results suggested that, in order to increase the cell density even further, a greater mass transfer or occupation of space at high cell density should be considered.

In terms of the fresh cell weight, the maximum cell density obtained by perfusion was 488 gFCW/l, whereas it was 394 gFCW/l in the batch culture (Fig. 2). The fresh cell weight as well as the dry cell weight is also an important parameter for monitoring high-density culture of plant cells, because the mass transfer or attainable maximum cell density is determined by the fresh cell weight in the practical operation of a bioreactor [17]. With our results, we thought that the maximum attainable fresh cell weight in *N. tabacum* cell culture was about 500 g/l. In order to increase the dry cell weight, another strategy such as the reduction of FCW/DCW ratio would be necessary. The FCW/DCW ratio has been used as a cell size index in plant cell suspension cultures. Figure 3 shows the change in the cell size index during the culture period. Generally, a reduction of the cell size to maintain homeostasis within a new environment, including the high osmotic pressure of a fresh medium, was required during the lag period [13]. A gradual increase in the cell size was observed with a decrease in the medium osmolality, which could be related to rapid uptake of sugars and salts by the cells. For this reason, the dry mass could not be increased by more than 14.3 gDCW/l in the control culture, although the fresh cell weight was continually increased over the same period. However, the influx of fresh medium by perfusion at the 6th day apparently reduced the cell size index from 26 to 18 and the FCW/DCW ratio was maintained at the lower level, *i.e.* below 20. Consequently, regulation of the cell size index could be a crucial factor in high-density cell cultures, and smaller cell size could be beneficial for overcoming the space limitations in a bioreactor at higher cell densities.

The goal of this study was to enhance the productivity of a recombinant protein via high-density cultivation. Therefore, the investigation of the protein production

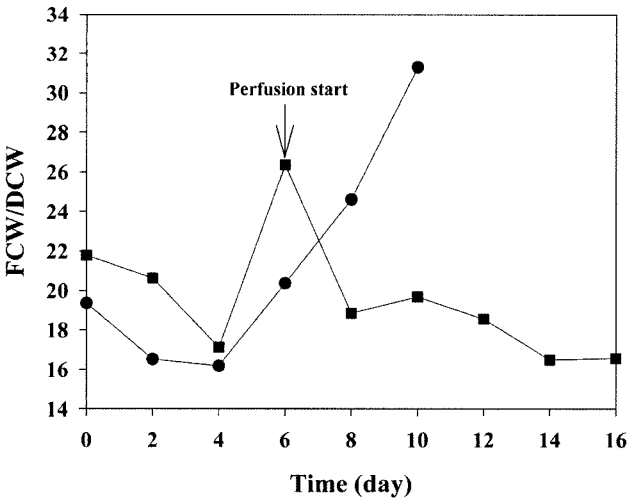

Fig. 3. Profiles of cell size index (FCW/DCW ratio) change according to perfusion.

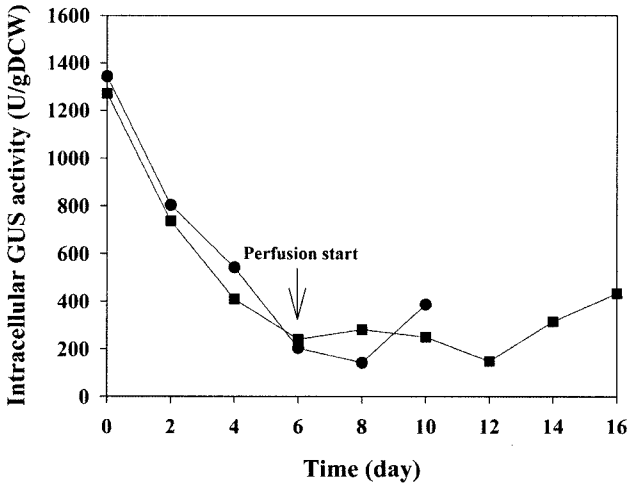

Fig. 4. Effect of medium exchange on GUS activity per unit cell.

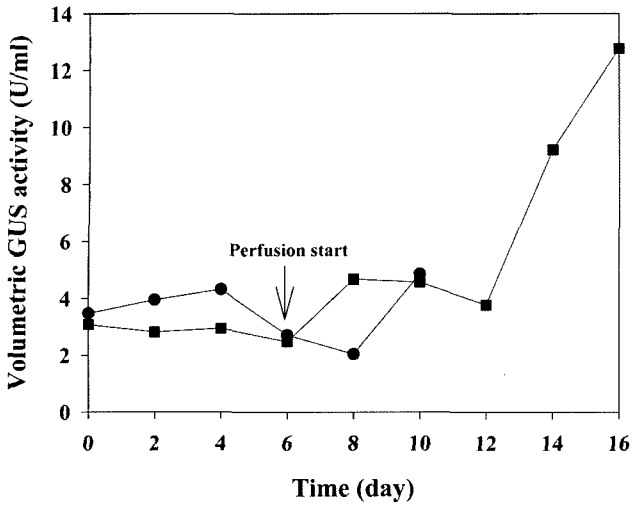

Fig. 5. Temporal change of volumetric GUS activity.

profiles at higher cell concentrations is indispensable. As shown in Fig. 4, the recombinant GUS contents varied by either culture type or culture age. The reason for this difference prior to the start of perfusion is not clear; however, it is evident that the GUS content with higher cell densities showed a pattern similar to that of a batch culture. Therefore, the volumetric GUS activity was drastically improved in the high-density culture, owing to the increase in the cell density with the same specific activity (Fig. 5). In the perfusion culture, a GUS activity of 12.8 U/ml could be obtained at day 16, which was 2.6-fold higher than the 4.9 U/ml in the batch culture. In order to achieve a higher productive process, a high-density of productive cells in the bioreactor should be considered [3]. Therefore, approaches to enhance the production of a target protein per unit cell should be accompanied by high-density cultivation strategies.

In conclusion, the data presented in this paper showed that perfusion culture with high-density transgenic plant cells could be a potentially by useful process for enhancing the productivity of useful foreign proteins, and that the versatility of a perfusion process could be amplified in the case of a secreted system [1, 4].

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