

Analysis of Pigment Accumulation Heterogeneity in Plant Cell Population by Image-Processing System

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Abstract: Plant-cultured cells are often highly heterogeneous in secondary metabolite productivity. The industrial application for large-scale metabolite production requires establishment of a stable high-producing cell line. In this study, image analysis of the individual cell is investigated as a method for evaluation of a heterogeneous cell population, and compared with the conventional method of estimation, which is based on average-cell productivity. Among strawberry cells producing anthocyanins, cells with a wide-range of pigment concentration were observed and maximum anthocyanin content was 10 times higher than the average value. In addition, a change of the frequency distribution was revealed in batch cultivation. © 2000 John Wiley & Sons, Inc. *Biotechnol Bioeng* 67: 493–497, 2000.

Keywords: plant cell culture; heterogeneous cell population; anthocyanin; image-processing system

INTRODUCTION

Recently, plant cell culture has been applied to the production of valuable secondary metabolites. This method is cost-effective, and has the added advantage of producing substances that are difficult to synthesize by alternative-chemical methods. However, the problem is how to increase the productivity of plant cell culture. In general, it is well-known that plant-cultured cells exhibit heterogeneity in secondary metabolite production. Zenk et al. (1977) showed that the range of yields of ajmalicine and serpentine were 0–0.85% and 0–1.4% of dry weight, respectively, in different clones from cell cultures of *Catharanthus roseus*. Dougall et al. (1980) indicated that anthocyanin accumulation in cultured cells of *Daucus carota* was not uniform for each

cell, and the amount of anthocyanin accumulation showed a wide distribution. That is, in the plant cell population, these secondary metabolites could be produced only by a fraction of the cells. Two main factors are thought to affect the heterogeneity of metabolite production. One is hereditary—the gene-expression levels related to the secondary metabolites differ among the individual cells. The other is physiological—each cell may undergo a different microenvironment in the medium of the culture because of a different position in a cell aggregate with different size.

A selection of high-producing cells from the heterogeneous culture often contributes to an enhancement of productivity. The amount of anthocyanin from cultured *Euphorbia millii* cells obtained after 24 selections was 7 times larger than that found in the original cells (Yamamoto and Mizuguchi, 1982). Similarly, the selection of a high-producing cell aggregate was reported to enhance anthocyanin accumulation in *D. carota* cells (Dougall and Vogeliën, 1990). However, anthocyanin accumulation in individual cells has not been analyzed, though such analysis would provide fruitful information for a further increase in productivity and the stability of high-producing cells. Among the various secondary metabolites, pigments have been studied widely because of their simplicity in the quantitative determination (Ibrahim et al., 1971; Ozeki, 1996; Straus, 1959). In most studies (Matsumoto et al., 1973; Sakamoto et al., 1994; Yamakawa et al., 1983; Zhong et al., 1991), only an average pigment content has been determined by measurement of absorbance; pigment accumulation in individual cells has not been estimated. Some researchers reported a ratio of pigmented cells according to an ambiguous definition (Nozue and Yasuda, 1985; Sato et al., 1996).

In this study, we estimated secondary metabolites production by an image-processing system, using a suspension culture of strawberry cells that produce the red pigments, anthocyanins. In this culture system, pigmented cells and

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non-pigmented cells exist simultaneously, and pigment-accumulation levels varied among cells, and distribution exhibited a wide-range. Smith et al. (1995) determined the average anthocyanin accumulation and cell growth by capturing the top and side view of a callus clump on solid medium. However, they evaluated productivity not for individual cells, but for cell aggregates. Regarding the color or hue of individual plant-cultured cells, no previous report has quantified their value for secondary metabolite production by image analysis. The image-processing system is suitable for estimating characteristics of individual cells' characteristics such as size, shape, and secondary metabolite content.

Herein, we discuss the evaluation method for anthocyanin accumulation in individual cells from strawberry cell cultures. The measurement of individual-cell characteristics is expected to facilitate the selection of high-producing cells and the estimation of their genetical stability.

MATERIALS AND METHODS

Plant Materials and Culture Conditions

Cultured strawberry cells (*Fragaria ananassa* cv. Shikinari) were induced from strawberry plant leaves and subcultured for more than 5 years (Mori et al., 1993; Mori and Sakurai, 1994). Suspension cultures of strawberry cells were grown in Linsmaier-Skoog medium (Linsmaier and Skoog, 1965) supplemented with 30 g sucrose, 1 mg 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg benzylaminopurine (BAP) per L of medium. The medium pH was adjusted to 5.7 before autoclaving. The cells were subcultured every week by inoculating 100 mL of fresh medium with 2-g fresh-cell weight (fcw) in inoculum cells (Mori et al., 1993) and kept under about 600 lx light irradiation not for accumulating pigment. The flasks were maintained at 26°C on a reciprocating shaker at 100 strokes per min. For anthocyanin production, the flasks under weak-light irradiation (600 lx) were transferred to under ca. 5000 lx light irradiation provided by fluorescent lamp (FL40SS, Toshiba Co., Tokyo, Japan).

Analytical Methods

Harvested cells were separated by vacuum filtration and weighed to measure the fresh-cell weight. For quantitative determination, anthocyanins were extracted from 100 mg of fresh cells in 4 mL methanol containing 0.1% of HCl at -20°C overnight. The anthocyanin content was calculated by the extinction coefficient ($E_{1\text{cm}}^{1\%} = 680$ at 530 nm), which was obtained using purified peonidin-3-glucoside from cultured strawberry cells as standard (Mori et al., 1993). As anthocyanin concentration in the culture medium was very low in our experimental conditions and could not be detected by extractive method, extracellular anthocyanins

were not taken account in this study. The experiment was performed in triplicate.

Aggregate Maceration

To estimate the characteristics of the individual cells, enzyme treatments of cell aggregates were carried out as preparation for image analysis. Enzyme treatment by solution consisted of 2% Cellulase Onozuka R-10 (Yakult Pharmaceutical Co., Ltd., Nishinomiya, Japan), 1% Macerozyme R-10 (Yakult Pharmaceutical Co., Nishinomiya, Japan), 0.01% Pectolyase (Sigma Chemical Co., St. Louis, MO), and 0.7M mannitol, which led to maceration of cell aggregates (Sato et al., 1996), then isolated cells were used as samples for image analysis. To adjust the pH of the enzyme solution to 5.6, all substances were dissolved in a buffer containing 20 mM morpholine ethansulfonic acid (MES) and 5 mM MgCl₂. Cells of 100 mg-fcw were suspended in 2 mL of the enzyme solution and incubated in the well of a plastic culture plate (Corning Coster Co., NY; Cat. #: 430343) for 2 h at 26°C in the dark on a reciprocating shaker at 100 strokes per min. After incubation, the cell suspension was filtrated by nylon mesh (pore size is 37 μm) and centrifuged at 60g for 2 min. The supernatant was substituted for a rinse solution containing 0.5M mannitol and 1 mM CaCl₂.

Image Acquisition and Analysis

The enzyme-treated cell suspension was placed on a hemacytometer and covered with a glass plate. Images of the cells were obtained by using a microscope (IX-70, Olympus Optical Co., Tokyo, Japan) with a charge-coupled device (CCD) camera (DXC-151, Sony Co., Tokyo, Japan). The magnification of the image was always 10× objective. The video signal from the CCD camera was digitized through a video-capture board, and the brightness data for each of the red (R)-green (G)-blue (B) components were captured in a personal computer (Endeavor Pro-330S, Epson Direct Co., Japan). The image-analysis software were Scion Image for Windows (Scion Co., MD, USA), based on the NIH Image, and an original program in Borland Delphi (Inprise Co., CA, U.S.A.). The resolution of this image was 640 pixels (horizontal) by 480 pixels (vertical). In this system, each pixel had 8-bit color. The color characteristics of anthocyanins produced by strawberry cell culture were as follows. The red component value (R) was much larger than the green (G) or blue (B) component values, and G was almost equal to B. Then, the difference between R and G, the (R-G) value, was defined as an index for pigment accumulation in this study. Each cell size (cell region) and the average (R-G) value of a cell were determined by using the image-processing system. The initial step for determination of cell region was edge detection of the monochromatic image, and the next step was to count the pixels of the area including interior holes. In both calculations for cell region and average (R-G) value, the individual cells were detected after the

appropriate thresholds had been decided. We defined a pigmented cell as a cell that had a portion composed of pixels containing a value of the (R–G) value larger than the threshold, and all others were considered non-pigmented cells. Average (R–G) value of whole-cell population was determined by calculating the (R–G) value for pixels composed of all cell regions over the whole data matrix (640 × 480 pixels).

RESULTS AND DISCUSSION

Cell Growth and Pigment Accumulation in Strawberry Cells

The average characteristics of strawberry cells during a batch suspension culture are shown in Figure 1. Figure 1A shows cell growth based on fresh-cell weight and that based on dry-cell weight. The vigorous cell growth occurred after about 1 d of lag phase. Within 24 h after inoculation with light irradiation, anthocyanin accumulation was observed at an average content of 0.36 mg/g-fcw determined by the extractive method as shown in Figure 1B. However, the average anthocyanin content decreased to 0.15 mg/g-fcw at day 5, and subsequently increased to about 0.5 mg/g-fcw at day 7. Consequently, Figure 1C shows a steep rise between day 4 and day 6 in the specific anthocyanin-accumulation rate calculated by average anthocyanin content and fresh-cell weight. An earlier increase of specific anthocyanin accumulation rate was also observed on day 1. The reason for this phenomenon is unclear, but might relate with the change in light condition or dilution effect in the inoculation.

Evaluation of Anthocyanin Accumulation by Image Analysis

Yamamoto and Mizuguchi (1982) showed the frequency distribution of cell-clumps of mm size with various pigment contents using extractive method. As they discussed the effect of clump selection on the distribution, their evaluation was limited only for cell-clumps, not for individual cells. Therefore, we elucidated the anthocyanin content of individual cells using an image-processing system, while the conventional extraction method indicates only the average anthocyanin content among the cultured cells. Figure 2 shows a time course of the pigmented-cell ratio determined by image analysis. Here, the pigmented-cell ratio was calculated by dividing the number of pigmented cells by the number of total cells using an image-processing system. More than 400 cells were analyzed at any cultivation time. The standard errors in image analysis were shown, and they were of the order of a few %. It is found that the maximum pigmented-cell ratio was not more than 50%, and that ratio changes were consistent with the anthocyanin content as shown in Figure 1B. The relationship between average anthocyanin content by extractive method and average (R–G) value by image analysis is shown in Figure 3. To obtain

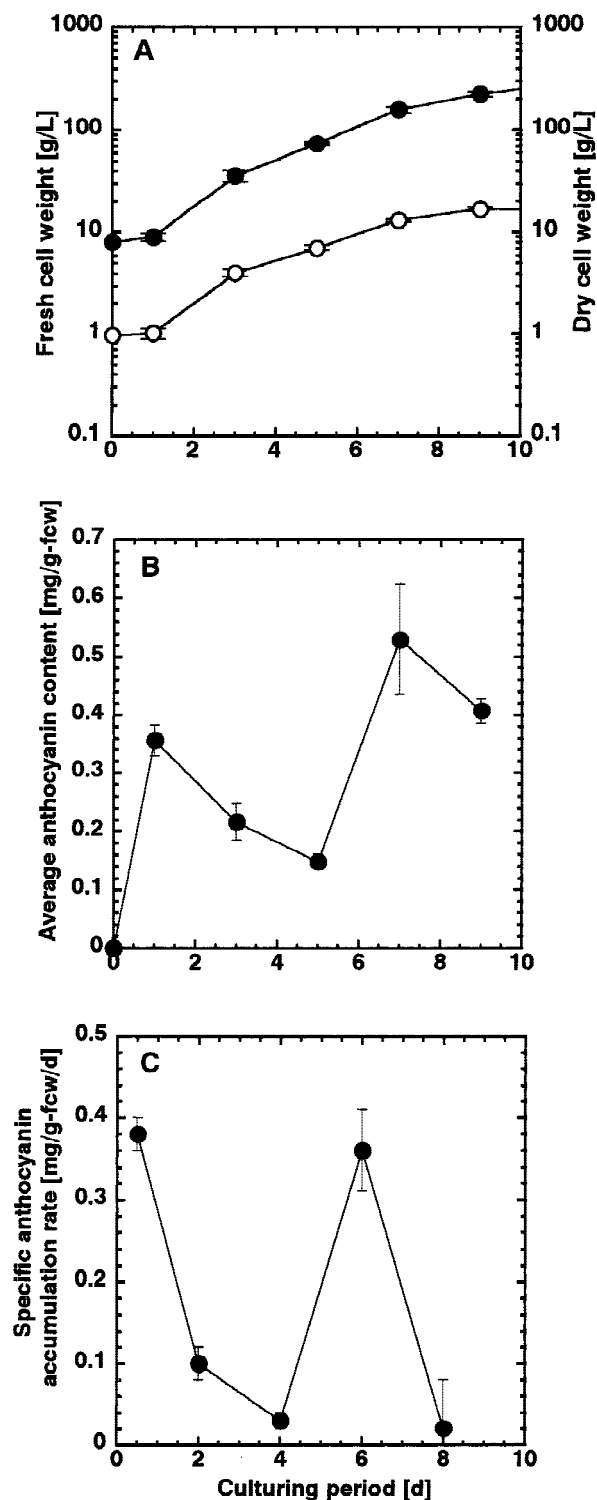


Figure 1. Batch suspension culture of strawberry cultured cells. (A) Cell growth based on fresh-cell weight (●) and that based on dry-cell weight (○). (B) Average anthocyanin content in cells. (C) Specific anthocyanin accumulation rate. Straight bars represent the standard error for three replicates.

these data, cell suspensions were first cultivated under various conditions for producing anthocyanin. The pigment was extracted for determining average anthocyanin content from absorbance. Also, the cells were analyzed by the image-

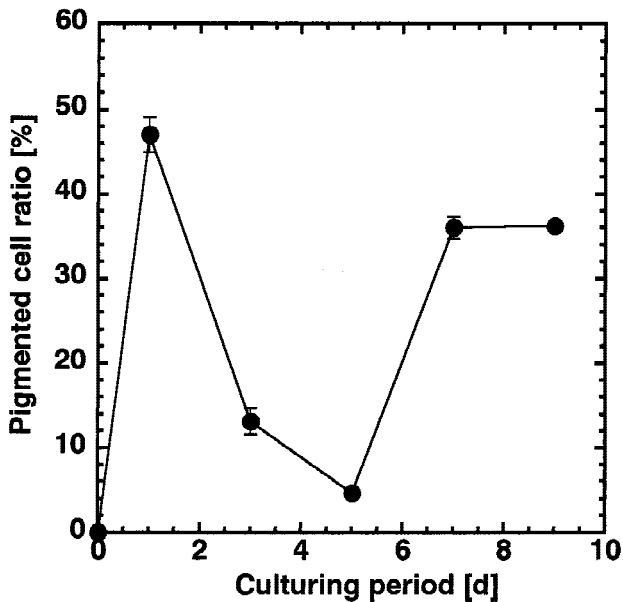


Figure 2. Time course of pigmented-cell ratio by image analysis.

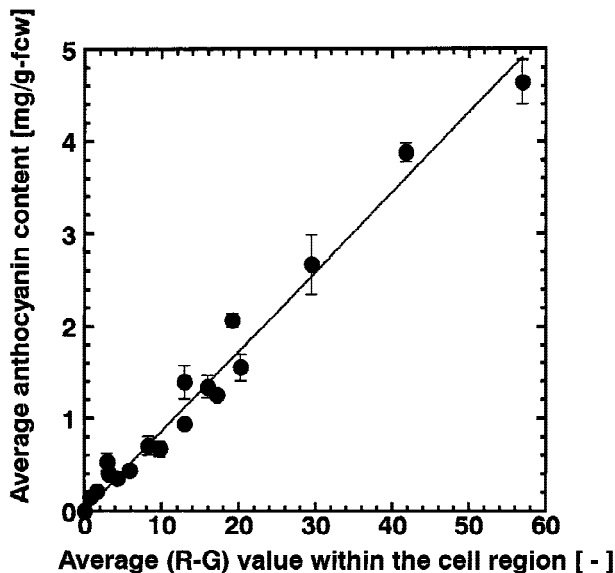


Figure 3. Relationship between average anthocyanin content determined by extractive method and average (R-G) value within the cell region calculated by image analysis.

processing system, and the average (R-G) value was calculated simultaneously. Both data have a good linear correlation with $R^2 = 0.98$. Smith et al. (1995) claimed that RGB information is not appropriate for color analysis in anthocyanin-producing cells. As they claimed, RGB data could not provide adequate determination independently. In this study, however, a combination with RGB data could provide quantitative analysis without conversion to HSI (hue, saturation, and intensity). The anthocyanin concentration accumulated by strawberry cultured cells ranged below the concentration limit that the linearity of (R-G) values held.

From image analysis, we found the cell size was ranged

approximately from 40 μm to 50 μm at any cultivation time. Berzin et al. (1999) claimed that morphology analysis is not less important than the color analysis of the object. Our results, however, suggested that the effects of the cell-size distribution on the image analysis were rather small, because (R-G) values per pixel were transformed into pigment concentration not into pigment amount per individual cell.

The correlation shown in Figure 3 was used for calculating a frequency distribution of anthocyanin content and distribution of cell concentration with various anthocyanin contents. The frequency distribution of anthocyanin content is shown in Figure 4 and demonstrates that some portion of non-pigmented cells always existed under light-irradiation conditions, and shows pigmented cells accumulating a content of anthocyanin more than 10 times higher than that of the average value shown in Figure 1B. Figure 5 shows the distribution of cell concentrations with various anthocyanin contents. It can be seen in this figure that from day 1 to day 5, the pigmented cell concentration was very low, while the number of non-pigmented cells increased. After day 7, the number of pigmented cells increased with a change that corresponded closely to change in specific anthocyanin accumulation rate as shown in Figure 1C. These results indicated that change in the distribution profile of anthocyanin accumulation correlates to changes in the cell growth in a batch-suspension culture.

It was often observed that the pigmented-cell ratio was increased by changing culturing conditions, such as phytohormone (Yamamoto et al., 1989), sucrose (Do and Cormier, 1990), or inorganic components (Sato et al., 1996). These phenomena suggest that non-pigmented cells also have an ability to accumulate anthocyanin if they are surrounded with the proper environmental conditions for pigment accumulation. In general, anthocyanin content varies not only in terms of the pigmented-cell ratio but also in terms of the amount of accumulation in pigmented cells. Many researchers, however, have reported only pigmented-

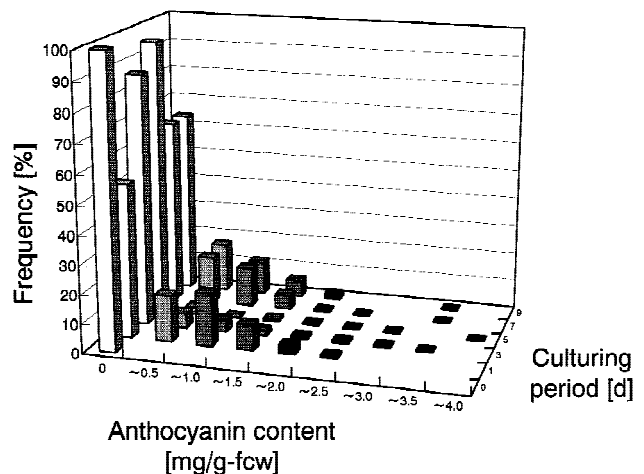


Figure 4. Frequency distribution of anthocyanin content in cultured strawberry cells during the culturing period.

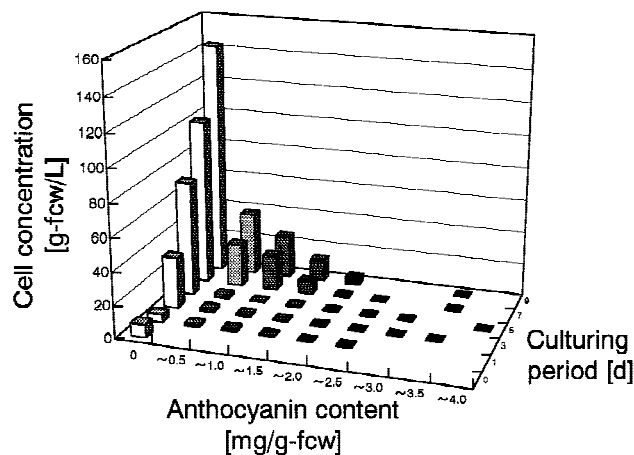


Figure 5. Distribution of cultured strawberry cell concentrations at various anthocyanin content during the culturing period.

cell ratios estimated with an optical microscope by counting the number of pigmented cells in various plant tissue cultures (Do and Cormier, 1990; Nozue and Yasuda, 1985; Sato et al., 1996; Yamakawa et al., 1983). In these studies, all the pigmented cells with various pigment contents have been defined simply as the “red” cells. Strength of pigmentation in the individual pigmented cells, that is, the spectrum of pigmentation including pale pink, red, and crimson cells, etc. has heretofore not been evaluated. Moreover, the boundary between the pigmented cell and the non-pigmented cell has been obscure, and results based on visual determinations could be influenced by strength of background (light source) or the criterion of each observer. Image analysis can track changes in the pigment profile of each cell, and the results are reproducible irrespective of the number of observers. However, in the application of the image-processing method shown here to the other secondary metabolites determination, further study must be done because characteristic features of RGB values depend on the species of pigment produced.

In conclusion, anthocyanin content in individual pigmented cells can be evaluated by an image-processing system. We demonstrated a profile of the anthocyanin content and the pigmented-cell ratio. A wide distribution of anthocyanin content was found in each cell, and the highest pigment content in a cell was more than 10 times higher than the average value determined by extractive method. To enhance secondary metabolite productivity, further experiments are necessary to investigate the effects of high-accumulating cell-selection and the stability of these cells.

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