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Large-scale production of anthocyanin by *Aralia cordata* cell suspension cultures

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Abstract. The suspension culture of high anthocyanin-producing *Aralia cordata* cell lines, which grow and produce anthocyanin without light irradiation, was scaled up from flasks to a 10-l glass jar fermentor, a 95-l stainless steel jar fermentor, and finally a 500-l pilot-scale jar fermentor. By the administration of CO_2, cell damage was completely prevented and the anthocyanin content was kept as high as 7.0–17.2% (w/w) of the dried cells. In one of the operations of the 500-l jar fermentor, cells were cultivated for 16 days. During this operation, cell mass was increased by more than 26 times (cell yield: 69.2 kg fresh wt.) and the amount of anthocyanin increased by more than 55 times (anthocyanin yield: 545 g, anthocyanin content: 17.2% of the dried cells).

Introduction

Anthocyanins are widely found in various plant species. Since they are natural pigments and are very safe, they have a high potential utility value as a food additive and marker, and research institutes and food manufacturers are trying to produce them from various plant cell cultures. Anthocyanins in cultured plant cells, however, usually accumulate in only small amounts and their production generally requires light irradiation. We found a highly productive cell line of *Aralia cordata* by continuous cell-aggregate cloning (Sakamoto et al. 1993). The outstanding feature of this cell line is that light irradiation is not essential to maintain high anthocyanin productivity, which was as high as 15% (w/w) of the dried cells. Only a few species of plant cell culture: *Ajuga reptans* (Callebaut et al. 1990), *Daucus carota* (Dougall et al. 1980; Kinnersley and Dougall 1980), *Vitis* hybrid (Yamakawa et al. 1983) and *Buphleurum falcatum* (Hiraoka et al. 1986), have been reported to produce anthocyanins in the dark, though the anthocyanins levels were very low.

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The possibility of anthocyanin production at commercially viable levels was examined using 10-l, 95-l, and 500-l jar fermentors. In these experiments, we found that the administration of CO_2 into culture vessels was the key for successful scale-up. Similar results were obtained in previous experiments on immobilized cells of *Thalictrum minus* by one of the authors (Kobayashi et al. 1991a). Using such a technique, we could establish an effective scale-up process for the anthocyanin-producing *Aralia cordata* cells.

Materials and methods

Plant material. The callus of *A. cordata* Thunb. (Sakamoto et al. 1993), which were obtained from young leaves and stems, were maintained on Murashige and Skoog (MS) agar medium (0.9% w/v, pH 5.8, Murashige and Skoog 1962) and supplemented with 1.0 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/l of kinetin, and 3% sucrose, at 25° C in the dark, subculturing every 3 weeks.

Cell suspension cultures. In order to establish a cell suspension culture, cells (1 g fresh wt.) from the MS agar medium were cultured in 100-ml Erlenmeyer flasks containing 30 ml of modified MS liquid medium with different hormonal or nutrient concentrations. The cultures were agitated on a rotary shaker at a speed of 75 rpm at 25° C in the dark.

The effects of elevated CO_2 level on cell damage in flask cultures was examined by using a two-tier culture vessel consisting of two vertically connected flasks (Street 1977). Cells (1.0 g fresh wt.) were inoculated in the upper flask (30 ml medium) connected by a duct to the lower flask containing 2 M carbonate/bicarbonate buffer (50 ml) in order to modify the CO_2 concentration within the flask to approx. 2%. The effects of polyvinylpolypyrrolidone (PVPP) were examined by adding 1 g PVPP into a 30 ml culture medium before autoclaving. After autoclaving, cells (1.5 g fresh wt.) were inoculated into the medium.

Once a stable cell suspension culture of *A. cordata* was obtained by the above processes, cells (2 g fresh wt.) were inoculated into 100 ml medium in a Erlenmeyer flask and subcultured every 10 days.

Cultivation in jar fermentors. In these experiments, jar fermentors of three different sizes were used: 10 l (medium volume: 8 l, produced by Takasugi Seisakusho), 95 l (medium volume: 50 l, produced by Takasugi Seisakusho) and 500 l (medium volume: 300 l,

produced by KF Engineering). In each fermentor, the agitation rate was adjusted to 30 rpm and the temperature was 25° C. The aeration rate was 0.1 vvm (volume/volume of medium) in the 10-l jar, and 0.2 vvm in the 95-l and 500-l jar fermentors.

The CO_2 content in the air flow was adjusted with a gas mixing unit (Koflak). The CO_2 content was adjusted to 1% in the 10-l jar, and 0.3% in the 95-l and 500-l jars.

Measurement of cell growth and determination of anthocyanin content. Callus growth was measured by determining fresh and dry wt. (dried in an oven at 80° C for 3 days). Anthocyanin was extracted from fresh cells (100 mg) in 0.1% (v/v) HCl-methanol (10 ml) overnight. After centrifugation at 2000 rpm for 10 min, 1 ml of the supernatant was used for the measurement of absorbance at 530 nm with a spectrophotometer. The amount of anthocyanin was estimated by a standard curve, which was obtained by using cyanin chloride as the standard sample, and the content (percentage of dry cell wt.) was calculated.

Results and discussion

In the process of establishment of a cell suspension culture from callus culture, severe cell browning occurred, causing a decrease in both cell growth and anthocyanin production, especially when cells were cultured in the light. In order to prevent such damage, the effects of various concentrations of 2,4-D, kinetin, sucrose, phosphate, nitrogen sources and copper salts and the effects of different ratios of nitrate to ammonium were examined. However, any of these changes could not diminish such damage (data not shown). In the next step, the effects of CO_2 and PVPP were examined (Fig. 1). In general, enhanced CO_2 levels are known to be effective in prolonging the storage period of vegetables and fruits and preventing them from turning brown (Smock 1979; Isenberg 1979). PVPP is known to adsorb polyphenolics, which are oxidized to cause cell browning. The experiments showed that both CO_2 and PVPP could improve cell growth. However, as for anthocyanin production, PVPP completely inhibited pigment formation. PVPP might have adsorbed not only the precursor of browning substances but also the intermediate of anthocyanin biosynthesis. On the other hand, in the culture with CO_2 administration, anthocyanin production increased with the increase in cell growth.

The outstanding feature of this cell line is that light irradiation is not essential for maintaining high anthocyanin productivity (Fig. 1). For the commercial production of anthocyanins, cells having such a feature are much more favorable than other cells that require light irradiation for anthocyanin production. Thus, we tried to produce anthocyanin on a large scale. As shown in Fig. 2, CO_2 administration was also an important key for the successful scale-up from flasks to jar fermentors. In the control culture, *A. cordata* cells in a 10-l jar fermentor hardly grew and turned brown, even when sufficient O_2 was supplied. Such cell damage could be caused by enforced aeration, which purged CO_2 from the culture vessels (Kobayashi et al. 1991a). In the flask cultures, the level of CO_2 was much higher than that in the atmosphere because of cell respiration. In the fermentors, however, enforced aeration into the

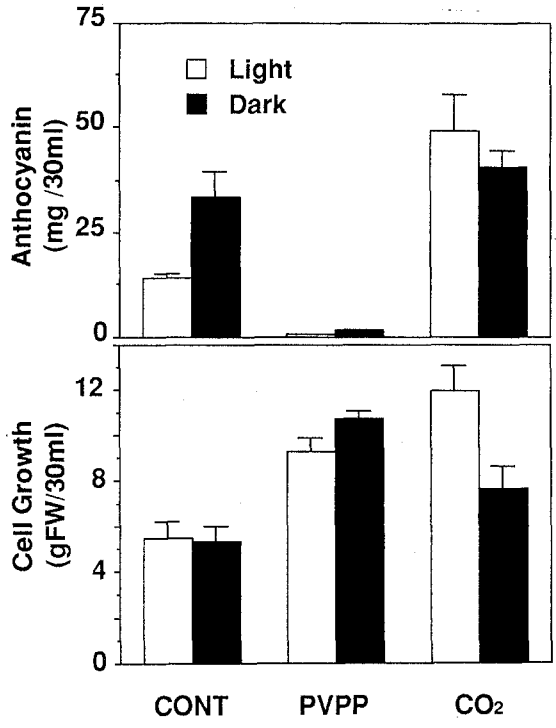

Fig. 1. The effects of polyvinylpolypyrrolidone (PVPP) and CO_2 on cell growth and anthocyanin production of *Aralia cordata* cells with or without light irradiation. Fresh cells (1.0 g) were cultured in the upper flask of a two-tier flask containing 30 ml culture medium. In the control (CONT) flasks, the lower flask contained 50 ml water. In order to test the effects of PVPP, 1 g PVPP was added into the culture medium. CO_2 was administered by adding 50 ml of 2 M carbonate/bicarbonate buffer: □, light; ■, dark; FW, fresh weight

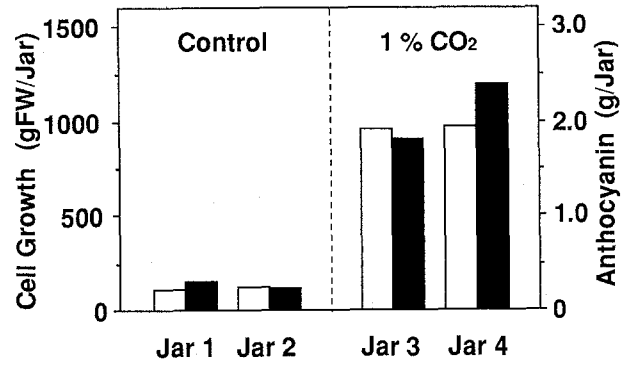

Fig. 2. The effects of 1% CO_2 administration to a 10-l jar fermentor on cell growth and anthocyanin production: medium volume, 8 l; aeration rate, 0.1 vvm; agitation, 30 rpm; □, cell growth; ■, anthocyanin

culture medium rapidly purged the CO_2 from the culture vessels. This deprivation of CO_2 might be the cause of cell damage. Such damage could be prevented by aeration with elevated CO_2 levels (Kobayashi et al. 1991a). In fact, the culture with 1% CO_2 administration showed improved cell growth and anthocyanin production. Furthermore, as shown in Fig. 3, both excellent cell growth and high anthocyanin productivity (7–10% of dried cells) were successfully maintained

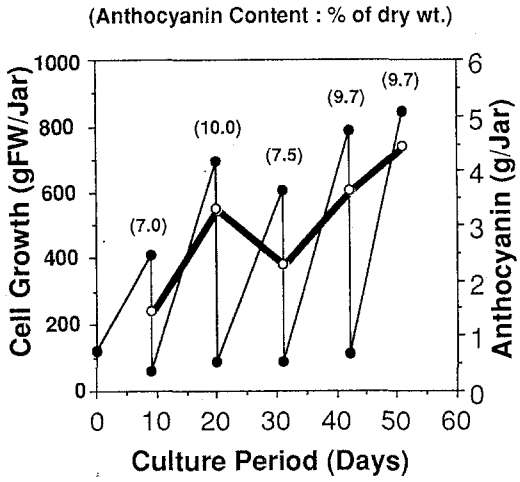

Fig. 3. Semi-continuous culture of *A. cordata* cells in a 10-l jar fermentor containing 8 l medium. Every 9 to 10 days, cells were harvested with 7 l spent medium and cell growth and anthocyanin yield were determined. Then 7 l fresh medium was added to the rest of the 1 l spent medium and subcultured. These processes were repeated for four times: medium volume, 8 l; aeration rate, 0.1 vvm; agitation, 30 rpm. The *figures in parentheses* represent the anthocyanin content (percentage of dry cell weight): —●—, cell growth; —○—, anthocyanin

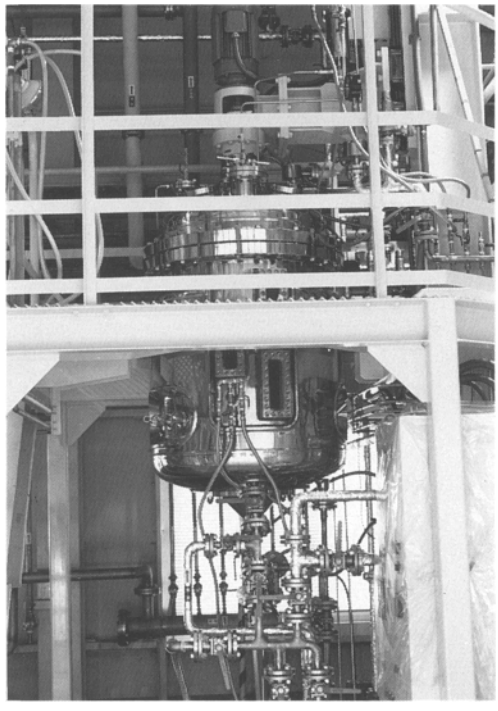

Fig. 4. A photograph of the 500-l jar fermentor used in these experiments. This fermentor is 80 cm in width and 129 cm in height

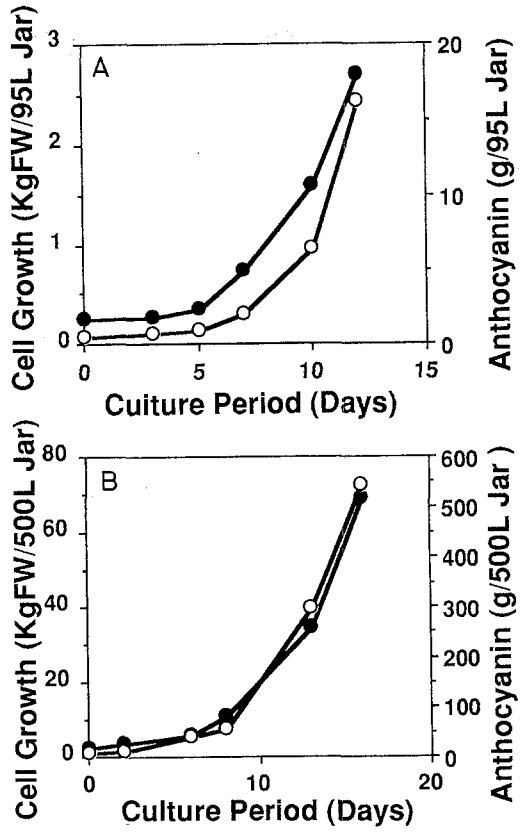

Fig. 5. **A** *A. cordata* cell culture in the 95-l jar fermentor containing 51 l medium inoculated with 261 g fresh wt. of cells: air flow rate, 0.2 vvm; CO_2, 0.3%; agitation, 30 rpm. **B** *A. cordata* cell culture in the 500-l jar fermentor containing 320 l medium inoculated with 2.60 kg fresh cells from the 95-l jar fermentor through a connecting stainless steel pipe: air flow, 0.2 vvm; CO_2, 0.3%; agitation, 30 rpm; —●—, cell growth; —○—, anthocyanin

during the whole period of semi-continuous culture (subcultured five times during 2 months).

Further scale-ups to 95-l and 500-l (Fig. 4) jar fermentors were also successful. In these operations, cells were at first cultured in 300-ml erlenmeyer flasks for 10 days, then transferred to a 95-l jar and cultured for 12 days before cultivation in a 500-l jar fermentor. In the first operation, 6.9 kg of fresh cells was transferred from two 95-l jars to a 500-l jar fermentor and cultured. After 10 days of culture in a 500-l jar fermentor with 340 l medium, the cell mass increased to 66.8 kg fresh wt. and the anthocyanin yield was 480 g. The anthocyanin content was 15.0% on a dry wt. basis and was as high as that found in highly productive callus culture obtained by continuous cell-aggregate cloning (Sakamoto et al. 1993). In the second operation, 261 g of fresh cells was inoculated into a 95-l jar and cultured for 12 days (Fig. 5A), then 2.60 kg fresh wt. of cells was transferred to a 500-l jar and cultivated for 16 days before harvesting (Fig. 5B). The amount of cell mass was 69.2 kg fresh wt. and the anthocyanin yield was 545 g. The content of anthocyanin was 17.2% on a dry wt. basis.

In conclusion, we succeeded in the large-scale production of anthocyanin using an *A. cordata* cell suspension culture. Manipulation of the CO_2 level within culture vessels is an important factor for successful scale-up. The main role of CO_2 might be the suppression of ethylene generation and its action (Smock 1979; Isenberg 1979; Kobayashi et al. 1991a). Before the determination of the amount of CO_2 administration, however, one should take into the consideration the fact that ex-

cessive CO_2 would also suppress cell respiration, causing the suppression of cell growth, or that ethylene might also have an essential role in the induction of certain secondary metabolites other than undesirable effects, as shown in the berberine production in *Thalictrum minus* cell cultures (Kobayashi et al. 1991b; Hara et al. 1991). In these experiments, suitable levels of CO_2 administration in the air flow were determined empirically. The suitable levels of CO_2 would differ in a different scale of fermentors because of changes in air flow rate (vvm), type of sparger, etc. The optimal dissolved CO_2 concentration in the medium still remains unclear. We are attempting to elucidate these problems.

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