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Continuous Production of Natural Colorant, Betacyanin, by *Beta vulgaris L.* Hairy Root

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Abstract It has been known that continuous cultivation of hairy root is difficult to maintain for a long period of time compared to the microbial and callus cultures. Chemostat cultivation was successfully carried out in order to economically produce a plant-based colorant, betacyanin, from red beet hairy root for more than 85 days in a 14-l fermentor. The result from the chemostat cultivation was compared to those of the batch and fed-batch cultivations of red beet hairy roots. It was shown that hairy root reached its steady state within 50 days of the cultivation, and then maintained for about 25-30 days in a wide range of dilution rates. Total betacyanin production from the continuous process was also calculated to be 2.65 g at 0.28 (1/d) of dilution rate, compared to 0.196 g from fed-batch cultivation. It was found that betacyanin production was a partially growth related process, yielding 0.376 mg/g-fresh wt. cell and 1.89×10^{-5} mg/g-fresh wt. cell/d, with 0.92 of correlation factor in a partial growth-product model. It was also shown that the cell growth required was relatively large for maintenance amount of energy at a low dilution rate. The growth of hairy root was inhibited by high light intensity in following a photo-inhibition model. The growth parameters were estimated to be 0.3 (1/d), 10.56 kcal/cm^2/h, and 35.81 kcal/cm^2/h for the maximum specific growth rate, half saturation light intensity, and inhibition light intensity, respectively.

Key words: Betacyanin production, *Beta vulgaris L.* hairy roots, continuous cultivation

Restrictions imposed on the use of artificial food colorants eventually led to a regained interest on natural pigments such as anthocyanin, betalain, and shikonin [2, 16, 17]. Betacyanin became important to the food industry as a color additive, because FDA banned the use of synthetic red pigments in food products [20]. Betacyanin is a widely distributed pigment in many plant species, especially in red beets (*Beta vulgaris L.*); nevertheless, to obtain stable pigments in large quantities throughout all seasons is a very difficult task. Studies on the red beets as a source of red pigments have been reported by many investigators [2, 3, 4, 15]. Many studies have shown the production of betacyanin in red beet cell culture [6, 7]. However, most attempts to economically produce the pigments *in vitro* from callus and suspension cultures have failed, because the cells could not produce the compounds in sufficient quantity or the yields were unpredictably variable [9, 21]. Consequently, there has been increasing attention on a hairy root culture induced by *Agrobacterium rhizogenes*, in order to overcome problems encountered in the plant cell culture [8, 14]. Compared to that of the original plant, hairy roots attracted attention because of their indefinite and fairly active proliferation in a hormone-free media fairly as well as their capacity to synthesize secondary metabolites in large amounts along with fast growth [5, 8, 12, 13]. Furthermore, secondary-metabolite production by transformed roots was shown to be stable for many generations [18].

Bioreactor studies have been tried to cultivate large quantities of hairy root cultures [19]. However, only a few authors have reported successful establishment of hairy root cultures in a large bioreactor [5, 10]. Maximum capacity of the reactor was reported of only up to 30 l for large-scale cultivation of hairy roots [11]. Scaling-up the cultivation process for hairy roots presents a unique problem due to their morphological structures. The nature of the hairy roots structure leads to the formation of interconnected heterogeneous material during the process of fermentation [13]. Little work has been carried out to overcome such problems and to maintain hairy root cells in a continuous cultivation process. The result presented in this study shows the growth characteristics of red beet hairy roots and economic production of betacyanin in a relatively large-scale reactor under continuous condition, giving a

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basis of scaling-up the culture process of growing hairy roots.

MATERIALS AND METHODS

Cell Line and Culture Medium

Red beet (*B. vulgaris L.*) hairy roots transformed with *A. rhizogenes* were obtained from the Chonnam National University of Korea, and 1/2 MS Medium (Murashige and Skoog) containing 3% sucrose was used. The composition of basal medium consisted of 25 ml of macro solution (NH_4NO_3 33 g/l; KNO_3 38 g/l; $CaCl_2 \cdot 2H_2O$ 8.8 g/l; $MgSO_4 \cdot 7H_2O$ 7.4 g/l; KH_2PO_4 3.4 g/l); 5 ml of micro solution (KI 166 mg/l; H_3BO_3 1.24 g/l; $MnSO_4 \cdot 4H_2O$ 4.46 g/l; $ZnSO_4 \cdot 7H_2O$ 1.72 g/l; $Na_2MoO_4 \cdot 2H_2O$ 50 mg/l; $CuSO_4 \cdot 5H_2O$ 5 mg/l; $CoCl_2 \cdot 6H_2O$ 5 mg/l); 5 ml of chelate iron solution ($FeSO_4 \cdot 7H_2O$ 5.56 g/l; $Na_2EDTA \cdot 2H_2O$ 7.46 g/l); 5 ml of organic supplement solution (myo-inositol 20 g/l; nicotinic acid 0.1 g/l; pyridoxine-HCl 0.1 g/l; thiamine-HCl 0.1 g/l; Glycine 0.4 g/l); distilled water 1 l. The pH of the media was adjusted to 5.7 with 0.5 N NaOH prior to sterilization [9].

Culture Conditions

One-tenth g (fresh cell weight) of hairy root tips were inoculated into a 100-ml Erlenmeyer flask containing 30 ml MS medium. It was subcultured at 25°C and 60 rpm under a 16 h-light, 8 h-dark photospheric condition with 0.8 klux (1.82×10^{-3} kcal/cm^2/h) of the light intensity by four 20 W white cool fluorescent lamps. The light intensity was measured by using a quantum sensor (LICOR Co. Coldsprings, U.S.A.). The roots were inoculated into a 14-l photo-bioreactor as shown in Fig. 1. The light intensity was adjusted by changing the distance between the lamps and the reactor. For a fed-batch cultivation, 0.5 g of fresh wt./l of the cells was first added into 4 l fresh medium and operated for batch cultivation until the cell growth reached an initiation phase of the exponential growth. Then, fresh medium was added into the fermentor for starting fed-batch cultivation. The fresh medium was intermittently added up to 14 l, depending on the cell growth. For a continuous cultivation, 0.5 g of fresh hairy roots was inoculated into a reactor (4 l of initial volume). Temperature, pH, and dissolved oxygen were automatically adjusted by a microprocessor as 25°C, 5.7, and 15% of air saturation level, respectively. When root density reached its maximum to represent the stationary phase of batch cultivation, fresh medium was added into the reactor for continuous cultivation by a peristaltic pump. The level of the medium in the reactor was automatically controlled by a level controller attached to the peristaltic pump. Feeding rate of the fresh medium was maintained constant until the root density remained relatively stable before the feeding rate was altered.

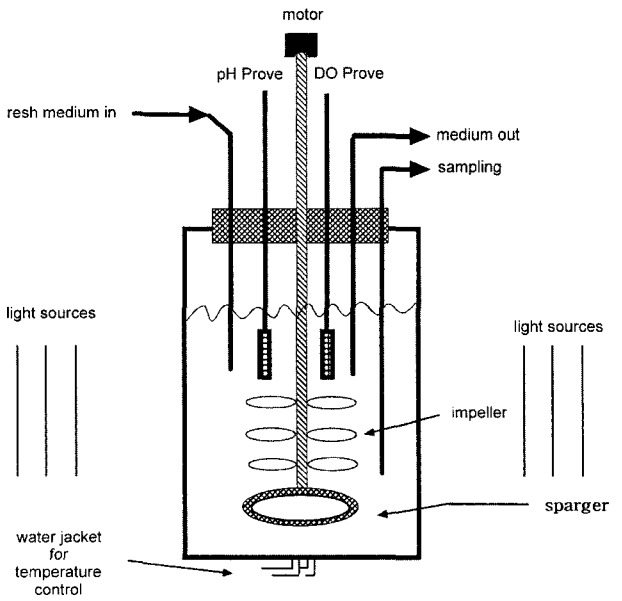

Fig. 1. The schematic diagram of a 14-l fermentor for the cultivation of hairy root.

Measurement of Root Weight and Betacyanin Concentration

The growth of hairy root was estimated by the changes in total weight of the reactor using the balance attached to the fermentor, since it was not possible to exactly measure the dry root weight by collecting the roots from the reactor, due to non-homogeneity of the bulky hairy roots. The initial weight of the reactor and medium each day were subtracted from the total weight of the reactor in order to calculate the increase of the total system, which corresponded to the total root growth for one day. For the measurement of betacyanin concentration, a total 3 g of fresh wt. of hairy roots was collected through a sampling port located in the middle of the reactor and washed three times with distilled water. They were first sonicated and centrifuged at 15,000 × g for 5 min. The supernatant was diluted with distilled water and absorbance was measured by a Spectrophotometer (Beckman DU-64, New Jersey, U.S.A.) at 535 nm [17] and compared with a standard betacyanin (Zipman Inc., Hannover, Germany).

Kinetics of Root Growth and Betacyanin Production

The root growth can be expressed as a function of light intensity under a continuous condition in a continuous pattern, employing the photo-inhibition model [1] as follows:

$$\mu = \frac{\mu_{max} \times I}{K_I + I + K_i I^2} \quad (1)$$

I is the light intensity (kcal/cm^2/h), K_I is the half saturation constant (kcal/cm^2/h), K_i is the inhibition parameter

(kcal/cm^2/h), μ is the specific growth rate (1/d), and μ_{max} is the maximum specific growth rate (1/d).

The betacyanin production from the hairy root cells can also be expressed by the following equation [2]:

$$q_p=\alpha \cdot \mu+\beta \quad (2)$$

where q_p (mg/g-fresh wt. cells/d) is a specific betacyanin production rate, α (mg/g-fresh wt. cells) is a maximum concentration of betacyanin produced per cell, and β (mg/g-fresh wt. cells/d) is a maximum specific rate of betacyanin production.

RESULTS AND DISCUSSION

Figure 2 illustrates the results of cultivating the hairy roots in batch and fed-batch cultivations in a fermentor. Here, only the fresh root density was counted in all cases, because it was impossible to precisely measure the dry root density by collecting the roots from the reactor due to non-homogeneity of the hairy roots within the reactor. As expected, a higher maximum cell density and betacyanin production were obtained in a fed-batch cultivation than in a batch cultivation; 246 vs 198 (g-fresh wt/l) and 14 vs 11 (mg/l), respectively. For the batch cultivation, the root growth sharply decreased when the root density reached its maximum value at 62 days of cultivation. The betacyanin production seemed to be closely related to the root growth and this was evidenced by a rapid drop of the production at the end of cultivation which was not typical for the secondary production in plant cells. This was possibly due to rapid deletion of the nutrients and also due to limitation of nutrient transfer into the center of the bulky hairy roots. The root growth gradually increased as the fresh medium was fed up to 56 days for the fed-batch cultivation. The product production showed a partially growth related process, yielding maximum production at the later periods of the cultivation. For the fed-batch cultivation, the operation day of the system extended up to 80 days, maintaining a relatively constant root density, whereas the betacyanin production dropped sharply at the end of the cultivation. This implied that the betacyanin production was regulated by the nutrient uptake rates of these hairy roots.

Figure 3 shows the kinetics of root growth and betacyanin production in the chemostat cultivation at 0.25 (1/d) of dilution rate in a 14-l fermentor. The system appeared to reach the steady state at 64 days of the cultivation, because of maintaining a constant root density. It showed that the cell growth reached the steady state very slowly, in comparison with that obtained by the conventional microbial cell growth. The betacyanin production also remained steady after the root growth was stabilized. The pattern of producing secondary metabolites from hairy roots was shown to be a partial growth related process, which was typical for plant cell cultures. The maximum betacyanin concentration was 12 mg/l which was higher than that of the batch cultivation, in spite of the fact that the maximum cell density was lower than that of the batch cultivation, such as 189 vs 198 (g-fresh wt/l). Higher production of betacyanin from the continuous process was

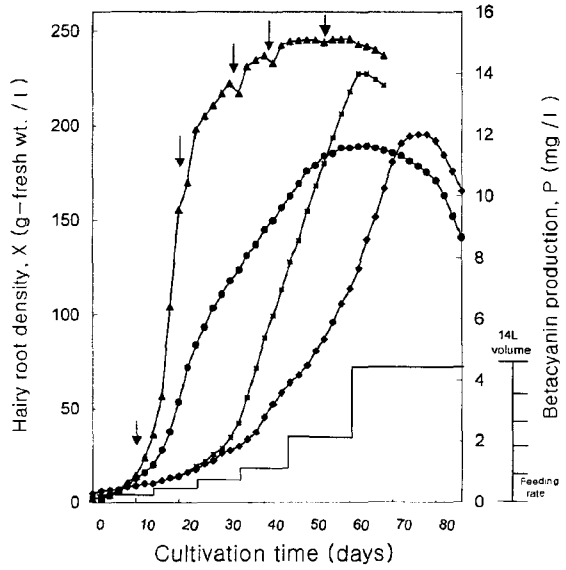

Fig. 2. Hairy root growth and betacyanin production from red beet for batch and fed-batch cultivations.
*Arrows are the points of feeding fresh medium. —●— batch, X; —▲— fed-batch, X; —◆— batch, P; —■— fed-batch, P.

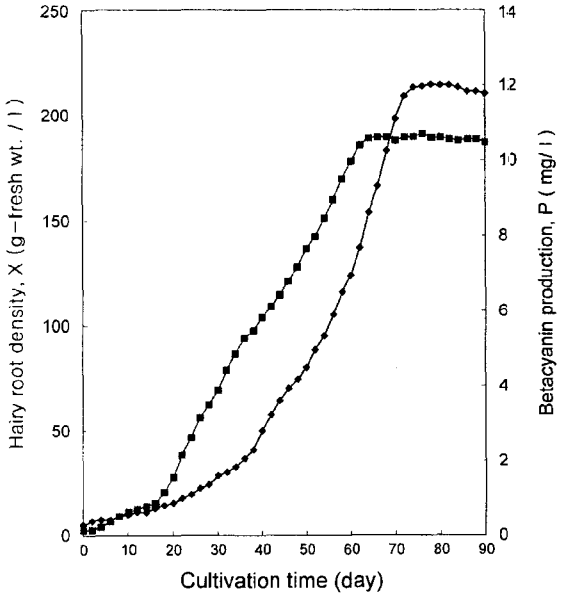

Fig. 3. Kinetics of growing red beet hairy root and betacyanin production under continuous conditions at 0.25 (1/day) of dilution rate.
—■— X; —◆— P.

possibly due to a balanced growth by continuous feeding of fresh medium. It was highly likely that the production of betacyanin from hairy roots was a partially growth related system. The operation of the system could be extended up to 89 days in the chemostat cultivation, compared to 65-80 operation days for batch and fed-batch cultivations. Table 1 compares the growth parameters of cultivating hairy roots in a fermentor by three different culture modes. Maximum root density can be obtained in a fed-batch cultivation as 246 g-fresh wt/l and also yields the highest specific production rate. This result obviously implies that the fed-batch cultivation is the best process to yield the highest cell growth and product production. Therefore, the fed-batch or other cultivation processes should be utilized for obtaining high root density and eventually producing large amounts of betacyanin, because it is very important to maintain high and active root density in order to produce intracellular products by making better environments for the growth of hairy roots. Maximum specific growth rate of 0.094 (1/d) was also high in fed-batch cultivation, compared to 0.045 (1/d) in batch cultivation. However, for a long-run cultivation, chemostat cultivation seemed to be economical if the process could maintain a certain level of active hairy roots, since the continuous process could obtain the largest quantities of betacyanin at the end of the process by continuously collecting the roots from the reactor. The chemostat cultivation can also be operated for about 88 days while batch and fed-batch cultivations operate for about 50-55 days. Even in the fed-batch cultivation, maximum product concentration was obtained.

Figure 4 demonstrates the effect of light intensity on the cell growth demonstrated by several different runs of the chemostat cultivation. The maximum specific growth rate, half saturation constant, and inhibition constant of the light intensity were estimated as 0.3 (1/d), 19.56 (kcal/cm^2/h), and 35.81 (kcal/cm^2/h), respectively, by employing a photo-inhibition model expressed as Eq. (1). It is obvious that the growth of hairy roots can be hampered at a high light intensity, which may be different from normal growth of hairy root in the field. In the range of low light intensity, specific growth rates slowly increased, and this meant that the growth of hairy roots required a relatively large maintenance energy [1]. Figure 5 shows a relationship between the root growth and betacyanin production based on Eq. (2). The root growth correlated very well to fit the specific product production rate by having 0.92 of the linear correlation factor. It is obvious that the betacyanin production from hairy root is a partially growth related process. The product model parameters were estimated as 0.376 mg/g-fresh wt. cell and 1.89×10^{-5} mg/g-fresh wt. cell/day, respectively. These values are relatively lower than those from other processes, such as microbial and animal cell growth [18]. However, 0.376 mg of the specific betacyanin production is relatively large for the secondary production from plant cells, when compared to the production of betacyanin from conventional callus cultures [23, 24]. This is the reason why the hairy root culture is receiving wide attention in spite of the fact that the culture of hairy roots is difficult to detect, when compared to other cultures, especially for maintaining a chemostat cultivation.

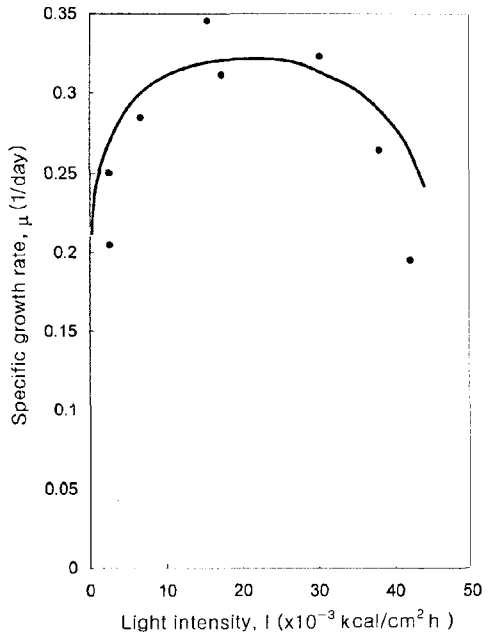

Fig. 4. The effect of light intensity on the specific growth rate of red beet hairy root cells.
A solid line is the result of fitting the data to a photo-inhibition model.

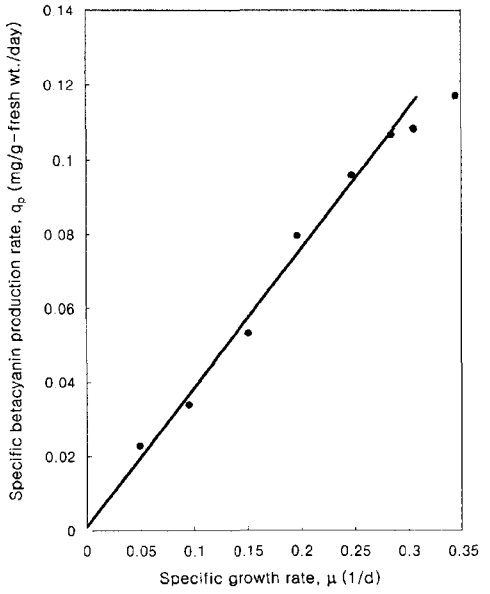

Fig. 5. The correlation between root growth and betacyanin production from chemostat cultivation data.
A solid line is the result of fitting the data to a product model.

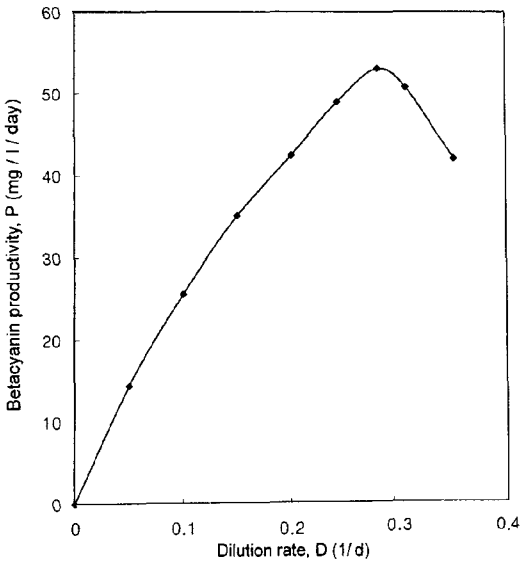

Fig. 6. The betacyanin productivity as a function of dilution rate.

Figure 6 shows the betacyanin productivity as a function of dilution rate. Fifty three mg/l/d of optimal productivity was obtained at 0.28 (1/d) of the dilution rate and its dilution rate was close to 0.3 (1/d) of the maximum specific growth rate, which was calculated by using Eq. (1). It also suggested that the betacyanin production was partially related to the root growth. By calculating the condition of the optimal dilution rate of 0.28 (1/d) with 53 mg/l/d of the product, about 2.65 g of betacyanin can be obtained after 30 days of steady state. This production is far greater than those from the batch and fed-batch cultivations, which yielded 0.154-0.2 g of the total betacyanin production. From the above results, it is concluded that the continuous cultivation of hairy roots should be used for plant cell cultures for producing products.

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