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Culture of Red Beet Hairy Roots in a Column-type Reactor Associated with Pigment Release

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In plant tissue cultures, the metabolites of interest are most frequently accumulated in cells without secretion, and thus the continuous production of the metabolites during cultures is greatly limited. To overcome this problem, several procedures to leak out the intracellular products from plant cells have been proposed including the addition of inorganic chemicals or organic solvents to cultures and exposure of cells to electricity[1,2]. Shimomura *et al.*[3] reported that in the culture of *Lithospermum erythrorhizon* hairy roots, the addition of liquid paraffin promoted the extracellular production of shikonin pigment. They also proposed an air-lift reactor equipped with an adsorbent column for the continuous production of the pigment by the hairy roots.

In our previous papers[4,5], it was reported that red beet hairy roots released pigment from the cells into medium when the roots were kept under O_2-deficient condition. In the present research, the hairy roots were cultivated using a column-type reactor where direct aeration was not conducted and instead O_2-enriched medium was circulated. As a gradient of dissolved O_2 (DO) concentration in medium is generated in this reactor through O_2 uptake by the hairy roots, pigment release is expected to occur from the roots existing at the location of reduced DO concentration in the medium.

In all experiments, the hairy roots of red beet (*Beta vulgaris* L.) induced by *Agrobacterium rhizogenes* A4 infection[4] were cultivated in Murashige-Skoog medium[6] containing 18 g/*l* fructose

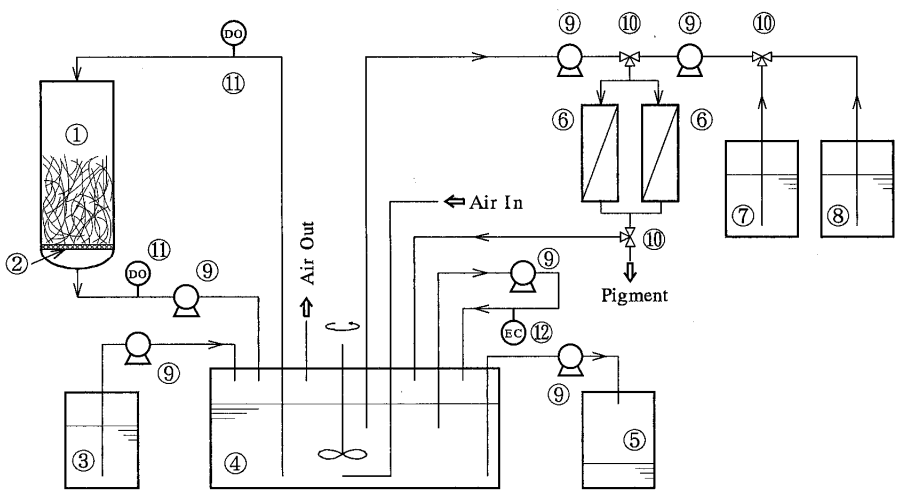

Fig. 1 Schematic diagram of culture equipment.
1: Column for hairy root growth, 2: Stainless mesh, 3: Reservoir of fresh medium, 4: Stirring vessel for medium aeration kept at 25°C, 5: Reservoir of culture broth, 6: Column for pigment adsorption, 7: Reservoir of water, 8: Reservoir of 30% (v/v) ethanol, 9: Pump, 10: 3-port valve, 11: DO probe, 12: Conductivity cell.

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at 25°C. **Fig. 1** shows a schematic diagram of the culture equipment which consisted of a glass column for root growth (6 cm in diameter by 26 cm in height) and stirring vessel for medium aeration (Model TBR-2 fementor, Sakura Seiki Co.). The total volume of medium in this culture system was 2 *l* (0.7 *l* in the growth column and 1.3 *l* in the aeration vessel). Inoculated hairy roots of *ca.* 25 g fresh mass (*ca.* 2 g on a dry weight basis) were anchored to a stainless mesh attached at the bottom of the column. Medium subjected to O_2 enrichment in the aeration vessel was made to flow from the top to the bottom of the column at a flow rate of 130 or 270 m*l*/min.

On occasion, part of the culture broth was replaced with an equal volume of fresh medium to avoid the depletion of nutrients in the medium. To recover the released pigment, the culture broth was circulated through an adsorbent column (3 cm in diameter by 20 cm in height) packed with 25 g of Sepabeads SP 207 (Mitsubishi Kasei Co.), which was previously selected as a resin for the adsorption of pigment[5]. Two columns were alternately used after eluting the adsorbed pigment with 30% (v/v) aqueous ethanol solution and washing the resin with water.

During culture, the amount of hairy roots in the reactor was estimated by the measurement of electric conductivity of the medium as reported previously[5]. At the end of culture, the hairy roots in the reactor were harvested and the amount of hairy roots was also determined gravimetrically after drying the roots at 80°C for 2 days. Under the experimental conditions in the present work, the error inherent in the conductometric estimation of root mass, X_{con}, was about 9.2% compared with the gravimetrical determination of root mass, X_{gra}. DO concentration at inlet or outlet of the growth column was in-line measured with a DO probe (Type AN-L, Oriental Denki Co.). Fructose concentration was determined as reported earlier[5]. The amounts of intracellular and extracellular pigments were analyzed according to the method described elsewhere[5]. Although betanin and vulgaxanthin-I were mainly detected in pigment samples, the amount of betanin was employed in the present study. Unless otherwise noted, the amounts of root mass and betanin were expressed on the basis of unit volume of medium in both the growth column and aeration vessel.

In culture of the hairy roots with the column-type reactor, the relationship between the amount of estimated root mass, X_{con}, and medium DO concentration at outlet of the growth column, DO_{out}, was first examined under the conditions of medium flow rate F=130 m*l*/min. and medium DO concentration at inlet of the column DO_{in}=8 ppm. As shown in **Fig. 2**, it was found that DO_{out} gradally decreased with increasing root mass, and the value became approximately zero when X_{con} was about 9 g-dry roots/*l*.

Fig. 3 shows the time course of the hairy root culture associated with the pigment release using the reactor. During the early period of culture time until 260 h, O_2-enriched medium (DO_{in}=8 ppm)

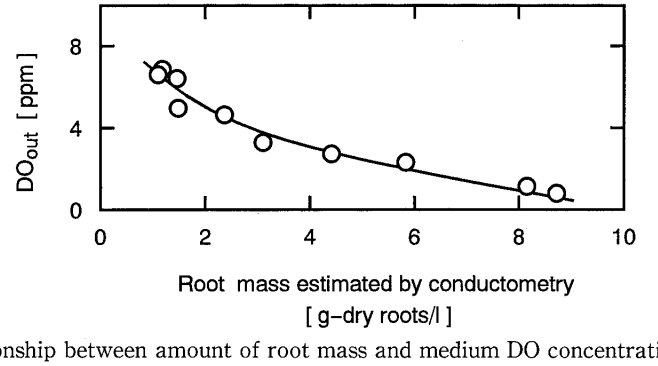

Fig. 2 Relationship between amount of root mass and medium DO concentration at outlet of growth column.
Red beet hairy roots were cultivated for 432 h at medium flow rate of 130 m*l*/min.

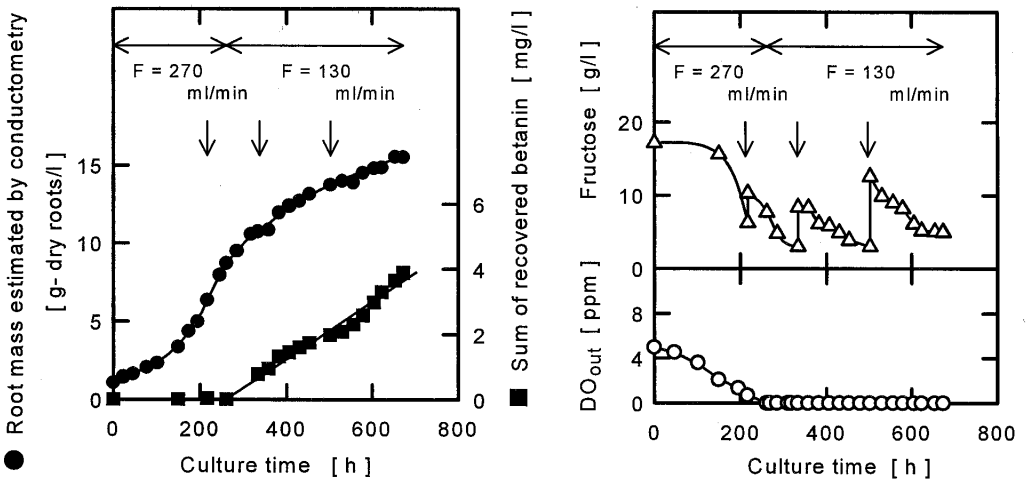

Fig. 3 Time course of red beet hairy root culture associated with pigment release. The arrows show the times of medium replacement and the total volume of replaced medium was 3 *l* during the culture.

was introduced into the growth column at F=270 m*l*/min. to ensure active growth of the hairy roots by sufficient supply of medium saturated with O_2. When the value of X_{con} reached about 9 g-dry roots/*l* at culture time of 260 h, the flow rate of medium changed from 270 to 130 m*l*/min. In response to the change in F, the hairy roots began to release betanin into the medium and DO_{out} became nearly zero. During the subsequent culture period of 260-672 h, the successive release of betanin occurred and released betanin was recovered by circulating the culture broth through the adsorbent columns.

At the end of the culture period of 672 h, the whole hairy roots in the growth column (X_{gra}=14.2 g-dry roots/*l*) were harvested and divided into three parts according to distance from the bottom of the column, Z; *i. e.*, lower part (Z=0-3 cm), middle part (Z=3-7 cm) and upper part (Z=7-11 cm). The average content of betanin retained in the hairy roots for each part was as follows: 2 mg/g-dry roots for the lower part, 7 mg/g-dry roots for the middle part and 6 mg/g-dry roots for the upper part. The hairy roots harvested from the lower part exhibited the reduced content of betanin, suggesting that the betanin release into medium mainly occurred from the hairy roots kept under O_2-deficient condition in the lower part of the column. The gross amounts of root mass and betanin obtained from the culture at 672 h were as follows: 28.4 g of dry roots, 16.3 mg of betanin retained in the roots and 8.0 mg of betanin recovered from the culture broth. In the culture of red beet hairy roots with the reactor, larger amount of betanin was produced in comparison with the result obtained in shake flask culture[4].

In conclusion, it was possible to cultivate red beet hairy roots in the column-type reactor with medium circulation, accompanying the successive pigment release from the roots into the medium.

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