GROWTH CHARACTERISTICS OF PLANT HAIRY ROOTS AND THEIR CULTURES IN BIOREACTORS

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Plant hairy roots from horseradish, carrots, etc. transformed by the soil bacterium *Agrobacterium rhizogenes* showed active growth in the phytohormone-free Murashige–Skoog medium with sucrose as a carbon source. Reticulate polyurethane foam was an appropriate support for the immobilization of hairy root cells. The hairy roots of 11 kg-dry cells·m^{-3} (110 kg-fresh cells·m^{-3}) were obtained in a 31-d culture in an air-lift column bioreactor with immobilized horseradish cells. A linear relationship was observed between the dry cell mass grown and medium conductivity decrease for hairy root cultures. Thus, it was possible to monitor cell mass concentration during the hairy root culture by on-line measurement of conductivity in the bioreactor system.

Introduction

The large-scale production of economically valuable biochemicals such as enzymes, pigments and alkaloids by fieldgrown plants has been limited mainly due to low propagation rate, restricted cultivation area and climate dependency. The techniques of plant cell culture with callus have been expected to minimize these disadvantages.[13,22] In spite of intensive investigations, however, few processes based on callus culture have been developed on an industrial scale, except for production of shikonin derivatives (pigmented anti-inflammatory drug) by *Lithospermum erythrorhizon*.[5,7] In general, the cultivation of undifferentiated cells (callus) is accompanied by several problems which involve the unstable production of desired metabolites owing to genetic and/or biochemical heterogeneity, and the difficulty of adequate oxygen supply in the bioreactor system.

Recently, so-called plant "hairy roots" have become of interest becuase of their indefinite and fairly active proliferation in phytohormone-free media[11,18] and their capacity to synthesize the products at levels similar to that of the original plants.[4,9] It is recognized that the root-inducing (Ri) plasmid in the soil pathogenic bacterium *Agrobacterium rhizogenes* causes the transformation of dicotyledonous plant cells by introducing the T-DNA region of the plasmid into genomic DNA of plant cells, and that the transformed plant cells give rise to hairy roots with the phenotype even after bacterium elimination and long-term subcultures.[3,25]

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Despite the outstanding properties of hairy roots, few investigations have been performed in biotechnological aspects. In the present paper, we describe the growth characteristics of hairy roots and bioreactor systems suitable for their cultures with the heterogeneous, structured and entangled nature of fibrous roots.

1. Experimental

1.1 Plant hairy roots

The hairy roots of *Armoracia rusticana* P. Gaert., B. Meyer *et* Scherb. (horseradish) and *Daucus carota* L. (carrot) were used. These hairy roots were induced and established as reported previously.[15,23] The hairy roots of *Cassia torosa* Cav. (stinkweed) was also used.[16] The hairy roots were maintained on a Murashige-Skoog (MS) medium[14] containing sucrose (30 kg·m^{-3}), agar (10 kg·m^{-3}) and no phytohormone, and subcultured once a month.

1.2 Medium and cultures of hairy roots

For experiments, the hairy roots were cultivated in liquid MS medium with 30 kg·m^{-3} sucrose, otherwise noted. The medium was autoclaved at 121°C for 20 min prior to use. The cultivations were carried out at 25°C in the dark, using the reactors described below.

1) Rotary shaking with Erlenmeyer flask For routine work or precultures, a 200-cm^3 Erlenmeyer flask with 50 cm^3 medium was shaken at 100 rpm on a rotary shaker (Model SR-12, Shibata Hario Glass Co.).

2) Stirred tank A glass vessel (working volume: 300 cm^3) was used as a reactor. The reactor was agitated with an impeller having two flat blades at 100 rpm and sparged with humidified air at 2.1 dm^3·h^{-1}.

3) Air-lift column A glass column of 4.2 cm ϕ × 35 cm (working volume: 300 cm^3) was used as a reactor. Humidified air was introduced into the column through a sintered glass sparger at the bottom (air flow rate: 2.1 dm^3·h^{-1}). For the immobilized cell system, reticulate polyurethane foam (Ester type MF-18 with 18 pores per 2.5 cm, Inoue MTP Co.) was used as a cell support as shown in **Fig. 1**. The hairy root cells were aseptically anchored to several positions on the polyurethane foam rod (0.8 × 2.0 × 20 cm) by forceps.

4) Other reactor systems Two other reactor systems were examined for hairy root cells immobilized on the polyurethane foam. The basal configurations of these reactors were similar to those of the air-lift column (Fig. 1) except that the cells were directly in contact with air phase during most of the cultivation period and forced air supply was not used. The medium was circulated by a timer-connected pump at 6-h intervals and was intermittently contacted with the cells on the support in the following ways. A) "Medium-trickling column": The medium was trickled through a sprinkler (at top of reactor) connected to a reservoir column by a siphon. B) "Medium-filling and drawing column with siphon": The reactor was filled with the medium, followed by immediate drawing of the medium by siphon (filling and drawing operation time: about 15 min).

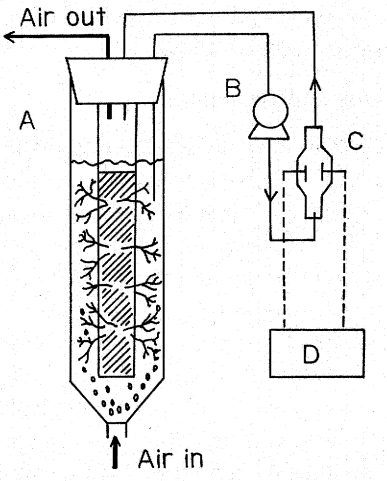

Fig. 1. Schematic diagram of air-lift column bioreactor with hairy root immobilization on polyurethane foam. A: air-lift column; B: peristaltic pump; C: conductivity cell; D: conductivity meter. The shaded part in the column indicates polyurethane foam

In all cultivations, the inoculum size was 0.3–0.5 kg-dry cells·m^{-3}.

1.3 Analyses

For cell mass determination, the hairy roots were harvested by paper-filtration and rinsed with a large amount of water. Dry cell weight was gravimetrically determined after drying the roots at 60°C for 24 h. Glucose concentration was measured with a glucose analyzer (Model 27, Yellow Springs Instrument Co.). The amounts of sucrose and fructose were determined by a Tri Rotar-V HPLC (Japan Spectroscopic Co.). equipped with an interference refractometer (Shodex RI SE-11, Showa Denko Co.) as a detector, and a PNH_2-10/S2504 column (Shimadzu Co.) with a precolumn (Amino Spheri-10 H2-GU, Brownlee Labs). Running conditions were as follows; eluent CH_3CN: H_2O=75:25 (v/v %) and eluent flow rate 60 cm^3·h^{-1}.

The batchwise measurement of medium specific conductivity was carried out at 25°C using a digital conductivity meter, Model CM-20A, and a dip-type conductivity cell, Type CG-201 PL (Toa Electronics Co.). In the cultivation with the reactors, specific conductivity was measured *in situ* by incorporating a handmade conductivity cell into the medium circulation system (see Fig. 1). This cell consisted of a pair of platinum electrode plates in parallel enclosed in a glass tube (electrode surface area: 1 cm^2; electrode

distance: 1.5 cm) and the surface of the electrode was coated with platinum black.[17]

2. Results and Discussion

2.1 Effects of carbohydrates on growth of hairy roots

In general, sucrose, glucose or fructose is the carbon source giving good growth in plant cell cultures. However, the growth responses of plant cells to carbon sources frequently depend on the plant species and clone of interest.[21]

At first, hairy roots of *A. rusticana* and *D. carota* were cultivated in the medium with various sugars as carbon sources, as shown in **Table 1**. Both these kinds of hairy roots exhibited the highest specific growth rate in the sucrose medium. Low growth rates were observed in the medium with glucose or fructose, which are component monosaccharides of sucrose. In the subsequent experiments, sucrose was used as the carbon source for the hairy roots.

2.2 Estimation of hairy root cell mass by medium conductivity

The methods generally adopted for cell mass determination in plant cell cultures are gravimetric and volumetric measurements on a wet or dry basis, or the microscopic counting of cells after protoplast-forming treatment.[8] Hairy roots normally develop in branched, filamentous organs, which make it impossible to obtain a homogeneous sample of the roots, or to employ the routine methods mentioned above in reactor cultures and especially in immobilized cell systems (see below).

As reported previously, conductivity measurement of culture broth was a convenient tool for cell mass determination in plant cell suspension (or callus) culture. The effect of environmental change on medium conductivity was almost negligible under plant cell culture conditions.[24] In the present study, estimation of the hairy root cell mass was tried on the basis of conductometry. **Figure 2** shows the relationship between cell mass grown and decrease in the medium conductivity during the cultures of *D. carota*, *A. rusticana* and *C. torosa* hairy roots. For each root culture, straight lines were obtained as

$$\Delta\kappa = \alpha \cdot (\Delta X) \qquad (1)$$

where X: dry cell mass concentration [$kg \cdot m^{-3}$], α: empirical coefficient [$S \cdot m^2 \cdot kg^{-1}$] and κ: specific conductivity [$S \cdot m^{-1}$]. The values of α were 0.046, 0.047 and 0.037 $S \cdot m^2 \cdot kg^{-1}$ for *D. carota*, *A. rusticana* and *C. torosa*, respectively. Thus, it is possible to estimate the cell mass concentration from conductivity measurement.

Figure 3 presents the cultivation results for these hairy roots in Erlenmeyer flasks on the rotary shaker. It was confirmed that the cell mass concentrations on the gravimetric basis were in good agreement with those calculated by Eq. (1) with the respective α values.

Table 1. Effects of carbohydrates (carbon sources) on the specific growth rates of *A. rusticana* and *D. carota* hairy roots

Sugar	Specific growth rate [d^{-1}]	
	A. rusticana	*D. carota*
None	NG	NG
Glucose	0.10	0.15
Fructose	0.15	0.26
Galactose	NG	0.15
Xylose	NG	NG
Glycerol	NG	NG
Maltose	0.10	0.17
Lactose	NG	NG
Sucrose	0.31	0.24

NG: no growth. The cultivations were carried out with Erlenmeyer flasks containing the MS media with 30 $kg \cdot m^{-3}$ carbohydrates.

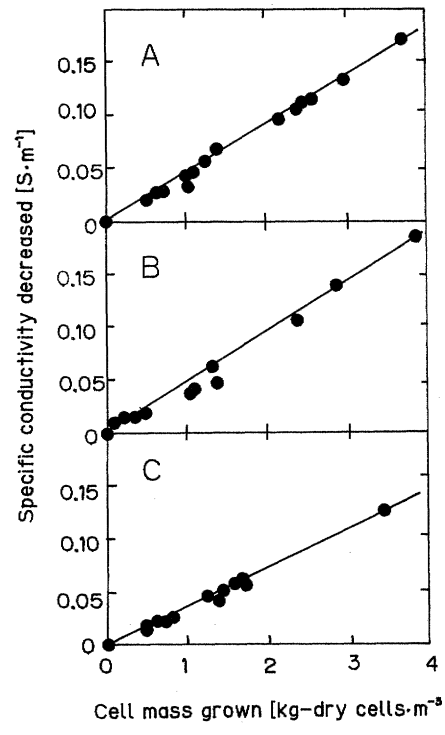

Fig. 2. Relationship between cell mass grown and medium conductivity decrease during cultures of *D. carota* (A), *A. rusticana* (B) and *C. torosa* (C) hairy roots

Hahlbrock *et al.*[6] demonstrated that specific growth phases and concomitant peaks in some enzyme activities of cells could be determined during plant cell suspension cultures based on conductometry. In the present work, we proposed the quantitative monitoring of the hairy root cultures by measuring medium conductivity. The decrease in medium conductivity appeared to reflect the amount of electrolytic or inorganic nutrients (mainly NH_4^+ and NO_3^-) consumed by the cells. Therefore, the α value has to be

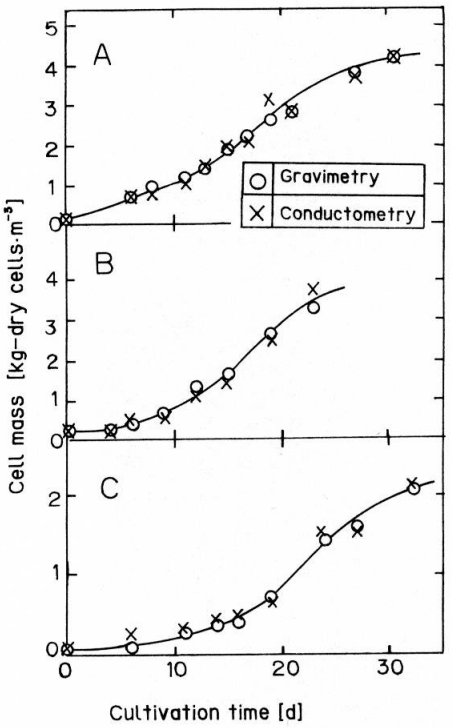

Fig. 3. Cell growth estimation for cultures of *D. carota* (A), *A. rusticana* (B) and *C. torosa* (C). The cultivations were carried out with Erlenmeyer flasks

experimentally predetermined for individual plant cells.

2.3 Hairy root cultures in reactors

Owing to the unique configuration of the hairy root cells, some problems to be solved associate with their culture in fermentors which were exploited on the basis of microbial culture techniques. There are few investigations of the bioreactor concerning hairy root cultures, except for works by Rhodes *et al.*[19,26]

In the present study, the bioreactor system was investigated for hairy root cultures using *A. rusticana* as a representative. As shown in **Table 2** (upper column), the cultivations with stirred tank and air-lift column were unsuccessful in the case of the free cell system. Problems in these runs were irregularity and stoppage of impeller rotation caused by clumps of growing hairy roots (stirred tank), and the thrusting out of most cells above the medium by air bubbles (air-lift column). In the latter case, moreover, serious cell disruption was observed and the amount of cells harvested was small. Only in this case was a discrepancy observed between the cell mass concentrations determined by gravimetry and conductometry.

Next we tried to immobilize (or anchor) the hairy roots on a reticulate support. **Figure 4** demonstrates *A. rusticana* cultures (20 d) in free and polyurethane-immobilized states. The amounts of cell mass were 7.5 (free) and 9.5 (immobilized) kg·m^{-3}, and no morphological difference was observed between them. Mavituna *et al.*[12] and Rhodes *et al.*[20] proposed

Table 2. Cultivation results of *A. rusticana* in various reactors

Type of reactor	Culture time [d]	Final cell mass [kg-dry cells·m^{-3}]	
		Gravimetry	Conductometry
Free cells			
Erlenmeyer flask (Rotary shaking)	25	7.7	7.6
Stirred tank	25	4.8	5.1
Air-lift column	25	2.8	5.1
Immobilized cells			
Air-lift column	31	11	10
Medium-trickling column	48	6.8	7.1
Medium-filling and drawing column with siphon	34	4.7	3.8

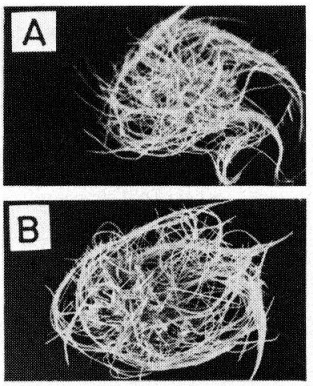

Fig. 4. *A. rusticana* hairy roots cultivated in free (A) and polyurethane-immobilized (B) states. The cultivations were carried out with Erlenmeyer flasks for 20 d

polyurethane foams as matrices for undifferentiated plant cells or calli, and polyurethane foam material was found to be a good support also for hairy plant cells.

For the immobilized cells of hairy roots, some culture systems were examined as listed in Table 2 (lower column). It was recognized that the air-lift column (see Fig. 1) gave the best cultivation result, comparable to that with the Erlenmeyer flask culture. Though the reason is not clear for the unsuccessful results in the other two systems with intermittent medium supply, it is possible that increased ion strength at the cell surface due to water vaporization had a negative effect on cell growth.

Figure 5 shows the time course of the air-lift column culture of immobilized *A. rusticana*. Sucrose was first hydrolyzed, followed by accumulation of glucose and fructose, and then the hydrolyzed sugars were almost consumed in 31 d, which is generally observed in callus cultures.[10] By on-line measurement of medium conductivity as shown in Fig. 1, it was possible to estimate the cell mass concentration *in situ* during the

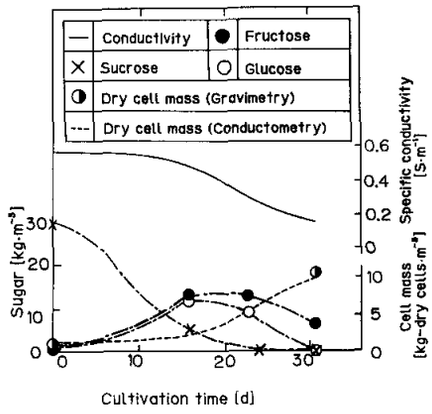

Fig. 5. Time course of air-lift column culture of immobilized *A. rusticana* hairy roots. The dotted line shows the dry cell mass concentration calculated by Eq. (1)

culture using Eq. (1) with the α value for *A. rusticana*. Good agreement was observed between the final cell mass concentrations on the gravimetric and conductometric bases. As also indicated in **Fig. 6**, the hairy roots showed good growth with the lapse of time and the column was closely packed with hairy roots after 31 d. The final amount of hairy roots was 11 kg-dry cells $\cdot m^{-3}$ (110 kg-fresh cells $\cdot m^{-3}$). Thus, it was confirmed that the air-lift column with cell immobilization was a superior cultivation system for the hairy roots.

In most cases, plant cells such as calli and hairy roots produce metabolites intracellularly.[13] In plant culture systems, therefore, it is desirable that the products be released from the cells into medium without cell harvest after full cell growth. Brodelius *et al.*[1,2] reported that treatment with some solvents was effective for the liberation of products from immobilized plant cells while leaving the cells in a viable state. In a preliminary experiment, we recognized that the hairy roots leaked metabolites into the medium by treating the cells with sonication and that the treated cells had the ability to grow again. Thus, these strategies can be applied to the hairy root culture presented in this paper, which will make it possible to develop a bioreactor system consisting of repeated cell growth (product accumulation) and product release phases.

Conclusion

1) The hairy roots transformed by *A. rhizogenes* exhibited active growth in MS medium with sucrose as a carbon source.

2) A linear relationship was obtained between the dry cell mass grown and medium conductivity decrease for the hairy root cultures.

3) Reticulate polyurethane foam was an appropriate support to immobilize the hairy root cells. A closely packed hairy root culture was achieved in the air-lift column bioreactor with immobilized *A. rusticana* cells.

4) By on-line measurement of medium conductivity, it was possible to monitor the cell mass concentration during the hairy root culture in the bioreactor system.

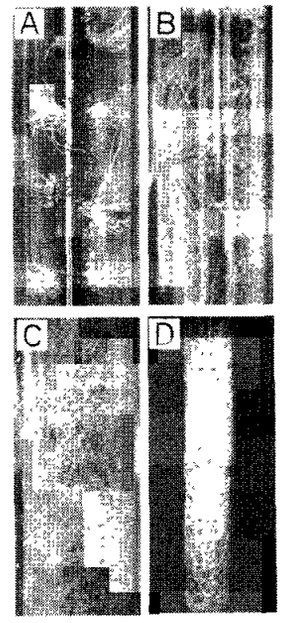

Fig. 6. Photographs of air-lift column culture of immobilized *A. rusticana* hairy roots. A: 0 d; B: 16 d; C: 31 d (in column); D: 31 d (out of column)

Acknowledgments

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