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Bioprocess Study on Chemical Production in Culture of Plant Hairy Roots

1995

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Bioprocess Study on Chemical Production in Culture of Plant Hairy Roots

（植物毛状根培養による有用物質の生産プロセスに関する研究）

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Preface

This dissertation work was carried out under the direction of Professor Setsuji Tone at Department of Chemical Engineering, Faculty of Engineering Science, Osaka University from 1989 to 1995.

The objective of this theses is to develop the processes of chemical production by plant hairy roots. The interest is focused on the selection of cell lines for metabolite production, modification of medium constituents, kinetic analyses of growth and chemical production, development of culture operation, design of bioreactor and optimization of culture system. The author hopes that the findings obtained in this work would make a significant contribution to the construction of effective bioprocesses for phytochemical production.

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General Introduction

Plants have played an important role in supplying human beings with many physiologically active substances since ancient times. Industry has long understood the importance of the production of plant substances not only as ingredients in beverages, but also in medicines, pigments, food additives and natural preservatives. Recently, with advances in plant tissue culture techniques, the methods for supplementing plant substances have become more diversified. In particular, by using a method which can control biological processes, the production rate of plant-derived metabolites allows for more economical industrial production (Fowler, 1981; Crutin, 1983; Rokem and Goldberg, 1985).

The advantages of this method include an access to a stable supply not susceptible to natural influences such as seasonal variation and abnormal weather, and the high productivity of chemicals through the arrangement of environmental conditions. The economical use of these biochemical potentials of plant cells long ago reached the stage of industrial utilization and in 1984 the initial commercialization of the industrial production of plant-derived metabolite was carried out in the production of shikonin by *Lithospermum erythrorhizon* (Tabata and Fujita, 1985). Down to the present day, however, there have been few cases of constructing industrial processes for chemical production by plant cells since several problems involving low content and unstable productivity of desired metabolites often attend the cultures of these cells.

The successful commercial use of plant cell derived products requires knowledge of the principles of plant cell genetics, physiology, metabolic regulation, and bioprocess engineering. Many problems remain to construct a culture system which realizing high yields of both biomass and the desired metabolites. The procedure for developing the process of chemical production

in culture of plant cells consists of the states shown in **Fig.1**. Secondary metabolite production often does not appear at all in the absence of prior ortanotenesis, or production trittered by ortanotenesis exceeds that of the correspondint ortan in the fully differentiated plant. Thus various approaches have been used to increase the yield of desired metabolites from cell cultures. Especially, the induction of cell lines, which have ortanotenesis to possess a stable- and hith-yieldint of desired metabolites, is one of the most important approaches (Yamamoto and Mizutuchi, 1982).

Most notably, plant hairy root transformed by a soil bacterium, *Agrobacterium rhizogenes*, which involves the intetration of a root-inducint plasmid into the plant cell tenome, has become an object of treat interest as a material for the production of plant-derived metabolites (Hamill *et al.*,1987; Toivonen, 1993). The resultant root tenerally exhibits active propatation in a pytohormon-free medium and has a metabolite content comparable to the oritinal plant root (Mano *et al.*, 1986; Bhadra *et al.*, 1993).

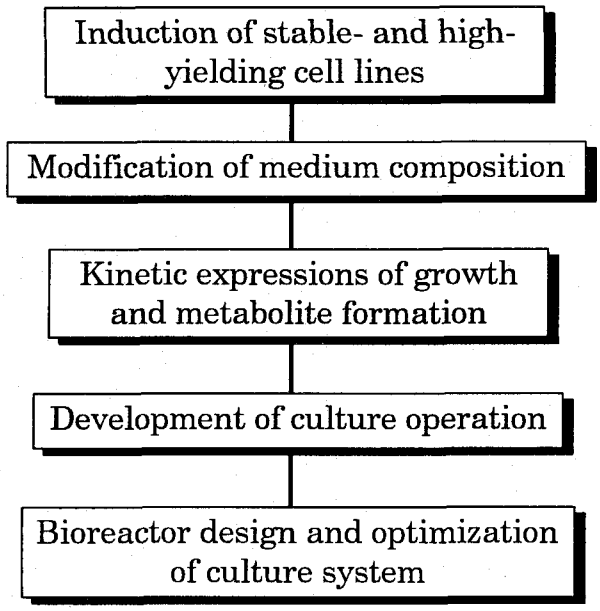


Fig.1 Procedure for developing process of chemical production in culture of plant cells

Moreover, high productivity in the culture of plant cells that include hairy roots can be achieved as the cell culture tends to respond in biological and biosynthetic behavior to slight changes in culture conditions (Panda *et al.*, 1992). Achieving this purpose requires the enhancement of growth rates and product yields through modifying the composition of the medium. Thus far, various key components such as carbon, nitrogen and phosphorus sources were found in hairy root cultures (Toivonen *et al.*, 1990; Jung *et al.*, 1992). Furthermore, when considering influence of the key components, the description of their kinetics should be conducted. Hairy roots have the morphological property of branching growth in which lateral roots branch and elongate, and there is a need to construct a mathematical model which is different from models applied to the culture of suspension cells.

In terms of the development of culture operations, the growth rate of plant cells that include hairy roots is slower than that of microorganisms. This leads to the technological importance of sustaining high cell mass density in a bioreactor and of utilizing the cell mass over a desirable period of culture time in the plant cell cultures. For this purpose, it is desirable that the products of concern are released from the plant cells into a medium since most of the products are intracellularly accumulated without secretion. Several excretion methods have significant potential to improve the feasibility of processes for producing favorable products from plant cell tissue culture. These involve, among others, the exposure of plant cells to organic or inorganic compounds, electricity and ultrasound (Brodelius and Nilsson, 1983; Uozumi *et al.*, 1992; Pu *et al.*, 1989; Kilby and Hunter, 1990). In these methods, however, it seems likely that the products are contaminated with the additives and that the heterogeneity of cellular distribution in the bioreactor prevents the effective release of the products.

Achieving a high concentration of plant cells in bioreactors is usually difficult due to insufficient mass transfer. Especially, a fundamental challenge of aerobic culture is supplying adequate oxygen (due to low oxygen

solubility in liquid nutrient medium). Therefore, a great deal of research that focuses on the effects of oxygen transfer on bioreactor performance has been conducted (Tanaka *et al.*, 1983). Fermentors for cell suspension cultures can not be applied to hairy root cultures since hairy roots with a highly branched morphology are easily destroyed by physical shock like shear stress. Therefore, in developing a fermentor suitable for the hairy roots, it is preferable to regard the fermentor as the reactor for a three phase system of gas-liquid-solid that includes immobilized catalyst. Several researchers have reported that hairy roots can be satisfactorily cultivated in fermentors where they were anchored to a support and nutrients were supplied by means of medium flowing (Kondo *et al.*, 1989; Hilton and Rhodes, 1990; Rodriguez-Mendiola *et al.*, 1992; Curtis, 1993; Ramakrishnan and Curtis, 1994). Furthermore, it is required to optimize the culture system which is integrated with individual stages as shown in Fig.1.

This paper includes five chapters, each dealing with specific aspects of a chemical production using plant cell culture. Five coherent themes are covered from the selection of cell lines for chemical production to bioreactor design and the optimization of the culture system as shown in Fig.1. All of these studies are integrated into the main theme of the development of a bioprocess relevant to the chemical production by plant hairy roots.

In Chapter 1, which comprises the basic study, the preponderance of hairy roots as the materials for the secondary metabolite production will be described by a comparison of the desired metabolite content between various kinds of hairy roots and their original plants. Moreover, as variation in the concentration of medium nutrients usually affects the productivity of the secondary metabolite, an on-line monitoring method for estimating the concentration of major inorganic nutrients by measuring conductivity and pH in the medium will be presented.

Chapter 2, covering the stage of improvement in environmental culture conditions, describes a strategy for the enhancement of the productivity of a

desired metabolite through the discovery of important factors (carbon, nitrogen and phosphorus sources) which affect the growth and metabolite formation. In particular, the extent of the effect of medium modification on pigment productivity will be discussed in the culture of red beet hairy roots.

Chapter 3 covers the kinetic description of growth and pigment formation in the hairy roots. In particular, the kinetic model available to the culture of red beet hairy roots is constructed, taking into account their morphological property and the effect of the media components obtained in Chapter 2.

Chapter 4, discussing the culture operation for the effective production of pigment in red beet hairy root culture, examines the culture with repeated processes of growth and *in situ* pigment recovery. The culture treatment by oxygen starvation in the medium was adopted for releasing pigment into the medium. Moreover, kinetic descriptions of pigment release and growth ability during oxygen starvation will be shown through the evaluation of the viability of hairy roots.

Chapter 5, which considers oxygen transfer between the medium and the hairy roots, will describe the design of a specialized bioreactor for the culture of the red beet hairy roots in which the oxygen supply is enhanced by the medium circulated through hairy roots. Moreover, as a result of integrating the culture system that includes medium modification, the construction of a kinetic model, the development of culture operations and the bioreactor design for the pigment production by red beet hairy roots, a description of the optimization of culture with periodic operations of growth and pigment release will be offered.

Chapter 1 Utilization of Hairy Roots for Production of Chemicals and Estimation of Nutrient Concentrations in Hairy Root Cultures

1.1 Introduction

In vitro cultures of plant cells have become increasingly important for the production of industrially valuable biochemicals. Hitherto, the majority of studies have dealt with undifferentiated cells (callus tissues) in either a dispersed or immobilized cell culture system (Tanaka, 1987; Lindsey and Yeoman, 1985). However, the cultures of such plant cells are often associated with several problems which involve low content and unstable productivity of desired metabolites.

Recently, plant hairy roots have become of interest as an alternative for cell culture because of their infinite and active proliferation in a phytohormone-free medium, and their ability to synthesize and accumulate valuable materials at a comparable level to the original plant (Mano, 1989). Moreover, in the term of bioreactor design, hairy roots have an adventitious property that they can be regarded as immobilized cells which are superior to endurance of damage as hairy roots grow with a highly branched morphology.

On the other hand, for *in vitro* cultures of plant cells, some inorganic compounds are important nutrients to sustain the propagation and metabolism of cells. From systematic examinations, Muranaka *et al.* (1992) reported that the modification of nitrogen source led to the increased secretion of scopolamine by *Duboisia leichhardtii* hairy root. Brown *et al.* (1976) recognized that a potassium level in medium had a pronounced effect on growth and embryogenesis of carrot cells. Thus the concentrations or ratios of the main inorganic nutrients are significant factors for the metabolite production and differentiation of various plant cells. Therefore it

is desirable to estimate and control the levels of these ionic nutrients during plant cell cultures. However, it is generally difficult to analyze the nutrients *in situ* in bioreactor and most of the conventional methods can be carried out only after withdrawing sample aliquots from the culture system.

As the basic study, the preponderance of hairy roots as materials for the chemical production is described by comparison of desired metabolite content between various kinds of hairy roots and their original plants. In particular, kinetics of growth and metabolite production through the culture of red beet, pak-bung and horseradish hairy roots are shown. Moreover, the main inorganic nutrient concentrations is estimated on the basis of conductivity measurement and the relationship between medium conductivity decrease and mineral consumption during plant cell cultures is described.

1.2 Experimental

A) Plant Cells The hairy roots of red beet (*Beta vulgaris* L. cv. Detroit dark red) (Taya *et al.*, 1992), pak-bung (*Ipomoea aquatica*) (Taya *et al.*, 1989a), horseradish (*Armoracia rusticana* P. Gaert., B. Meyer et Scherb.) (Noda *et al.*, 1987), and carrot (*Daucus carota* L.) (Tanaka *et al.*, 1985) were used, which were induced by inoculating the plant specimens with *Agrobacterium rhizogenes* A4 using the leaf disk method. The hairy roots of Madder (*Rubia tinctorum* L.) provided by San-Ei Gen F. F. I. Inc., Osaka, Japan were also used, which were induced by *Agrobacterium rhizogenes* ATCC 13332. Axenic hairy roots were maintained on Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) containing 30 kg/m^3 sucrose and 10 kg/m^3 agar, and subcultured every month.

B) Media and culture conditions Hairy roots were cultured using a 200 cm^3 Erlenmeyer flask with 40-80 cm^3 (carrot: 40cm^3, horseradish: 40 or 60 cm^3, pak-bung and madder: 60cm^3, red beet: 80cm^3) of liquid MS medium containing 20 kg/m^3 sucrose.

The medium pH was adjusted to 5.8 with 0.1 kmol/m^3 NaOH, followed by autoclaving at 121℃ for 20 min. The flask was shaken at 100 r.p.m. on a rotary shaker (Model HRS-24, Shibata Scientific Technology Ltd., Tokyo) at 25℃ in the dark, unless otherwise noted.

C) Analyses The cell mass was measured gravimetrically after the harvested hairy root was rinsed with distilled water followed by drying at 80℃ for 72 h.

For analyses of intracellular pigments of red beet, fresh cells of hairy roots (or materials excised from the original plant) were thoroughly homogenized in 50%(v/v) ethanol aqueous solution at ambient temperature using a mortar and pestle. The supernatant was obtained by centrifugation (24,000 x *g*, 15 min) at 25 °C and used as a pigment extract. The pigment concentrations in extract or medium were determined spectrophotometrically and the amounts of betacyanins and betaxanthins were expressed as those of betanin and vulgaxanthin-I, respectively, according to the method of Nilsson (1970).

The Hunter color property of the pigments was estimated with a color difference meter (S & M color computer SM-3, Suga Shikenki Co., Tokyo), and expressed in terms of L_H (lightness), a_H (redness), b_H (yellowness) and $\tan^{-1}(b_H/a_H)$ (color tone). The values were calibrated with a standard white tile.

The pigments of red beet were also analyzed chromatographically after pigment solution was pretreated through a Sephadex G-25 (Pharmacia Co., Sweden) column and then concentrated by an evaporator. High performance liquid chromatography (HPLC) was carried out with an LC-880 Series System (Japan Spectroscopic Co., Tokyo) under the following conditions: Column; Nucleosil 5C18 (4.6ϕ x 250 mm, Macherey & Nagel Co., FRG), eluent; 18% (v/v) methanol in 1 mM KH_2PO_4 (pH = 2.5) and detection; absorbance at 530 nm. Thin layer chromatography (TLC) was done with

silica gel plates (Kieselguhr F_{254}, Merck Co., FRG) using *n*-propanol/acetic acid/water (6:2:3 (v/v)) as a solvent system.

Agropine and mannopine (opines) in red beet hairy roots were identified by paper electrophoresis and alkaline silver nitrate staining (Tanaka, 1990).

For analysis of intracellular pigments of madder hairy roots, fresh hairy roots were homogenized in 70 % (v/v) ethanol aqueous solution at 50 ℃ using a mortar and pestle. The supernatant obtained by centrifugation (24,000 x *g*, 15 min, 25 ℃) was used as a pigment extract. The pigment concentration in the pigment extract was determined by high performance liquid chromatography (HPLC) with an LC-6A System (Shimadzu Co., Kyoto) under the following conditions. Column: Nucleosil 5C8 (Macherery-Nagel Co., Germany); detection: absorbance at 430 nm; eluent: a mixture of ethanol, water and 0.1 mol/dm^3 KH_2PO_4 (7:2:1, by volume) at a flow rate of 0.7 cm^3/min. Under these analytical conditions, the pigment mixture was eluted as two main peaks on a chromatogram with retention times of 3.7 min (peak No.1) and 4.9 min (peak No.2). In the present study, for convenience, peaks of Nos. 1 and 2 were evaluated by using authentic ruberythric acid (alizarin 2-*O*-β-primerveroside, retention time: 3.7 min) and authentic alizarin (1,2-dihydroxyanthraquinone, retention time: 4.9 min), respectively. The amounts of anthraquinone pigments were expressed as the sum of the alizarin and ruberythric acid quantities. Authentic alizarin and ruberythric acid were purchased from Tokyo Kasei Co., Tokyo and Carl Roth Co., Germany, respectively.

The superoxide dismutase (EC 1.15.1.1, SOD) activity in pak-bung and horseradish hairy roots were determined by methods of Asada *et al* (1974). First, fresh cells of hairy roots (or materials excised from the original plant) were thoroughly homogenized in 50 mM potassium phosphate buffer (pH 7.8) on ice using a mortar and pestle. The supernatant was obtained by centrifugation (24,000 x *g*, 15 min) at 5 °C and used as a enzyme extract. The

SOD activity in extract were determined at 25 ℃, pH = 7.8 by a system of xanthine-xanthine oxidase for generation of O_2^- and a ferricytochrome *c* (Sigma Chemical Co.) systems an electron acceptor being used. One unit of activity is defined as the quantity of enzyme required to produce 50% decrease in the rate of cytochrome *c* reduction.

The nitrogen contents of cells were measured with Nessler's reagent (Wako Pure Chemical Industries Co., Osaka) after the cell decomposition in a Kjeldahl flask with concentrated sulfuric acid. The following analyses were done using cell-free culture broth. The concentrations of ammonium, nitrate and phosphate ions were determined colorimetrically (JIS, 1974; Umbreit, 1957). The amount of potassium ion was determined by atomic absorption spectrometry (Model AA-880 Mark II, Japan Jarrell-Ash Co., Tokyo). The specific conductivity of medium or mineral solution was measured at 25℃ with a conductivity meter (Model CM-40S, Toa Electronics Co., Tokyo).

1.3 Results and Discussion

1.3.1 Chemical production by hairy roots

As a result of infecting the red beet specimens (leaf, petiole and root) with *A. rhizogenes*, many adventitious roots appeared, mainly at the vein cut-ends of leaf sections (Taya *et al.*, 1992). After repeated transfers and elongation of the root tips on MS agar medium, some axenic root clones were established (**Fig.1.1**). As shown in **Fig.1.2**, one clone (clone No.5) contained agropine and mannopine, which are synthesized as unique opines (amino acid derivatives) in transformants by enzymes encoded on the T-DNA of the Ri plasmid (Mano, 1989). Thus, clone No.5 was concluded to be a hairy root transformed with the plasmid. The hairy root exhibited active growth in liquid MS medium without phytohormones, even after successive subcultures.

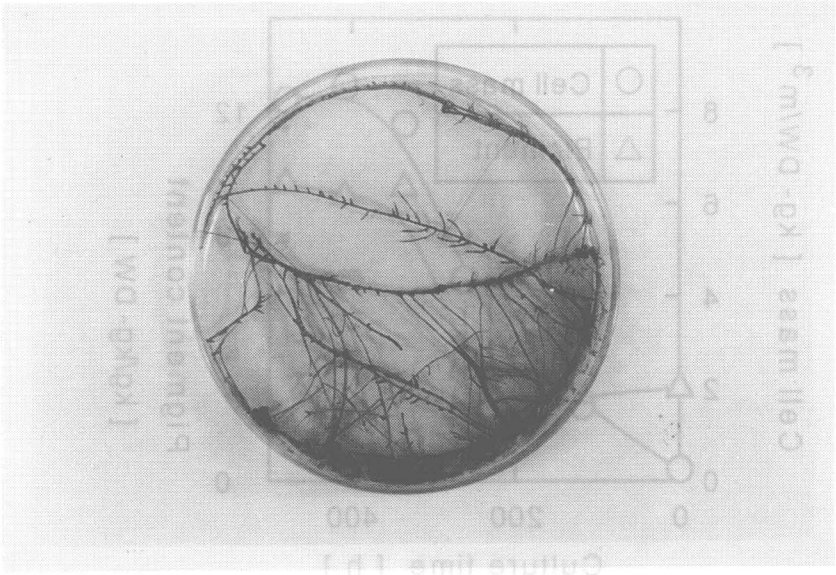


Fig.1.1 Photograph of red beet hairy roots on agar plate

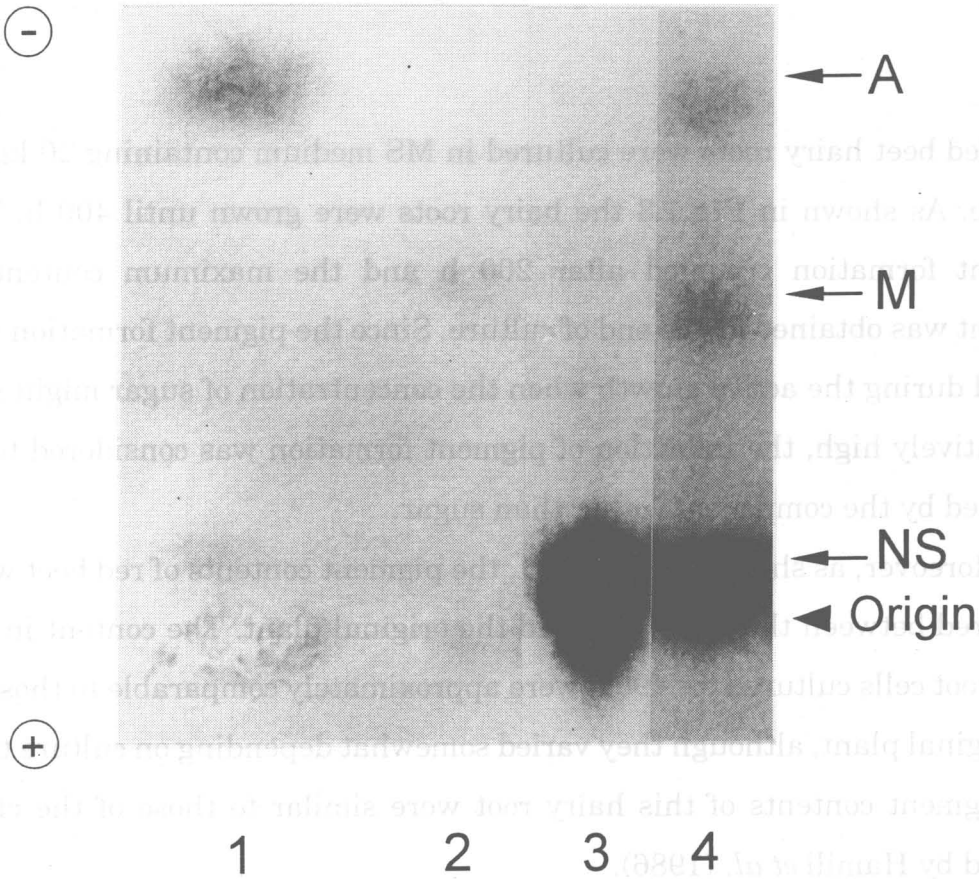


Fig.1.2 Electrophoretic analysis of opines in red beet hairy root (clone No.5)

Lane 1: Authentic agropine, 2: authentic mannopine, 3: original plant (root), and 4: hairy root. Abbreviations: A = agropine, M = mannopine, and NS = neutral sugars.

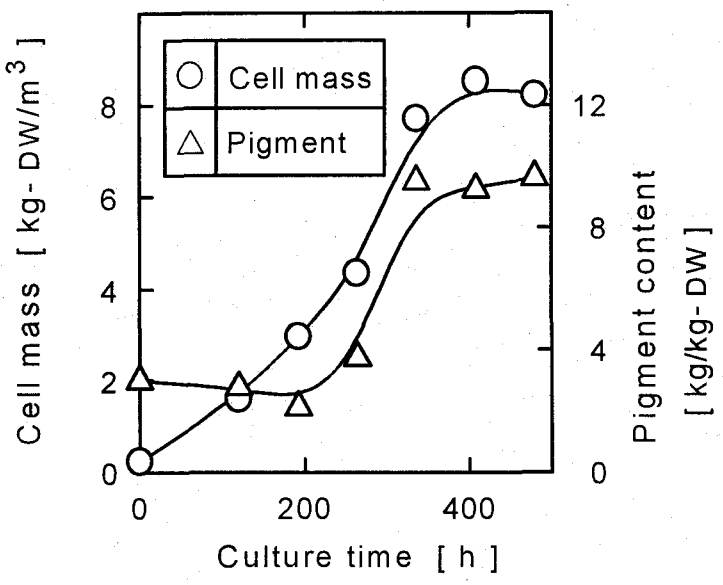


Fig.1.3 Course of culture of red beet hairy roots in MS medium containing 20 kg/m^3 sucrose

Red beet hairy roots were cultured in MS medium containing 20 kg/m^3 sucrose. As shown in **Fig.1.3** the hairy roots were grown until 400 h. The pigment formation occurred after 200 h and the maximum content of pigment was obtained at the end of culture. Since the pigment formation was started during the active growth when the concentration of sugar might still be relatively high, the induction of pigment formation was considered to be governed by the components other than sugar.

Moreover, as shown in **Table 1.1**, the pigment contents of red beet were compared between the hairy root and the original plant. The content in the hairy root cells cultured for 490 h were approximately comparable to those in the original plant, although they varied somewhat depending on culture time. The pigment contents of this hairy root were similar to those of the clone induced by Hamill *et al.* (1986).

The pigments produced by the hairy root culture were characterized. **Figure 1.4** shows HPLC profiles of the pigments in the hairy root cells and

Table 1.1 Comparison of metabolite production between hairy roots and original plants

Species	Products	Hairy root*	Original Plant		
			Leaf	Stem	Root
Red beet	Red pigment [10^{-3} kg/kg-DW]	9.0	4.4	6.3	5.5
Madder	Anthruquinone pigment [10^{-3} kg/kg-DW]	19	–	–	43
Horseradish	Superoxide dismutase [10^{6} U/kg-DW]	3.6	9.2	1.8	0.7
Pak-bung	Superoxide dismutase [10^{6} U/kg-DW]	2.9	2.4	0.1	0.6

* The figures in the column show the maximum values through the cultures with MS medium containing 20 kg/m^3 sucrose.

those in the original beetroot. The chromatographic pattern of the pigments in hairy roots was substantially identical to those extracted from the original roots. Here, the main peak of No.2 was identified as betanin shown in **Fig.1.5**; the others are unknown. The pigments in hairy roots were also confirmed to consist of identical components to the ones in original roots based on TLC analysis.

Table 1.2 shows the Hunter color properties of various pigments using a color difference meter. From this analysis, distinctive differences were not found among the pigments in the extracts from the cells (hairy and original roots) in terms of L_H, a_H, b_H, and $\tan^{-1}(b_H/a_H)$ values. Therefore, the pigments in extracts from hairy roots were regarded to be substantially the same as the original ones.

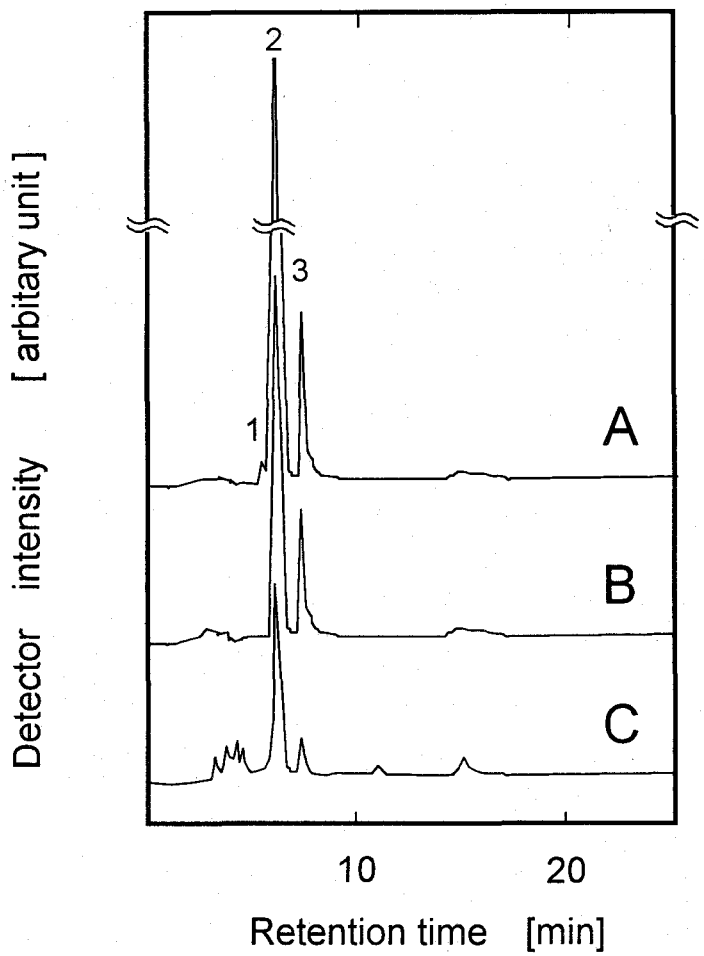


Fig.1.4 HPLC profiles of pigments from hairy root and original plant of red beet

A: Extract from original plant (root), B: Extract from hairy root cells, C: Culture broth with released pigments

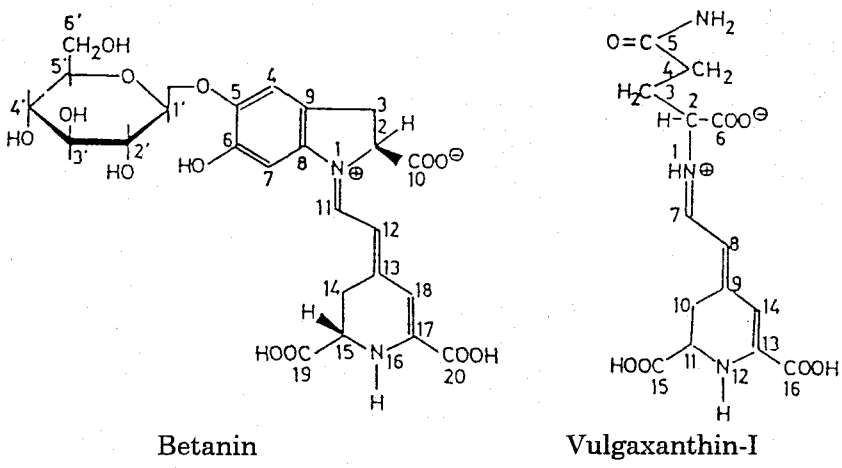


Fig.1.5 Structure of pigments in red beet hairy roots

Table 1.2 Hunter color properties of pigments from the original plant and hairy root of red beet

Pigment source	L_H	a_H	b_H	$\tan^{-1}(b_H/a_H)$
Extract from original plant (root)	68.7	36.1	12.0	18.4
Extract from hairy root cells	65.1	32.7	11.9	20.0

L_H: lightness (0=black and 100=white), a_H: redness, b_H: yellowness, and $\tan^{-1}(b_H/a_H)$: color tone.

For the investigation of capability for the chemical production by other kinds of hairy roots, comparisons of the contents of desired metabolites between hairy roots and their original plants were conducted.

Three species of plants described below were used; madder (anthraquinone pigment production), pak-bung and horseradish (SOD enzyme production). First of all, the pigment produced by the madder hairy roots was investigated in comparison with that in original plant roots. **Figure 1.6** shows the HPLC profiles of the pigment extracts obtained from the hairy roots (A) and original roots (B), and the mixture of the authentic pigments (D). In these two extracts, similar HPLC profiles were observed and the peaks of Nos.1 and 2 were identified as ruberythric acid and alizarin, respectively. Table 1.1 represents the pigment contents of hairy roots and original roots of madder. Both of them contained ruberythric acid as a main pigment (15 x10^{-3} kg/kg-DW in hairy root and 40 x10^{-3} kg/kg-DW in original root) with small amount of alizarin.

It is known that alizarin occurs in fresh madder roots in the form of ruberythric acid which includes alizarin as an aglycone (Vaidyanathan, 1985). The cultured madder hairy roots also contained ruberythric acid in larger quantity rather than alizarin. However, the total amount of pigments

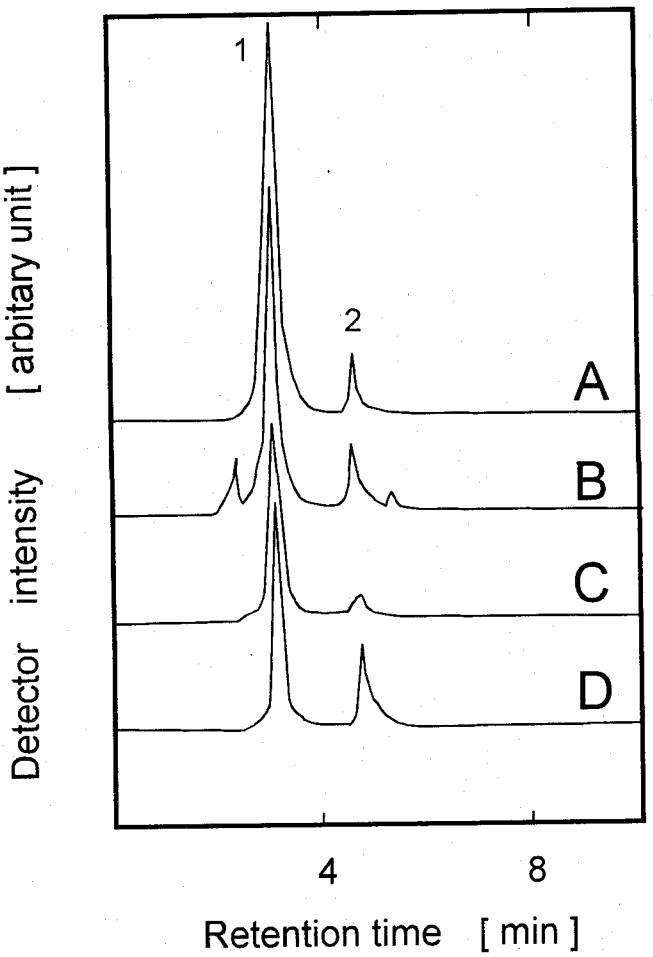


Fig.1.6 HPLC profiles of pigment extracts obtained from hairy root and original plant root of madder

A: Extract from hairy root, B: Extract from original plant root, C: Culture broth with released pigment, D: Mixture of authentic ruberythric acid and authentic alizarin

in the hairy roots is smaller than that in original plant roots, since the medium used in this culture of madder hairy roots was not adequate for the pigment production. Thus it was needed to investigate the influence of medium components on hairy root growth and pigment formation and improve the medium constituents.

On the other hand, SOD, which has an function to protect cells from superoxide, exists not only in various kinds of microorganisms but also in

plant cells, and it is of potential interest in pharmaceutical and food industries (Tsukuda and Soda, 1988). In this study, to investigate the ability of SOD productivity by hairy roots, the measurement of SOD activity in hairy roots of horseradish and pak-bung through the cultures and the comparison of SOD activities with those in original plant cells were conducted. As shown in Table 1.1, higher SOD activity in leaf cells of both original plants were observed. The SOD activities of both hairy roots were attained to about 3 x 10^6 U/kg-DW which was the value about 4 times higher than the one found in the roots of original plants. These results mean that horseradish and pak-bung hairy roots could be the promising materials for the production of SOD.

Moreover, as shown in **Fig.1.7**, the increased activity of SOD was observed after the logarithmic growth state, reaching for both pak-bung and horseradish hairy roots about 6 x 10^6 U/kg-DW. These values were considered to be of the same level or higher as found in other plant cells (Matkovics, 1977) or microorganisms (Taniguchi *et al.*, 1989).

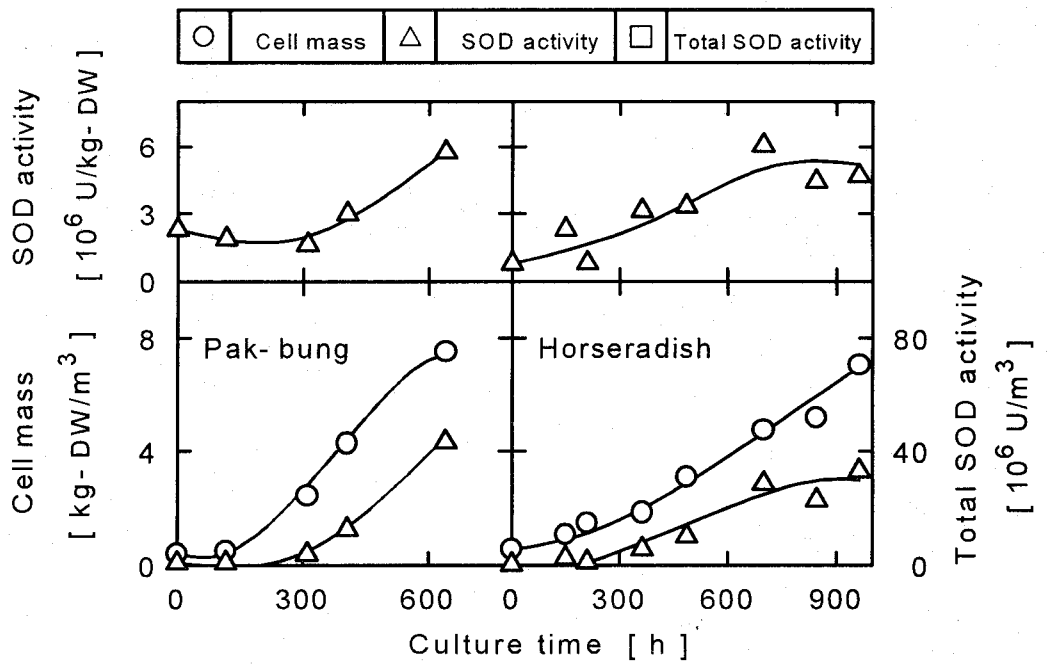


Fig.1.7 Time courses of cultures of pak-bung and horseradish hairy roots

1.3.2 Conductometric estimation of main inorganic nutrient concentrations in plant cell cultures

A) Relationship between specific conductivity and inorganic components of mediumIn plant cell cultures, main inorganic nutrients are important for the propagation and metabolite production of plant cells and it is needed to make on-line estimation of their concentration. Here, it was tried to estimate main inorganic nutrient concentrations using a elector conductivity method. First, the relationship between specific conductivity and inorganic components of medium was investigated.

Molar ionic conductivity for a given ion species is defined by Eq.(1.1) (Barrow, 1966).

$$\lambda_i = \frac{\kappa_i}{[I_i]} \tag{1.1}$$

Although the value of λ_i decreases with increased concentration of I_i owing to mutual interactions among ions, molar ionic conductivity at infinite dilution ($\lambda_{i,\infty}$) is a constant which may be obtained from literature (Lange, 1979).

In the present study, modified molar ionic conductivity, λ_i' is introduced for an ion species. The λ_i' value is assumed to be constant in the limited range (ion concentrations in plant cell culture: 0-40 mol/m^3), and to be obtained by Eq.(1.2).

$$\lambda_i' = \varphi \cdot \lambda_{\infty,i} \tag{1.2}$$

The specific conductivity of electrolyte solution is expressed as the sum of each ion contribution, based on Kohlrausch's law of independent migration of ions.

$$\kappa = \sum_i \lambda_i'[I_i] \tag{1.3}$$

As shown in **Fig.1.8**A, the specific conductivity of KCl and NH_4NO_3 solutions (0-50 mol/m^3) was measured. The solid and broken lines were

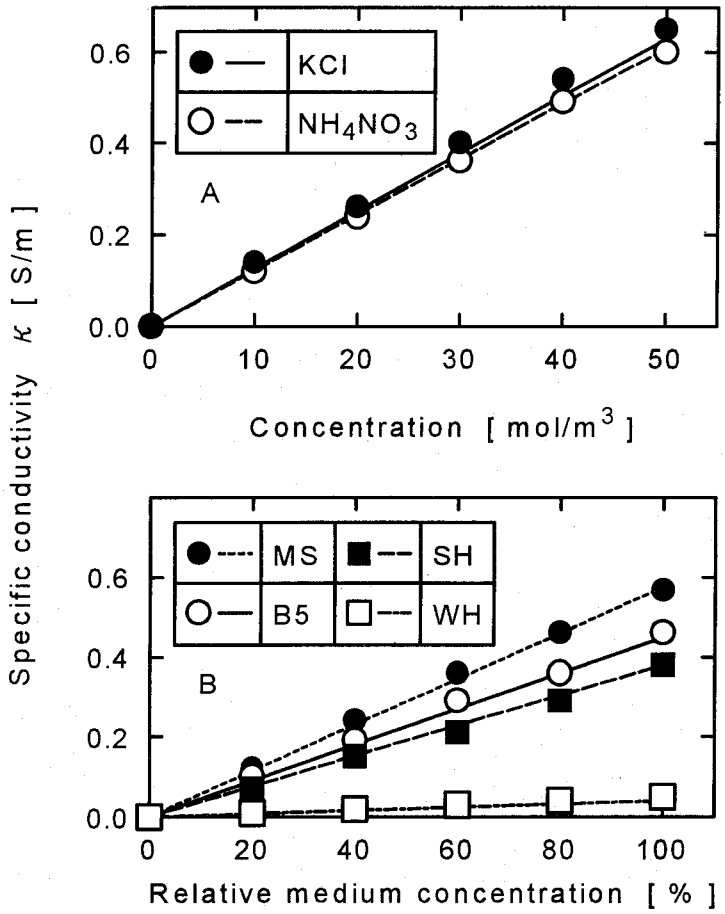


Fig.1.8 Relationships between specific conductivity and mineral concentration

A: KCl and NH_4NO_3 solutions; B: major compartment mixture of four media (lines show the calculated values. 100% relative medium concentration is corresponded to concentrations of minerals in Table 1.3)

calculated by Eqs.(1.2) and (1.3), and the φ value was evaluated as 0.84 by fitting these equations to the experimental data using the $\lambda_{\infty,i}$ values of 7.35 (for K^+), 7.35 (for NH_4^+), 7.64 (for Cl^-) and 7.14 (for NO_3^-) mS·m²/mol (see **Table 1.3**).

The specific conductivity of electrolyte mixture consisting of major medium components for plant cell culture was also evaluated. The major components of four media (Murashige-Skoog medium (denoted as MS), Gamborg B5 medium (denoted as B5), Schenk-Hildebrandt medium (denoted as SH), White medium (denoted as WH)) are shown in Table 1.3. In this case, the following association/dissociation reactions were considered because of

Table 1.3 Major inorganic components of plant cell culture media and values of modified molar ionic conductivity for calculation

Ion	Concentration [mol/m^3]				Modified molar ionic conductivity [10^{-3} S•m^2/mol]	
	MS	B5	SH	WH		
K^+	20.5	24.7	24.7	1.66	6.17	(7.35)
Na^+	0	1.09	0	1.53	4.21	(5.01)
NH_4^+	20.6	2.02	2.61	0	6.17	(7.35)
Ca^{2+}	2.99	1.02	1.36	1.27	10.0	(11.9)
Mg^{2+}	1.50	1.01	1.62	2.92	8.91	(10.6)
Cl^-	5.98	1.02	2.72	0.872	6.42	(7.64)
NO_3^-	39.4	24.7	24.7	3.33	6.00	(7.14)
$H_2PO_4^-$	1.25	1.09	2.61	0.120	2.77	(3.30)
HPO_4^{2-}					9.58	(11.4)
SO_4^{2-}	1.50	2.02	1.62	4.33	13.4	(16.0)
H^+	-	-	-	-	29.4	(35.0)
OH^-	-	-	-	-	16.7	(19.9)

The values in the parentheses show molar ionic conductivity at infinite dilution cited from the literature (Lange,1979).

medium pH values (initial pH: 5.6 and pH change during culture: 4.5-8).

$$H_2PO_4^- = HPO_4^{2-} + H^+, \quad K_{P2} = [HPO_4^{2-}][H^+]/[H_2PO_4^-] \quad (1.4)$$

$$NH_4^+ = NH_3 + H^+, \quad K_A = [NH_3][H^+]/[NH_4^+] \quad (1.5)$$

$$H_2O = H^+ + OH^-, \quad K_W = [H^+][OH^-] \quad (1.6)$$

where K_{P2} = 6.23 x 10^{-8} kmol/m^3, K_A = 5.68 x 10^{-10} kmol/m^3 and K_W = 1.01 x 10^{-14} kmol^2/m^6.

As shown in Fig.1.8B, the specific conductivity of the four media could be described well by Eqs.(1.2) and (1.3), using the φ value of 0.84 (see the lines in the figure). The λ_i' and $\lambda_{\infty,i}$ values for all the ions are summarized in Table 1.3.

B) Estimation of main organic nutrients in plant cell cultures From these results, the estimation of main inorganic nutrients was made as follows. For various plant cell cultures, linear relationships were observed between the changes of dry cell mass and medium conductivity (Taya *et al.*, 1989b).

$$\Delta X = -\beta \cdot \Delta\kappa \qquad (1.7)$$

Assuming that the nitrogen content of a plant cell line is constant and that the cell's nitrogen originates only from ammonium and/or nitrate in medium, the following equation is obtained from Eq.(1.7) on the basis of mass balance for nitrogen.

$$1000\beta \cdot C_N \cdot \Delta\kappa/14 = [NH_4^+] + [NH_3] + [NO_3^-] \qquad (1.8)$$

With respect to the main inorganic nutrients of interest (NH_4^+, K^+, NO_3^-, $H_2PO_4^-$ and HPO_4^{2-}), $\Delta\kappa$ is expressed by Eq.(1.3) , on the assumption that the other ion uptakes by plant cells have negligible contributions to medium conductivity decrease.

$$\Delta\kappa = \lambda'_{PO} \cdot \Delta[K^+] + \lambda'_{AM} \cdot \Delta[NH_4^+] + \lambda'_{H} \cdot \Delta[H^+] + \lambda'_{NI} \cdot \Delta[NO_3^-]$$
$$+ \lambda'_{OH} \cdot \Delta[OH^-] + \lambda'_{P1} \cdot \Delta[H_2PO_4^-] + \lambda'_{P2} \cdot \Delta[HPO_4^{2-}] \qquad (1.9)$$

From the electric neutrality in medium,

$$[K^+] + [NH_4^+] + [H^+] + [Na^+] + 2[Ca^{2+}] + 2[Mg^{2+}] = [NO_3^-] + [OH^-]$$
$$+ [H_2PO_4^-] + 2[HPO_4^{2-}] + [Cl^-] + 2[SO_4^{2-}] \qquad (1.10)$$

Based on the result that the consumption of Na^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-} is negligible in plant cell cultures, Eq.(1.10) can be rearranged as follows.

$$[K^+] + [NH_4^+] + [H^+]$$
$$= [NO_3^-] + [OH^-] + [H_2PO_4^-] + 2[HPO_4^{2-}] + \gamma_0 \qquad (1.11)$$

where, $\gamma_0 = [Cl^-] + 2[SO_4^{2-}] - [Na^+] - 2[Ca^{2+}] - 2[Mg^{2+}]$. γ_0 = 0 mol/m^3 for MS and SH. γ_0 = -0.09 mol/m^3 for B5. γ_0 = -0.38 mol/m^3 for WH.

In preliminary cultures of the hairy roots, it was found that phosphate ions were almost consumed at very early stage of the cultures. This

Table 1.4 Value of constants used in Eqs. (1.7) and (1.8) (MS medium)

Plants	C_N [kg/kg-DW]	β [kg-DW/(S•m^2)]
Hairy roots		
Carrot	0.089	22
Horseradish	0.089	22
Calli		
Tabacco	0.062	28
Coffee	0.046	36

phenomenon on quick phosphate uptake has been reported in other plant cell cultures (Kato, 1977). Phosphate appears to be stored and utilized in the plant cells. In subsequent calculation, therefore, the values of $H_2PO_4^-$ and HPO_4^{2-} concentrations were regarded as zero except for initial culture time.

The values of C_N and β were determined for the various hairy roots and calli in MS medium culture (**Table 1.4**). The cultivation results of carrot and horseradish hairy roots are presented in **Fig.1.9**. It was found that the concentrations of K^+, $NH_4^+$and NO_3^- during the cultures could be satisfactorily estimated by using Eqs.(1.4)-(1.6), (1.8), (1.9) and (1.11) on the basis of conductivity and pH measurement.

Figure 1.10 summarizes the relationships between the concentrations of these ions and dry cell mass based on conductometric method, and those based on direct analytical method during the plant cell cultures. In this figure, the calculation results using parameter values shown in Table 1.4 are also included for the cultures of tobacco and coffee calluses (Taya *et al.*, 1989b). A good linearity was observed and the regression line (see the solid line) obtained by least-squares method agreed closely with diagonal in the figure. The value of correlation coefficient was 0.96.

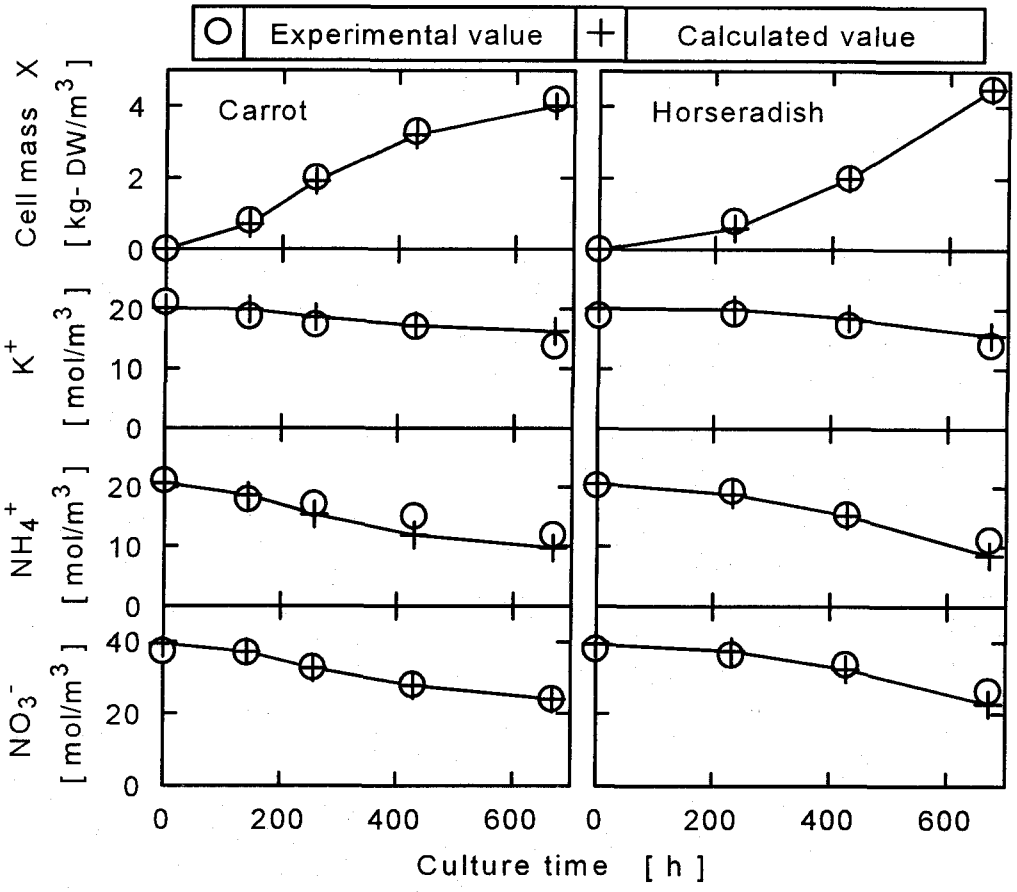


Fig.1.9 Cultivation results for hairy roots of carrot and horseradish

It is known that the concentrations of NH_4^+ and NO_3^- are important factors for the production of secondary metabolites by plant cell cultures (Fujita *et. al.*, 1981). Therefore, the real-time estimation is desired for these inorganic nutrients. In this study, the convenient method for simultaneous estimations of these main ions is presented by measuring medium conductivity and pH. It is possible to determine medium conductivity *in situ* in plant cell bioreactors, and this will enable us to perform the on-line estimation and control of plant cell culture processes.

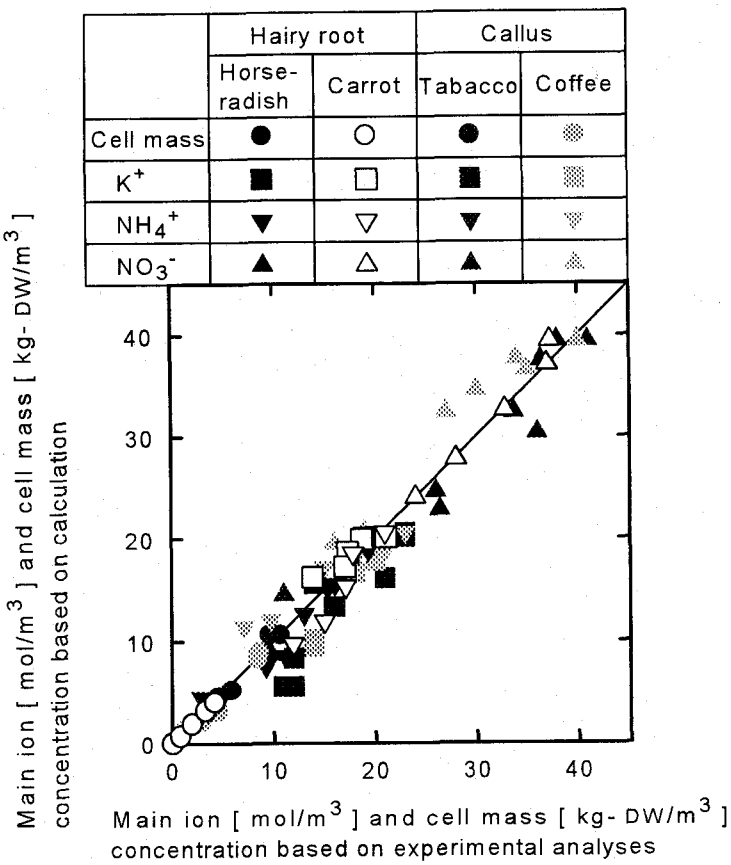


Fig.1.10 Relationship between ion and cell mass concentrations on calculated and experimental analytical bases

1.4 Summary

The feasibility of chemical production by the hairy roots of red beet (pigment production), madder (pigment production), horseradish (SOD production) and pak-bung (SOD production) was investigated and it was found that except madder the hairy roots had the levels of chemicals comparable to those in corresponding roots of original plant and thus they can be considered as promising materials for the chemical production. Especially, in the culture of red beet hairy roots, the pigments in hairy roots were examined using the profiles of HPLC and Hunter color properties and were confirmed to substantially identical to those extracted from original

plant cells of red beet. Moreover, the induction of pigment formation was suggested to be governed by medium components other than sugar. While, pigment content in madder hairy roots was lower than that in original plant roots and the necessity of enhancement of the pigment content in madder hairy roots occurred.

Furthermore, it was found that the main inorganic nutrients which are important for the propagation and metabolite production of plant cells can be determined using electrical conductivity method. The specific conductivity of medium could be expressed as the total contribution of its electrolyte components in terms of modified molar ionic conductivity. On the assumption that the medium conductivity decrease is exclusively attributable to the consumption of potassium, ammonium and nitrate ions, and that plant cells do not secrete ionic products into medium during the growth, the concentrations of these ions could be calculated from electrical neutrality and nitrogen balance equations. It was confirmed that the calculated amounts of the ions were in fair agreement with the experimentally analyzed data during the culture of calluses (coffee and tobacco) and hairy roots (carrot and horseradish). The conductivity methods proved to be suitable for the real-time monitoring of the main inorganic nutrients in these plant cell cultures.

Chapter 2 Effect of Medium Components on Growth and Chemical Production in Hairy Root Cultures

2.1 Introduction

Plant hairy roots induced by *Agrobacterium rhizogenes* infection have gained attention in recent years as *in vitro* cultured cells which produce valuable biochemicals as well as callus tissues (Signs and Flores, 1990; Tanaka *et al.*, 1992). The advantage of hairy root cultures is that the hairy roots proliferate in a defined medium without hormones while retaining high metabolite biosynthetic capabilities which are frequently comparable to those of the original plant roots.

In many plant cell cultures, it is well known that medium constituents (carbon and nitrogen sources, phosphate, metals, vitamins, and regulators) have significant effects on cell growth and metabolite formation (Rokem and Goldberg, 1985). For example, in shikonin production by suspension culture of *Lithospermum erythrorhizon* cells, a medium without ammonium is suitable though a medium containing both ammonium and nitrate was favorable for cell growth (Fujita *et al.*, 1981). Moreover, it was reported that the limitation of phosphate in the medium caused an increase in metabolite formation by cultured plant cells; *e.g.* anthocyanin production by *Vitis* callus tissue (Yamanaka *et al.*, 1983) and solavetivone production by *Hyoscyamus muticus* hairy roots (Dunlop and Curtis, 1991)

In this chapter, the influence of medium constituents on accumulation of the red pigment betanin in red beet hairy roots (*Beta vulgaris* L. cv. Detroit dark red) is investigated, and the components causing the enhanced pigment production by the hairy root culture are identified. The influence of medium constituents on the growth and metabolite formation of the other kinds of hairy roots (madder and pak-bung hairy roots) are also investigated.

2.2 Experimental

A) Hairy roots, medium and culture conditions The hairy roots of red beet, madder and pak-bung described in section 1.2 were used. Hairy root cultures for maintenance and inoculum preparation were carried out at 25°C for 10 d (red beet) or in Murashige-Skoog (MS) liquid medium (Murashige and Skoog, 1962) containing 30 kg/m^3 sucrose and no hormones. For the experiments, the hairy roots were cultivated with an inoculum of 2-3 kg-fresh root mass per m^3 at 25°C in the dark using 200-cm^3 Erlenmeyer flasks (medium volume: 60 (madder and pak-bung) or 80 cm^3 (red beet)) shaken on a gyratory shaker at 100 r.p.m. As a basal medium, hormone-free MS medium (pH=5.7) was used after being autoclaved at 121℃ for 20 min.

To examine the effect of medium constituents on the red beet hairy root cultures, the nutrients in MS medium were divided into four groups as indicated in **Table 2.1**; *viz.*, carbon source (component A), major inorganic nutrients (component B), trace elements (component C) and organic supplements (component D). Normal MS medium as a control consisted of all the nutrients at the concentrations shown in Table 2.1. The solution containing monosaccharide or the other components was prepared. These solutions were autoclaved separately at 121℃ for 20 min and mixed together after cooling them to ambient temperature.

The influence of nitrogen source in MS medium was investigated in the cultures of madder and pak-bung hairy roots, and the initial concentrations of NH_4^+ and NO_3^- are shown in **Table 2.2**.

B) Analyses The dry weight of root mass (DW) and the amount of the red pigment betanin in the roots were measured as described in section 1.2. These analyses of the hairy root cultures were conducted in triplicate. The concentration of phosphate in the medium was determined by the ammonium molybdate method (Umbreit *et al.*, 1957), The phosphorus content in the roots was analyzed by the same method after the roots were

completely digested in a Kjeldahl flask with 98% H_2SO_4.

Sugar concentrations were determined using an LC-6A high performance liquid chromatograph and an RID-6A refractive index detector (Shimadzu Co., Kyoto). The analytical conditions were as given by Kim *et al.* (1990).

Table 2.1 Four groups of components of MS medium

Component A (Carbon source)
fructose: 111 mol/m^3

Component B (Major inorganic nutrients)
NH_4NO_3: 20.6 mol/m^3, KNO_3: 18.8 mol/m^3, $CaCl_2 \cdot 2H_2O$: 3.0 mol/ m^3, $MgSO_4 \cdot 7H_2O$: 1.5 mol/m^3, KH_2PO_4: 1.25 mol/m^3

Component C (Trace elements)
$FeSO_4 \cdot 7H_2O$: 100 mmol/m^3, EDTA$\cdot 2H_2O$ (disodium salt): 100 mmol/ m^3, H_3BO_3: 100 mmol/m^3, $MnSO_4 \cdot 4H_2O$: 100 mmol/m^3, $ZnSO_4 \cdot 7H_2O$: 30 mmol/m^3, KI: 5.0 mmol/m^3, $Na_2MoO_4 \cdot 2H_2O$: 1.0 mmol/m^3, $CuSO_4 \cdot 5H_2O$: 0.1 mmol/m^3, $CoCl_2 \cdot 6H_2O$: 0.1 mmol/m^3

Component D (Organic supplements)
myo-inositol: 555 mmol/m^3, glycine: 26.6 mmol/m^3, nicotinic acid: 4.1 mmol/m^3, pyridoxine-HCl: 2.4 mmol/m^3, thiamine-HCl: 1.5 mmol/m^3

Table 2.2 Nitrogen source compositions of media

Medium	Concentration [kg/m^3]			Nitrogen molarity [mol/m^3]
	KNO_3	NH_4NO_3	NH_4Cl	
MS	1.90	1.65	-	60
MS-A	4.04	-	0-3.21	40-100
NI	6.07	-	-	60
AM	-	-	3.21	60

The other components are the same as those of MS medium.

2.3 Results and Discussion

2.3.1 Influence of medium constituents on growth and pigment formation of red beet hairy roots

A) Selection of carbon source in culture of red beet hairy roots

In general, sucrose, glucose or fructose is the carbon source giving good growth responses of plant cell cultures. However, the growth responses if plant cells to carbon sources frequently depend on the plant species and clone of interest (Dougall, 1980). Thus in preliminary experiments, red beet hairy roots were cultivated using MS basal medium containing various sugars for the choice of carbon source suitable for the growth. The selection of used sugars obeyed the previous report (Taya *et al.*, 1989c). As shown in **Table 2.3**, the values of cell mass grown were the higher in the cultures using sucrose and fructose as carbon sources which were practically equal in the pigment productivity. The difference of these carbon sources was not recognized through the time course cultures (data not shown). Therefore, the

Table 2.3 Effect of carbon sources on cell mass growth and red pigment formation in the culture of red beet hairy roots

Carbon source	Cell mass [kg-DW/m^3]	Red pigment content [10^{-3} kg/kg-DW]
Glucose	3.2	3.5
Fructose	9.1	7.5
Xylose	0.4	-
Galactose	0.4	-
Maltose	1.8	2.3
Lactose	1.3	3.8
Sucrose	6.4	9.9
No sugar	0.4	-

The cultivation was carried out for 290 h in an Erlenmeyer flask containing the normal MS medium with 20 kg/m^3 carbon source.

fructose was used as a carbon source throughout this chapter for the culture of red beet hairy roots.

B) Influence of medium constituents on enhancement of pigment production In preliminary experiments, when red beet hairy roots were cultivated in a diluted MS medium (containing all the MS medium constituents at one-eighth strength), the pigment content in the hairy roots was found to increase significantly in spite of the suppressed growth due to the possible deficiency of some nutrients. This fact suggested that the concentration(s) of certain constituent(s) in MS medium may have a positive effect on pigment production by the hairy roots.

In order to examine root growth and pigment formation in detail, red beet hairy roots were cultivated in medium in which the concentration of component A, B, C or D (Table 2.1) was reduced to one-eighth strength of normal MS medium. **Figure 2.1** shows the concentration of root mass, X [kg-DW/m^3], pigment content in the roots, B [kg/kg-DW], and the total amount of pigment production, A_B $(= X \cdot B)$ [kg/m^3], after 240 h of culture. It was found that reduction of component B (mixture of NH_4NO_3, KNO_3, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4) resulted in a remarkable enhancement of A_B value compared to that observed in the control culture using normal MS medium; *i.e.*, in medium with component B dilution, X = 4.0 kg-DW/m^3, B = 9.2 x 10^{-3} kg/kg-DW and A_B = 37 x 10^{-3} kg/m^3, whereas in the normal MS medium, X = 1.7 kg-DW/m^3, B = 3.0 x 10^{-3} kg/kg-DW and A_B = 5.1 x 10^{-3} kg/m^3.

Next, the effects of individual constituents of component B on the hairy root culture were investigated. In these cultures, the hairy roots were cultivated in medium in which one of the five inorganic nutrients was reduced to a quarter strength of normal MS medium. As shown in **Fig.2.2**, it was observed that phosphate was a key nutrient responsible for increasing pigment accumulation in red beet hairy roots.

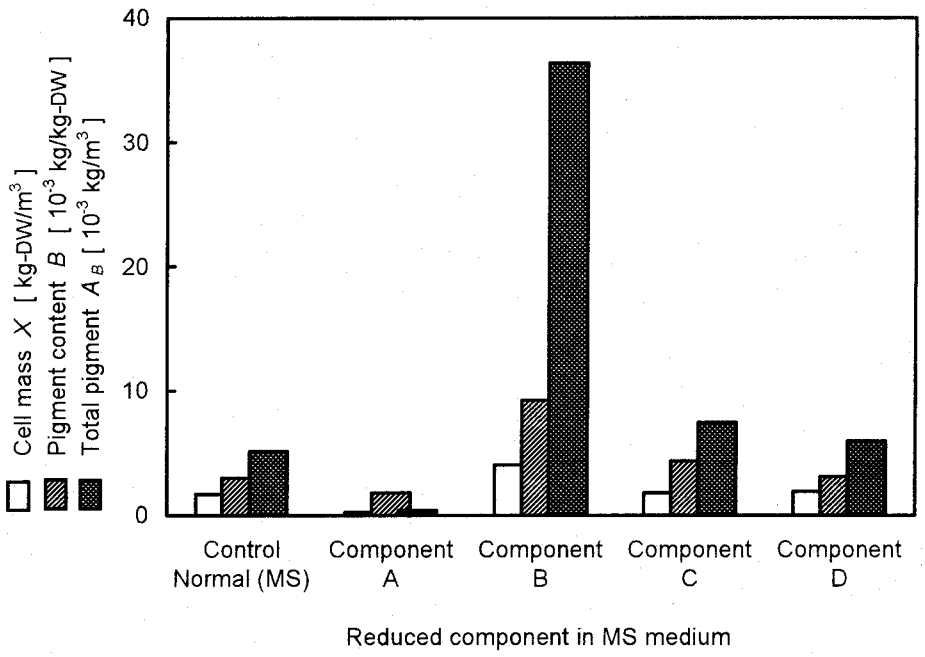


Fig.2.1 Growth and pigment formation of red beet hairy roots cultivated in media with reduced amounts of components

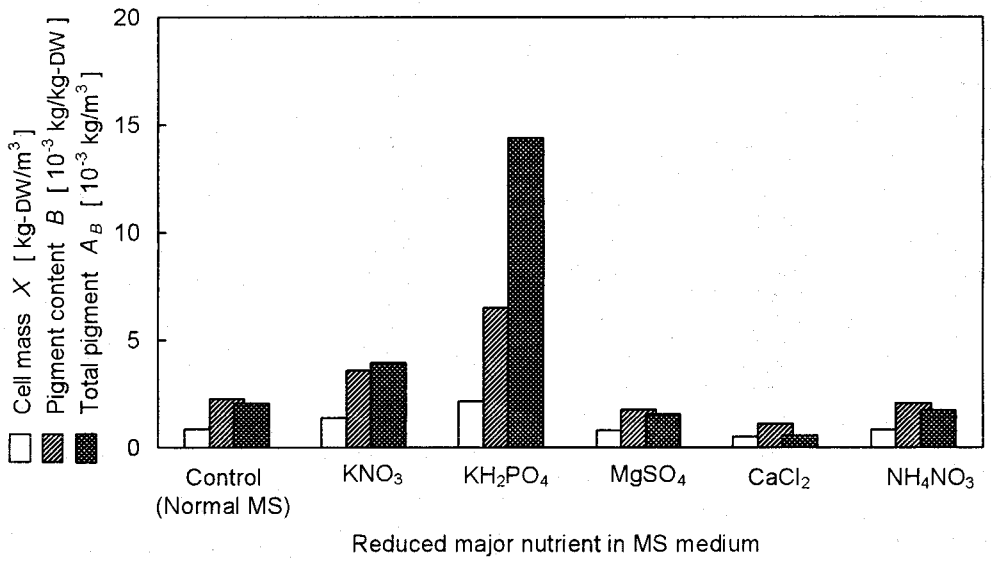


Fig.2.2 Growth and pigment formation of red beet hairy roots cultivated in media with reduced amounts of the major inorganic nutrients

C) Effect of phosphorus on hairy root growth and pigment production

The effects of the initial phosphate level in the medium on the hairy root culture were examined in detail. **Figure 2.3** presents the results after 240 h in the hairy root cultures using the media containing 0-2.5 mol/m^3 phosphate. The concentrations of the other nutrients were the same as those of normal MS medium. Higher B and A_B values were obtained at lower phosphate concentrations (range of 0-0.25 mol/m^3), while the highest values were achieved in phosphate-free medium (B = 11 x 10^{-3} kg/kg-DW and A_B = 19 x 10^{-3} kg/m^3). It was also observed that root growth did not decline in the phosphate-free medium; the value of X was 1.7 kg-DW/m^3 and it was even larger than the X = 1.2 kg-DW/m^3 of the normal MS medium with 1.25 mol/m^3 phosphate.

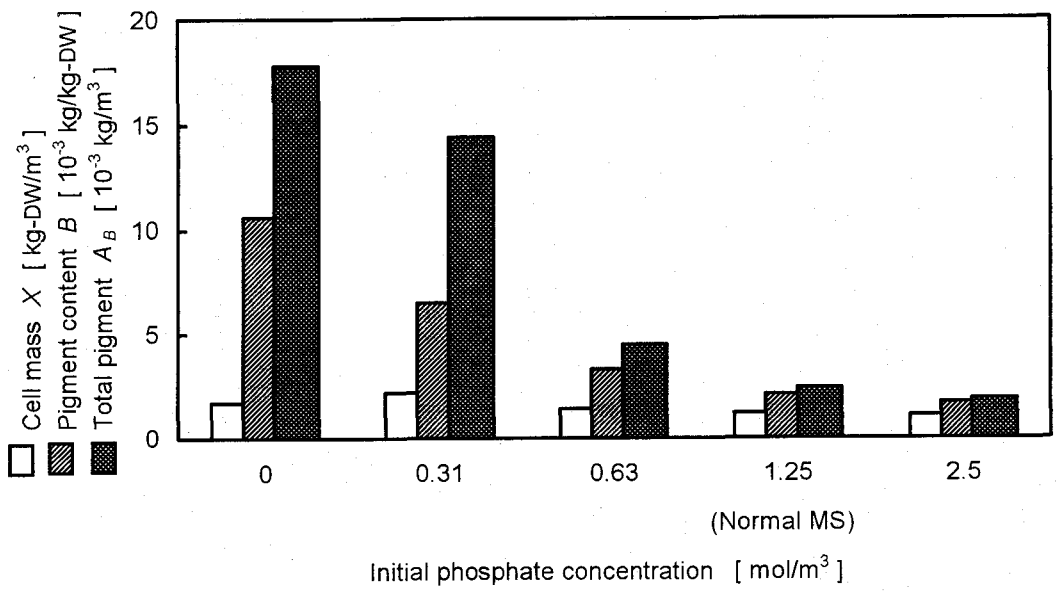


Fig.2.3 Growth and pigment formation of red beet hairy roots cultivated in media with various initial phosphorus concentrations

In higher plant cells, it is generally recognized that the cells store phosphorus in vacuoles or other locations in the form of polyphosphate, phytic acid and so on, and that the availability of stored phosphorus compounds supports the growth of plant cells even under phosphate-deficient culture conditions (Bieleski, 1973; Brodelius and Vogel, 1985). According to the assay of phosphorus in the hairy roots used as inocula in the present study, the phosphorus content was about 0.52 mol of phosphorus equivalent per kg-DW. This value was somewhat larger than the phosphorus contents of cultured plant cells in stationary phases (ca. 0.1 mol of phosphorus equivalent per kg-DW) as reported by Curtis *et al.* (1991). It is presumed that red beet hairy roots grown in phosphate-free medium utilize phosphorus compounds stored in the roots.

Figure 2.4 presents the time course of red beet hairy root cultures with medium containing 0 or 1.25 mol/m^3 phosphate (C_{P0}). In the phosphate-containing medium, root growth rate was lower than that in phosphate-free medium during the period from 0 to 312 h, a period which corresponded to the phosphate uptake period in the culture with the former medium. The suppressed root growth observed in the phosphate-containing medium may be attributed to an inhibitory effect of phosphate in the culture broth. The following results were obtained at the end of the culture (432 h). In phosphate-free medium, X = 3.5 kg-DW/m^3, B = 18 x 10^{-3} kg/kg-DW and A_B = 63 x 10^{-3} kg/m^3, and in phosphate-containing medium, X = 4.1 kg-DW/m^3, B = 3.1 x 10^{-3} kg/kg-DW and A_B = 13 x 10^{-3} kg/m^3.

Therefore, the exclusion of phosphate from the medium resulted in 5.8- and 4.8-fold increases in the B and A_B values, respectively, compared to those of the control culture at the 1.25 mol/m^3 phosphate level.

Although the metabolic role of phosphate (or phosphorus compounds) in plant cells is still unclear, there have been several studies on the enhanced production of secondary metabolites by callus tissues cultivated in

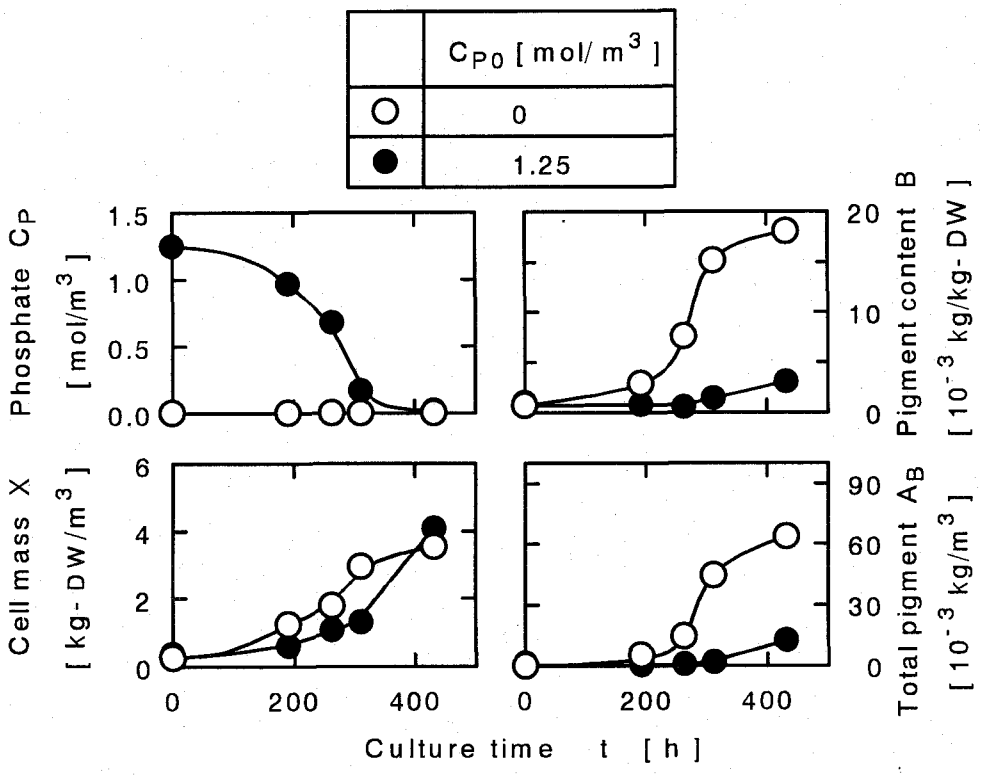


Fig.2.4 Time course of red beet hairy root culture in phosphate-free or normal MS medium

phosphate-limited media; such as the formation of alkaloids and phenolics by *Catharanthus roseus* cells, L-3,4-dihydroxyphenylalanine by *Stizolobium hassjoo* cells, harmalol and harmine by *Peganum harmala* cells, and anthocyanins by *Vitis* cells (Rokem and Gold berg, 1985; Yamakawa *et al.*, 1983; Dougall, 1980; Sakuta and Komamine, 1987). In hairy root culture, Dunlop and Curtis (1991) reported that *H. muticus* hairy roots exhibited a 28-fold higher content of sequiterpene in culture with a phosphate-free medium, compared to that in a control culture using medium with phosphate at 1.1 mol/m^3.

From these results, it is important to investigate the effect of medium components on growth and metabolite formation. The utilization of the phosphate-free MS medium is appropriate for the production of red pigment in the culture of red beet hairy roots which accumulated phosphorus in the preculture using MS medium in advance.

2.3.2 Influence of medium constituents on growth and pigment formation of madder hairy roots

A) Effect of carbon source To enhance the growth rate and pigment formation of madder hairy root, the influences of the main nutrients in medium were investigated. For the choice of carbon source suitable for the madder hairy root growth, the madder hairy root was cultivated using the MS basal medium containing various carbon sources. As shown in **Table 2.4**, higher growth was obtained in the culture with fructose than in that with glucose or sucrose which is usually used in plant cell cultures as a carbon source. **Figure 2.5** shows the courses of madder hairy root cultures with (A) sucrose, (B) fructose, (C) fructose and glucose, and (D) glucose. At 630 h culture time, the concentration of cell mass in fructose medium (9.7 kg-DW/m^3, Fig.2.5A) was attained. This value was 2.1 times higher than that in sucrose medium (4.6 kg-DW/m^3, Fig.2.5B).

Table 2.4 Effect of carbon source on growth and pigment content of madder hairy root

Carbon source	Cell mass [kg-DW/m^3]	Pigment content [10^{-3} kg/kg-DW]
Glucose	1.0	32
Fructose	3.5	20
Xylose	1.0	22
Galactose	0.6	32
Maltose	1.2	27
Lactose	0.9	20
Sucrose	1.5	26
No sugar	0.4	19

The cultivation was carried out for 290 h in an Erlenmeyer flask containing the normal MS medium with 20 kg/m^3 carbon source.

It is frequently observed that by invertase in plant cell wall, sucrose is first hydrolyzed to fructose and glucose which exist in medium, followed by the uptake of glucose and fructose into plant cells (Akazawa and Okamoto, 1990; Kato and Tsuji, 1981; Masuda *et al.*, 1988). A similar phenomenon of carbon source utilization was observed in the madder hairy root culture with sucrose medium (Figure 2.5B). In this case, glucose was preferentially consumed after the sucrose hydrolysis. In the culture with fructose and glucose mixture (Fig.2.5C), it was also found that glucose was preferentially consumed. Furthermore, the growth of hairy roots was very slow when glucose was utilized as a carbon source (Fig.2.5D). Thus, it was suggested that the low growth rate of madder hairy root in sucrose medium was due to the low consumption rate of glucose by the cells.

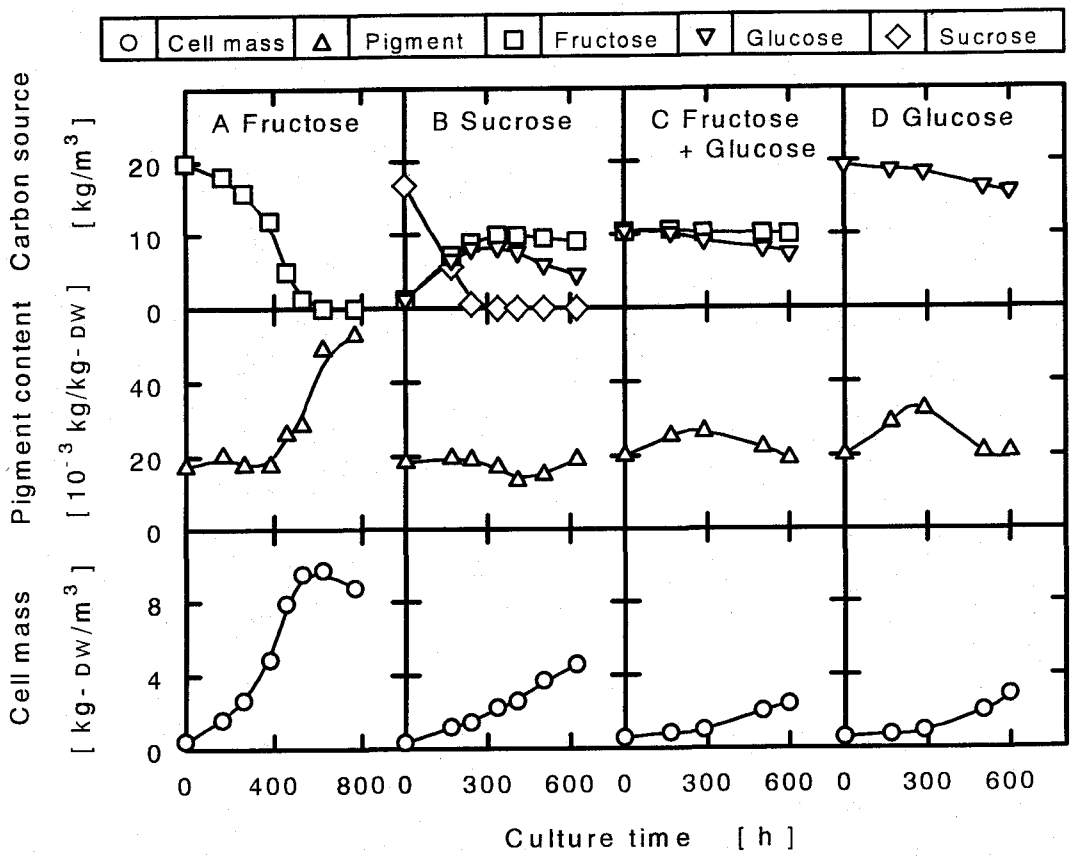


Fig.2.5 Time courses of madder hairy root cultures using fructose, sucrose, fructose and glucose, and glucose as carbon sources

With respect to the effects of carbon sources on the pigment content (Figure 2.5), no significant differences were recognized among these cultures while the hairy root grew (pigment content: 20-30 x 10^{-3} kg/kg-DW). However, after 456 h in the culture with fructose the pigment content increased remarkably and when the hairy root growth ceased (at 768 h), it reached 53 x10^{-3} kg/kg-DW.

Based on the findings described above, fructose was selected as a sole carbon source for the madder hairy root culture in this study. The use of a monosaccharide such as fructose will be advantageous in the hairy root culture because its concentration can be easily regulated in a culture such as the fed-batch system proposed by Uozumi *et al.*(1991).

B) Effect of nitrogen source Generally, hairy roots were cultured using media with combined addition of NH_4^+ and NO_3^- as main nitrogen sources such as Murashige-Skoog, Linsmaier-Skoog and Gamborg's B5 media. It is well known that the growth and metabolite formation in plant cells are influenced by the nitrogen sources in medium. Sargent and King (1974), and Ushiyama *et al.*(1984) reported that the presence of NH_4^+ in medium suppressed the growth of *Haplopappus gracilis* and *Panax ginseng* cells.

In preliminary experiments, the growth of various hairy roots was examined in the medium without NH_4^+ or with 0.36 kg/m^3 NH_4^+. The concentration of 0.36 kg/m^3 NH_4^+ corresponds to that of the standard MS medium used for hairy root cultures. As shown in **Table 2.5**, it was found that the cell mass concentrations in the culture of various kinds of hairy roots was decreased in the media with 0.36 kg/m^3 NH_4^+ in comparison with those in the media without NH_4^+. Especially, the growth of pak-bung and madder hairy roots were suppressed by NH_4^+ to a relatively high extent among the hairy roots tested. Bretefer and Siegerist (1984) reported that the supply of NH_4^+ to dwarf bean caused the lowering of NO_3^- uptake rate and

NO_3^- reductase activity of the root cells. Although the mechanism of NH_4^+ toxicity to the hairy roots is still unknown, presence of NH_4^+ in the medium may inhibit NO_3^- uptake and subsequent assimilation of nitrogen in the hairy root cells.

For further investigation for effect of nitrogen source on the growth and pigment formation of madder hairy roots, the madder hairy root was cultivated in medium varying concentrations of NH_4^+. As shown in **Table 2.6**,

Table 2.5 Effect of NH_4^+ on growth of madder, red beet and carrot hairy roots

Hairy roots	Cell mass [kg-DW/m^3] without NH_4^+	with NH_4^+
Red beet	6.0	4.4
Horseradish	4.3	2.6
Pak-bung	6.0	3.9
Madder	6.9	2.5
Carrot	4.5	3.3

The hairy roots were cultivated in MS-A medium without NH_4^+ or with 0.36 kg/m^3 NH_4^+ under the following conditions. Red beet hairy roots: X_0 = 0.9 kg-DW/m^3 and t = 301 h, horseradish and pak-bung hairy roots: X_0 = 0.5 kg-DW/m^3 and t = 190 h, madder hairy roots: X_0 = 0.7 kg-DW/m^3 and t = 264 h, and carrot hairy roots: X_0 = 0.5 kg-DW/m^3 and t = 384 h.

Table 2.6 Effect of NH_4^+ on growth and pigment content of madder hairy root

Run	NH_4^+ [kg/m^3]	Cell mass [kg-DW/m^3]	Pigment content [10^{-3} kg/kg-DW]
1	0	6.9	41
2	0.18	2.4	25
3	0.36	2.5	24
4	0.54	2.2	27
5	1.1	1.9	29

The hairy root was cultivated for 264 h in an Erlenmeyer flask containing the MS-A medium with indicated concentration of NH_4^+ (as NH_4Cl) and 20 kg/m^3 fructose.

the cell growth was inhibited in the medium containing NH_4^+ (Runs 2-5) whereas in the medium without NH_4^+ (Run 1) the maximum cell growth of 6.9 kg-DW/m^3 was achieved, which was 2.7 times higher than that in the medium containing 20 mol/m^3 NH_4^+ (corresponding to NH_4^+ concentration in MS basal medium, Run 3).

Figure 2.6 shows the course of madder hairy root culture in the NH_4^+-free medium (NI medium) containing 20 kg/m^3 fructose. The growth rate was

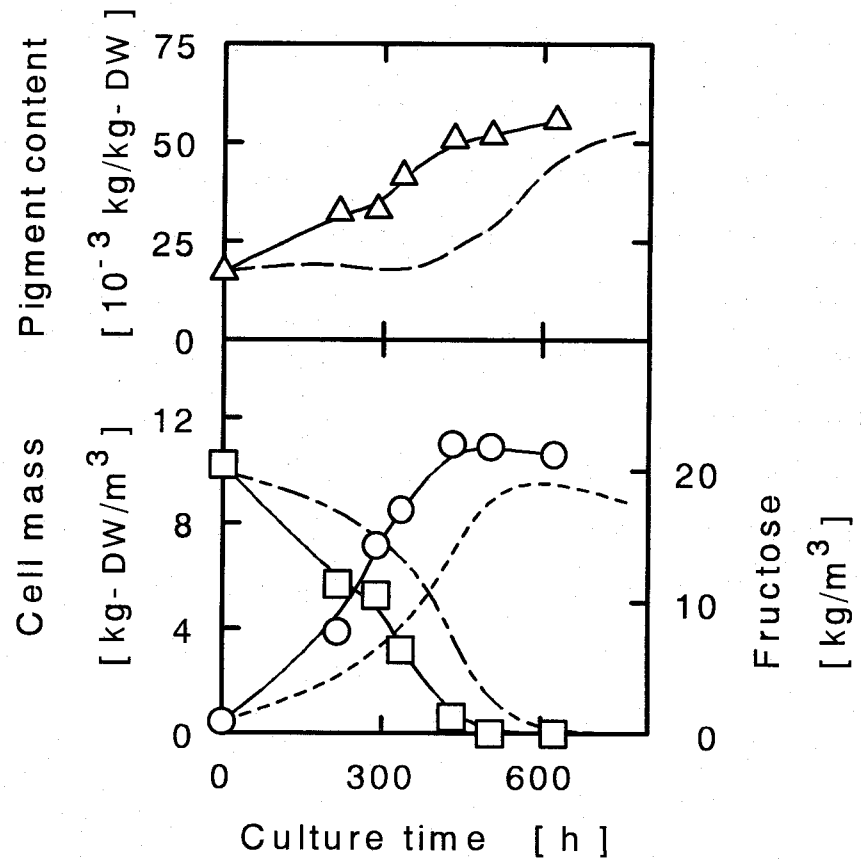


Fig.2.6 Time course of madder hairy root culture using nitrate as the sole nitrogen source

The hairy roots were cultivated in an Erlenmeyer flask containing NI medium with fructose as a carbon source. The symbols are the same as those in Fig. 2.5. The broken lines (- - - -, root mass; — - — -, fructose; and — — — -, pigment content in roots) show the results obtained in MS medium with fructose (Fig. 2.5A).

significantly enhanced and the concentration of cell mass reached 10.9 kg-DW/m^3 at 500 h culture time. The pigment content increased with culture time and the level was at about 50 x 10^{-3} kg/kg-DW in the stationary culture phase. This pigment content was comparable to that of original plant root (Table 1.1). The total amount of pigment ([cell mass concentration] x [pigment content]) in the culture with the NI medium containing fructose was 567 x 10^{-3} kg/m^3. This value represents a 10-fold increase over that obtained in the MS basal medium containing sucrose. From these results, the subsequent cultivation of the madder hairy root was done in the NH_4^+-free medium (NI medium) with 20 kg/m^3 fructose.

2.3.3 Influence of medium constituents on growth and enzyme activity of pak-bung hairy roots

As shown in Table 2.5, the growth of pak-bung hairy roots was inhibited by NH_4^+. For further investigation for influence on their growth and enzyme activity of superoxide dismutase (SOD) which was a main useful metabolite, pak-bung hairy roots were cultivated in media containing various NH_4^+ concentrations.

First, the effect of NH_4^+ on hairy root growth was investigated. As shown in **Table 2.7**, the values of cell mass grown were decreased with increasing NH_4^+ concentration and the value at NH_4^+ concentration of 0 kg/m^3 was 1.5 times higher than that at NH_4^+ concentration of 0.36 kg/m^3 (normal MS medium). From view point of the growth of pak-bung hairy roots, it is preferable to conduct the culture using medium without NH_4^+.

On the other hand, the effect on SOD activity was also investigated by cultures using the media of MS, NI and AM. As shown in **Table 2.8**, the maximum activity of SOD in the culture with AM medium was achieved and in comparison of the SOD activity at 275 h in NI medium which was preferable for the growth, the SOD activity in AM medium was enhanced 7.8 times and NH_4^+ promoted SOD production. It is known that SOD is produced

Table 2.7 Effect of NH_4^+ on growth of pak-bung hairy roots

NH_4^+ concentration [kg/m^3]	Cell mass grown [kg-DW/m^3]
0	6.0
0.18	4.4
0.36	3.9
0.54	3.3
1.1	2.8

The hairy roots were cultivated for 190 h in the MS-A medium with various amounts of NH_4Cl (see Table 2.2).

Table 2.8 SOD activity during cultures of pak-bung hairy roots with NI, MS and AM media

Culture time [h]	SOD activity in hairy roots [10^6U/kg-DW]		
	NI	MS	AM
0	3.9	3.9	3.9
125	3.6	4.2	11
275	1.2	4.0	9.3

for the elimination of superoxide caused by the exposure to stress such as disturbance of metabolic pathway or aging in cells, and in this case the stress of culture in AM medium inhibiting the growth was also considered to promote SOD production.

For further evidence of the effect of NH_4^+ on SOD activity, the influences of Cl^- (counter anion to NH_4^+) in AM medium and pH were

Table 2.9 Effects of Cl^- and pH on growth and SOD activity during cultures of pak-bung hairy roots

Run	Medium	pH Initial →Final	Cell mass [kg-DW/m^3]	SOD activity in hairy roots [10^{6}U/kg-DW]
1	MS	5.8→4.7	5.2	5.3
2	MS[a)]	5.5→4.4	5.5	6.2
3	AM	5.8→3.4	1.3	10
4	AM[b)]	5.8→4.9	1.3	14

a) MS medium with 5.0 kg/m^3 KCl, b) AM medium with 0.5 kg/m^3 KNO_3, Culture time : 340 h.

investigated. As shown in **Table 2.9**, in comparison with the culture in Runs 1 and 2 no difference in tendencies to growth and SOD activity of pak-bung hairy roots was recognized.

In the culture with AM medium (Run 3), the extent of pH declination was slightly large. Then, the culture with AM medium containing small amount of KNO_3 (Run 4) was conducted. It was found that pH declination didn't affect the growth.

Furthermore, the two-stage culture of pak-bung hairy roots was performed for the effective production considering the NH_4^+ effect. Two-stage culture was attempted under the following concept. In the first stage, the active growth is achieved (growth stage), and in the second stage high SOD activity is achieved (production stage).

In the time course of two-stage culture of pak-bung hairy roots in combination with NI and AM media (NI→AM, □) in **Fig.2.7,** the hairy roots were cultured in NI medium for 392 h (cell growth stage). The medium was exchanged with fresh AM medium and the culture was continued for 133 h (SOD production stage). The culture with single NI or MS medium (MS→MS,

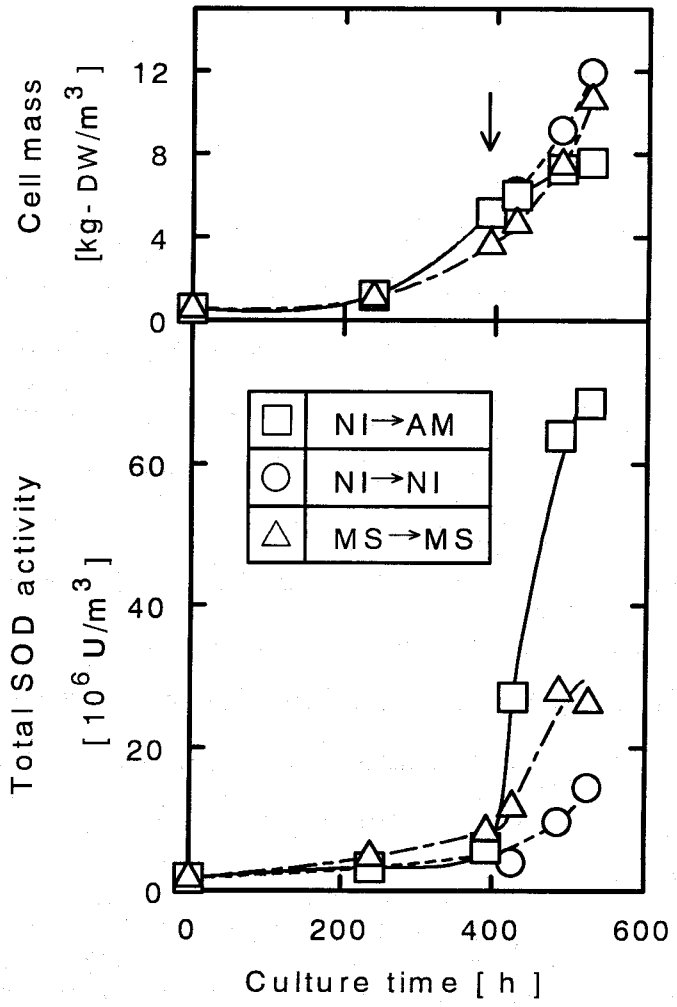


Fig.2.7 Time courses of two-stage culture of pak-bung hairy roots
Arrow shows the time of medium exchange.

△ or NI→NI, ○) was conducted as the control culture.

In the first stage for 392 h, the higher concentration of cell mass was obtained in the culture of NI medium (symbol; □ and ○). In the second stage for 133h, SOD activity was drastically enhanced in the culture of AM medium (symbol; □) and the amount of SOD activity (= [cell mass] x [SOD activity]) in the culture with NI→AM medium was 2-3 or 5-7 times higher than that with MS→MS (△) or NI→NI (○) medium, respectively. Here, the SOD activity at the end of culture with NI→AM medium (□) was attained at

9.1×10^6 U/kg-DW which was almost equal to that in the culture with AM medium in Table 2.8.

2.4 Summary

Effects of medium constitutes on growth and metabolite formation of red beet, madder and pak-bung hairy roots were investigated.

The cultures of red beet hairy roots were conducted with various modified media. Fructose or sucrose was selected as a carbon source and phosphate was identified as an important medium constituent which stimulated pigment formation in red beet hairy roots. That is, limiting the amount of phosphate in the culture medium led to a remarkable increase in pigment accumulation in the hairy roots, as long as the intercellular phosphorus source supported root growth. From these results, the amount of pigment production in the culture with phosphate-free MS medium containing 20kg/m^3 fructose was enhanced 4.8-fold in comparison with the normal MS medium.

The effects of major nutrients on the culture of madder hairy roots were studied with a view of increasing its growth and pigment production, and a modified MS medium which contained fructose and nitrate as carbon and nitrogen sources, respectively was developed. In the culture using the modified medium, the cell growth and pigment formation of the hairy root were remarkably enhanced. The total pigment amount produced for 500 h culture was increased by ten times in comparison with the culture in conventional MS medium with sucrose.

From the examination of the effect of nitrogen source in pak-bung hairy root cultures, it was shown that NH_4^+ in medium had an inhibitory effect on the hairy root growth. However, the presence of NH_4^+ in medium resulted in the enhancement of SOD activity in the hairy root cells. Thus, a two-stage culture of pak-bung hairy root was done: cell growth stage in the medium

with NO_3^- and SOD production stage in the medium with NH_4^+. In this culture system, the SOD productivity was 5-7 times higher than that in the culture with the medium containing only NO_3^- as a nitrogen source.

Chapter 3 Kinetic Expressions of Growth and Pigment Production in Hairy Root Cultures

3.1 Introduction

To produce valuable plant-derived metabolites, hairy roots have become of interest as a candidate for *in vitro* culture of plant cells or organs as described in Chapter 1. To gain the efficient production of metabolites by hairy root cultures, it is required to clarify the influence of medium components on metabolite formation in the course of root propagation. In the cultures of red beet hairy roots described in Chapter 2, limiting phosphate in medium caused an increase in pigment accumulation, as long as intracellular phosphorus source supported the root growth.

To design and control the hairy root culture system, the kinetics of growth and pigment formation must be characterized on the basis of its characteristic nature of proliferation (Uozumi *et al.*, 1995).

The aim of this chapter is first to propose kinetic modeling and expression of the hairy root growth process, and next, to propose a pigment formation model of red beet hairy roots on the basis of positional variation of pigment content along a root exhibiting linear extension at a tip meristem. The experimental data were obtained with respect to growth and pigment formation of the hairy roots containing various quantities of intracellular phosphorus. Moreover, the kinetic behavior of pigment accumulation in cells during hairy root culture was described by considering the effects of intracellular phosphorus on the growth and pigment formation of the roots.

3.2 Experimental

A) Hairy roots and media The hairy roots of red beet, madder, carrot, horseradish, senna and pak-bung were used. The hairy roots were maintained in Murashige-Skoog (MS) liquid medium (Murashige and Skoog,

1962) with 30 kg/m^3 sucrose and no phytohormone *via* subcultures at 25℃ as described in section 1.2 and 2.2

In experiments, MS liquid media with 20 kg/m^3 (carrot, horseradish and senna) or 30 kg/m^3 (pak-bung) sucrose were used. In the culture of red beet hairy roots, phosphate-free MS medium was used. The phosphate-free medium contained 0 mol/m^3 phosphate and 20 kg/m^3 fructose, and the other nutrients in the medium were the same as those of normal MS medium. The media were sterilized by autoclaving at 121℃ for 20 min after pH was adjusted at 5.7 with 0.1 kmol/m^3 NaOH.

B) Preparation of inocula of red beet hairy roots for cultures with phosphate-free medium To prepare inocula of red beet hairy roots containing different phosphorus contents for cultures with phosphate-free MS medium, the following procedure was conducted. About 2 g of fresh mass of the hairy roots were put into 500-cm^3 Erlenmeyer flasks (medium volume: 300 cm^3) incubated in darkness at 25℃ with shaking at 100 r.p.m. on a rotary shaker (Model HRS-24, Shibata Scientific Technology Ltd.). Here, the medium with 0.63, 1.25 or 2.5 mol/m^3 phosphate (pH after autoclaving = 5.3, 5.5, 5.7, respectively) was used. The hairy roots were cultured in the medium for a week during which the roots were allowed to absorb phosphate in the medium. Thus, the hairy roots possessing different contents of phosphorus (0.17 to 1.3 mol per kg of dry root mass) were obtained, and were used as inocula in subsequent experiments for the cultures in phosphate-free medium after being rinsed with distilled water.

C) Culture methods and conditions In shake cultures of various hairy roots, fresh root mass was inoculated in 200-cm^3 Erlenmeyer flasks containing 40 cm^3 of MS medium (carrot, horseradish, senna and pak-bung) or 80 cm^3 of phosphate-free MS medium (red beet). The triplet of whole cultures obtained from the flasks was used as a sample at prescribed culture time.

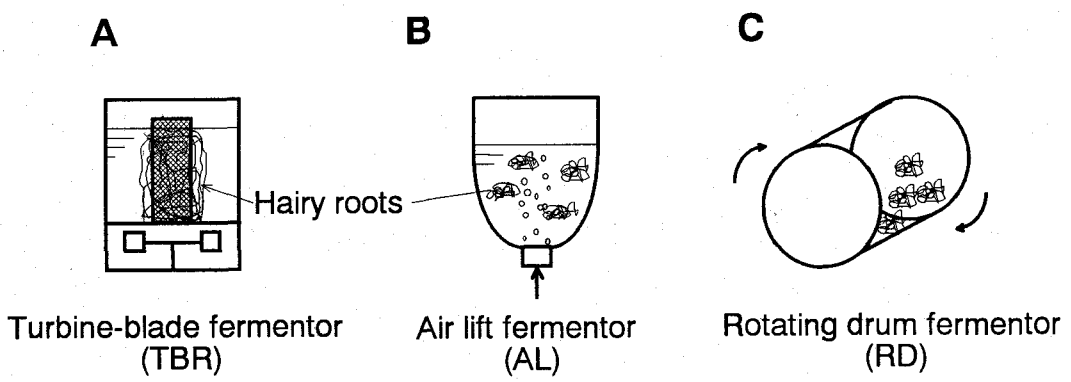


Fig.3.1 Experimental apparatus

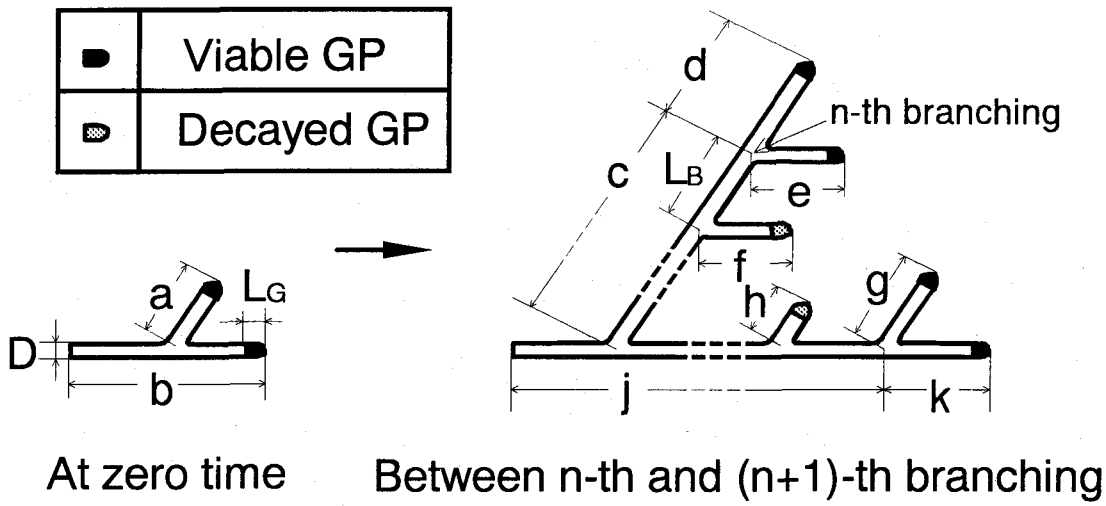
In cultures of red beet hairy roots with a fermentor, the hairy roots were grown at 25℃ using a turbine-blade fermentor (TBR, Model TBR-2, Sakura Seiki Co., Tokyo) containing 1 dm^3 of phosphate-free MS medium as shown in **Fig.3.1**A. The change of culture with elapsed time was determined from a series of runs operated under the same conditions. The culture runs were terminated at prescribed time and harvested roots were subjected to analyses. To compare the growth kinetics in the cultures with various fermentors, air lift (AL) and rotating drum (RD) were also used (Fig.3.1B and C)(Kondo *et al.*, 1989).

D) Analyses The dry weight of root mass (DW) and the contents of betanin and phosphorus in the roots were spectrophotometrically measured as described in section 2.2.

3.3 Results and Discussion

3.3.1 Kinetic expression of hairy root growth

A) Modeling of hairy root growth According to the morphologically



$L_0 = a + b$ $\qquad$ $L = c + d + e + f + g + h + j + k$

Fig.3.2 Schematic drawing of hairy root branching growth

properties of branching and elongation, the following assumptions were made for the modeling of hairy roots growth (see **Fig.3.2**) .

1) The hairy roots grows in the manner of one-dimensional extension at root tip meristem (growing point, denoted as GP) with length L_G .
2) The binary division of GP occurs within negligible time on hairy root branching. Each GP grows and forms "highly branched hairy root".
3) Environmental factors such as shear stress cause GP decay.
4) The root is regarded as a cylinder with diameter D and length L .

On the basis of assumption 1), a linear growth law for one GP was introduced:

$$dL/dt = \mu \cdot L_G \tag{3.1}$$

where μ is the specific elongation rate of GP. The time until next branching (division) of GP (Δt) is given by

$$\Delta t = \int_0^{L_B} dL/(\mu L_G) \tag{3.2}$$

Between the n-th and $(n+1)$-th branching of GP $(t_{I,n} \le t < t_{I,n+1})$, the initial conditions are

$$t = 0 \ : \ L = L_0, N = N_0 \qquad (n = 1) \tag{3.3}$$

$$t = t_{I,n} : \ L = L_{I,n}, N = N_{I,n} \qquad (n \ge 2) \tag{3.4}$$

where N shows the number of GPs and the subscripts 0, I and n denote zero time, initial time for n-th branching and n-th branching of GP, respectively.

The variation in viable GPs between the n-th and (n+1)-th branching is expressed as follows.

$$\begin{aligned} t_{I,n} \le t < \theta_1 \quad &: \ N = N_{I,n} \\ \theta_1 \le t < \theta_2 \quad &: \ N = N_{I,n} - 1 \\ \vdots \qquad &\quad \vdots \\ \theta_m \le t < t_{I,n+1} \quad &: \ N = N_{F,n} = N_{I,n} - m \\ i = 0, 1, 2, \cdots, m \end{aligned} \tag{3.5}$$

where the value of m shows the number of decayed GPs between the n-th and (n+1)-th branching.

Here, the decay rate of GP is expressed as follows.

$$dN/dt = -k_d N \tag{3.6}$$

Thus, i of GPs decay at $t = \theta_i$,

$$\theta_i = (1/k_d) ln\{N_{I,n}/(N_{I,n} - i)\} + t_{I,n} \tag{3.7}$$

At the $(n+1)$-th branching, the initial number of GPs $(N_{I,n+1})$ were calculated from Eq.(3.8).

$$N_{I,n+1} = 2N_{F,n} \tag{3.8}$$

Thus, overall root length L can be obtained by integrating Eq.(3.1) with respect to N of GPs.

The concentration of dry cell mass (X) can be calculated from Eq.(3.9).

$$X = \rho\pi(1 - W_C)LD^2/4V \tag{3.9}$$

<u>B) Growth kinetics of various hairy roots in various fermentors</u> So far,

no kinetic expression has been reported of hairy root growth. As mentioned above, the branching growth model was presented, based upon the linear growth law. This kinetic pattern is recognized for fungal growth pattern (Pirt, 1975). The filamentous growth of fungus seems essentially similar to that of the hairy roots.

As shown in **Fig.3.3**, various kinds of hairy roots (pak-bung, carrot, horseradish, and senna) were cultured in Erlenmeyer flask with MS medium containing 20 or 30 kg/m^3 sucrose.

In the experiment, sugar concentration was regarded as a limiting

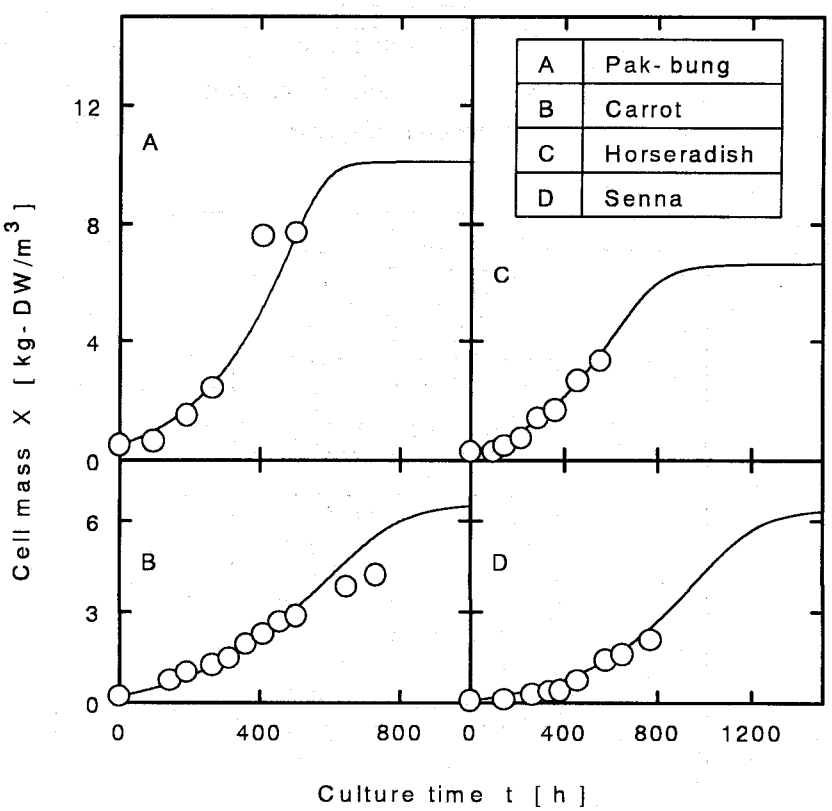


Fig.3.3 Simulation of hairy root cultures with Erlenmeyer flasks

The lines show the calculated results using the following values of X_0 [kg-DW/m^3] and S_0 [kg/m^3], respectively: 0.40 and 30 (pak-bung); 0.20 ad 20 (carrot); 0.25 and 20 (horseradish); and 0.10 and 20 (senna). The values of L_0 and N_0 were calculated from a given X_0 value, based upon Eq.3.9 and $N_0 = L_0/L_B$.

substrate and a Monod-type equation for μ was adopted.

$$\mu = \mu_{Sm} S / (K_S + S) \tag{3.10}$$

If the cell mass yield of hairy roots is assumed to be constant, the increase of cell mass is expressed by the decrease in sugar concentration as follows.

$$\Delta X = -Y_S \cdot \Delta S \tag{3.11}$$

From these equations the calculated value of X was obtained from the following procedure. As shown in **Fig.3.4**, starting with initial values of X_0 and S_0 which are experimental values, the values of L and X were

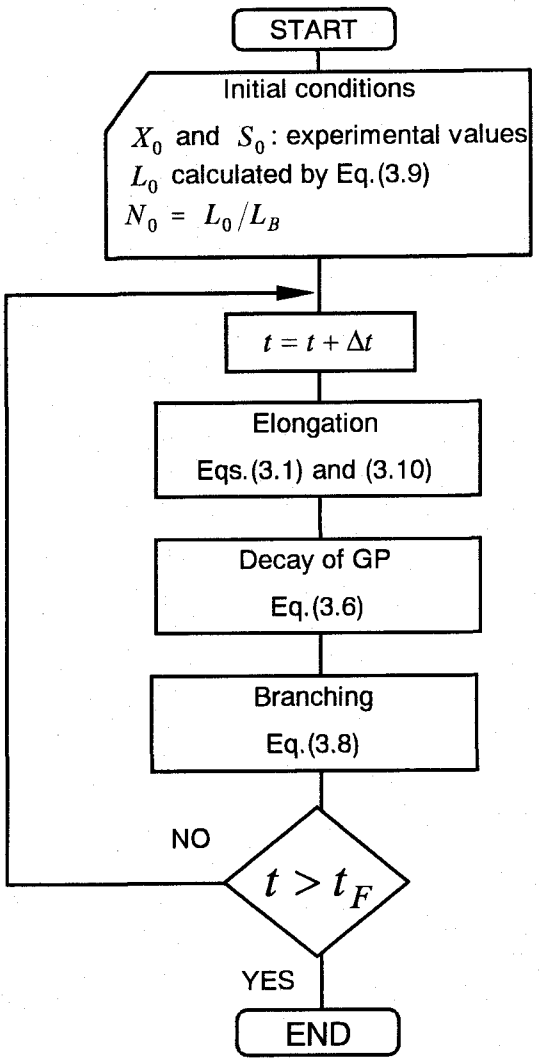


Fig.3.4 Calculation procedure for hairy root growth

calculated by considering the elongation at GPs, decay of GPs, branching of GPs.

Using the branching growth model mentioned above, the model parameters of μ_{Sm} and K_S in Eq.(3.10) were estimated by simulation with Eqs.(3.1) and (3.9)-(3.11) for Erlenmeyer flask cultures. From the results shown in Fig.3.3, these values of various hairy roots were obtained by a nonlinear least-squares method. The parameters and constant used for calculation are summarized in **Table 3.1**. From the μ_{Sm} values, it was estimated that a GP elongates by as much as 5-6mm per day. On the other hand, a wide range of K_S was reported for callus suspension cultures. Although it is difficult to compare the hairy root and callus directly, the K_S values obtained in our work are thought to be reasonable for highly organized plant cells.

Kondo *et al.* (1989) reported the cultures of carrot hairy roots with various fermentors as shown in Fig.3.1 A-C. The results obtained by Kondo *et al.* were compared with the growth model shown in **Fig.3.5**, and the decrease in the root growth rates seemed to be affected by physical damage to root cells such as shear stress and collision. The k_d values for reactors (Table 3.1)

Table 3.1 Parameter and constant values used for calculation

		Pak-bung	Carrot	Horseradish	Senna
μ_{Sm}	[h^{-1}]	0.47	0.43	0.43	0.40
K_S	[kg/m^3]	4.1	4.5	4.4	4.5
		Flask	TBR	AL	RD
k_d	[$10^{-3} h^{-1}$]	4.6	2.4	5.3	6.0

The other values: D = 1.0 x 10^{-3} m, L_B = 1.5 x 10^{-2} m, L_G = 5.0 x 10^{-4} m, V = 4.0 x 10^{-5} m^3 (flask), 1.0 x 10^{-3} m^3 (fermentor), W_C = 0.85, Y_S = 0.32, ρ = 1.01 x 10^3 kg/m^3.

were considered to reflect the extent of these effects.

In TBR, a stainless steel mesh is equipped to separate a culture space (0.6 dm^3) from an agitation space (0.4 dm^3) so that the hairy roots are not in contact with an impeller and the hairy roots are anchored and grown on a cylindrical stainless grid apart from a mechanical agitation unit. The use of this kind of structure minimize physical damages in the cultivation of hairy roots.

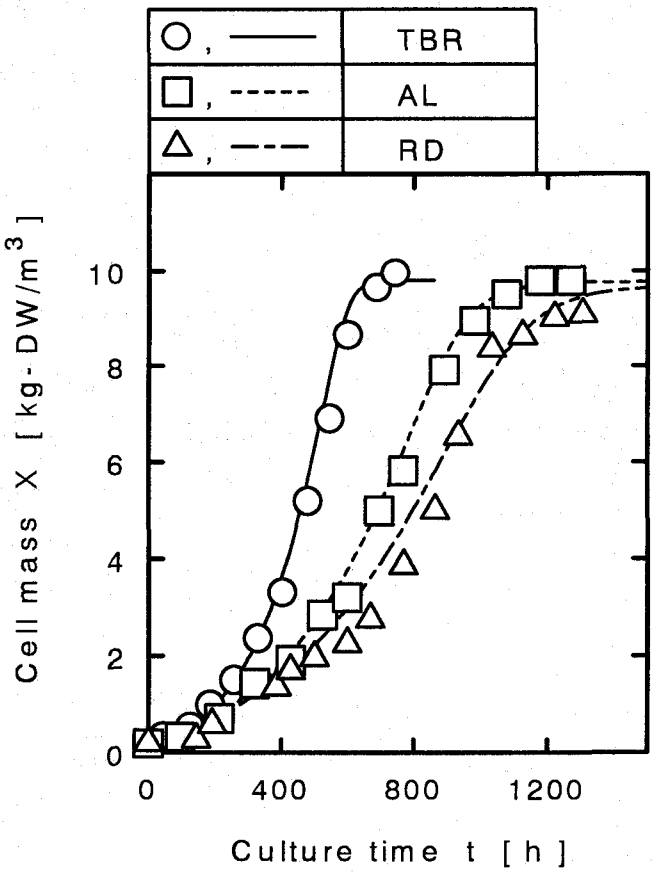


Fig.3.5 Simulation of carrot hairy root cultures with various fermentors
The lines show the calculated results using the following values of X_0 = 0.2 kg-DW/m^3 and S_0 = 20 kg/m^3, respectively. The other captions are the same as in Fig.3.3.

C) Growth kinetics considering effect of intracellular phosphorus

In Chapter 2, it was found that phosphorus stored in cells was an important factor exerting a remarkable effect on the production of pigment by the hairy roots of red beet. For further investigation, the hairy roots with various contents of intracellular phosphorus (P_0 = 0.17, 0.69 and 1.3 mol/kg-DW) were cultured in shake flasks with phosphate-free MS medium. As shown in **Fig.3.6**, the hairy roots propagated with the remarkable reduction of intracellular phosphorus content. As the intracellular phosphorus content

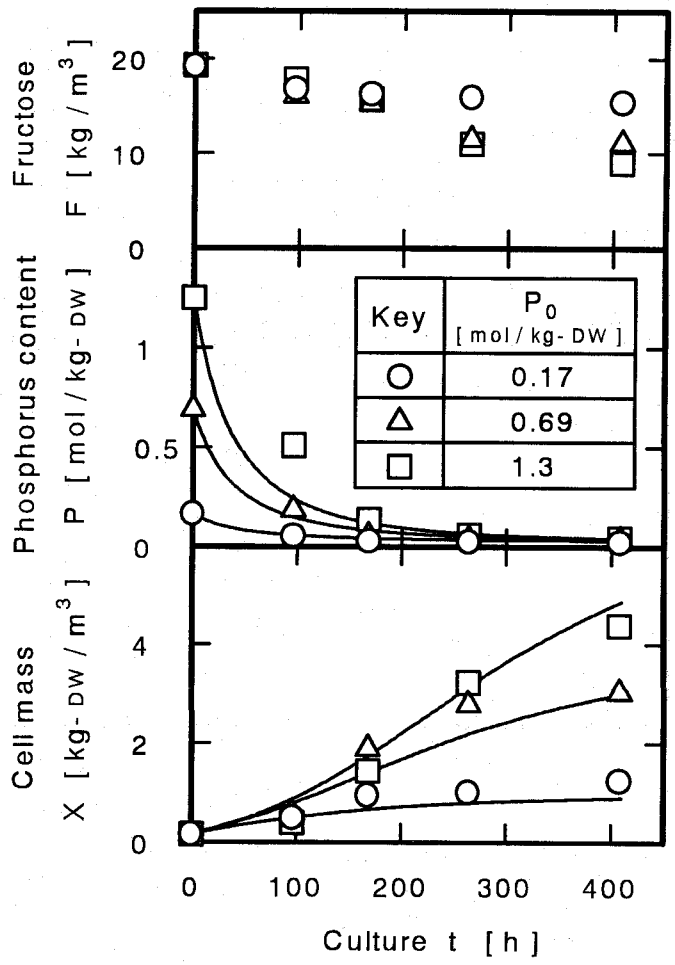


Fig.3.6 Time courses of flask cultures of red beet hairy roots with various P_0 values

Lines show the fitted lines.

decreased, the declination in growth rate was observed and the final amount of cell mass (X_F) at the culture time of t_F = 408 h depended on the P_0 values of the inoculated roots. It was also recognized that the remaining concentration of fructose in each culture at t_F = 408 h (F_F) was in the range from 9.0 to 15.1 kg/m^3. Under these experimental conditions, therefore, intracellular phosphorus was considered to be a limiting substrate for growth kinetics in the hairy root cultures.

Figure 3.7 indicates the plots of X_F values against $P_0 \cdot X_0$ values (total amount of phosphorus in the inoculated roots), which were obtained from the

	○	△	□	▽	◇	◎
t_F [h]	408	408	408	864	864	864
X_0 [kg- DW / m^3]	0.17	0.17	0.17	0.24	0.24	0.28
P_0 [mol / kg- DW]	0.17	0.69	1.3	1.1	1.5	1.4

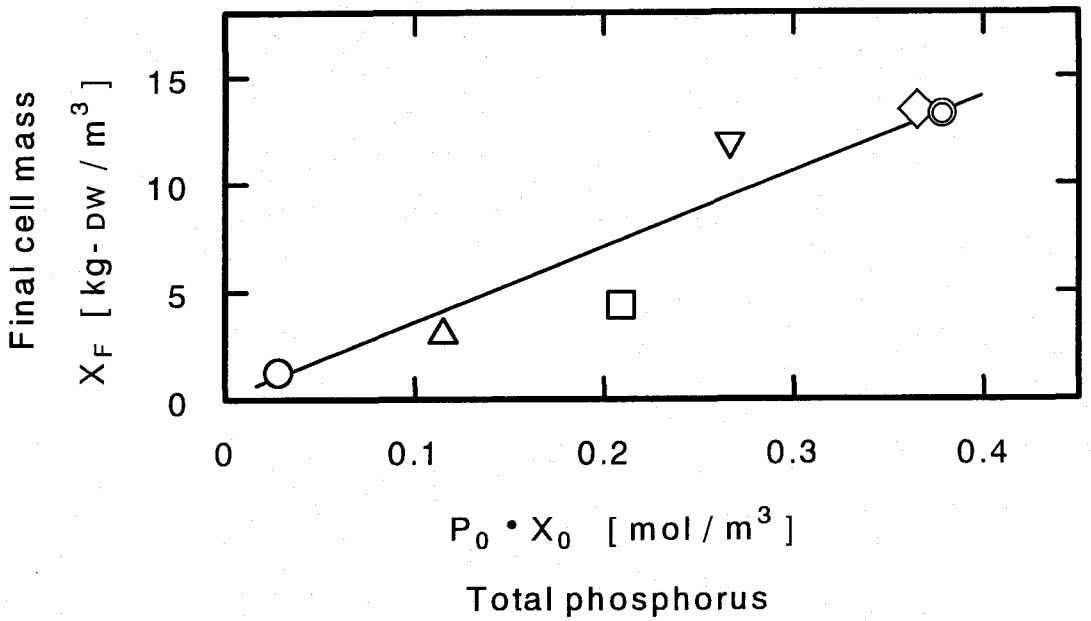


Fig.3.7 Relationship between X_F and $P_0 \cdot X_0$ values in flask cultures of red beet hairy roots with various P_0 values

Line shows the fitted line.

cultures of red beet hairy roots with different values of X_0 and P_0, including the data shown in Fig.3.6. A straight line was obtained by the least-squares method.

$$X_F = Y_P \cdot P_0 \cdot X_0 \tag{3.12}$$

where Y_P = 37 kg-DW/mol, indicating the cell mass yield based on intracellular phosphorus assimilated by the hairy roots. The value of $1/Y_P$ provides an apparent minimum content of intracellular phosphorus, which is incorporated into cellular elements like lipids, nucleic acid *etc*. The estimated value of $1/Y_P$ was 0.027 mol/kg-DW, and nearly equal to the values which were reported to be 0.033 mol/kg-DW for *Coffea arabica* callus (Bramble and Graves, 1991) and 0.025 mol/kg-DW for *Hyoscyamus muticus* hairy roots (Dunlop and Curtis, 1991).

When the value of $1/Y_P$ is presumed to be constant during the hairy root culture, the net content of phosphorus available for the root growth (free phosphorus, P_f) is presented as follows.

$$P_f = P - 1/Y_P \tag{3.13}$$

From a mass balance of intracellular phosphorus, moreover, the value of P is given by the following equation.

$$P = P_0 \cdot X_0 / X \tag{3.14}$$

Here, the specific elongation rate (μ) is expressed by a Monod-type formulas as a function of limiting substrate, which is intracellular phosphorus available for root growth in the culture of red beet hairy roots with phosphate-free MS medium.

$$\mu = \mu_{Pm} \cdot P_f / \left(K_P + P_f\right) \tag{3.15}$$

For the estimation of μ_{Pm} and K_P values, the calculated values were fitted to the experimental ones shown in Fig.3.6 by using a nonlinear least-squares method. The calculated values indicated by the solid lines were obtained using Eqs.(3.1)-(3.10) and (3.13)-(3.15), and the values of constants

Table 3.3 Parameter and constant values of red beet hairy roots used for calculation

Parameters			
$\hat{B}_b$ =	2.5 x 10^{-3} kg/kg-DW	$\hat{B}_m$ =	26.0 x 10^{-3} kg/kg-DW
k =	3.0 x 10^{-2} h^{-1}	K =	3.21 x 10^{2} (mol/kg-DW)$^{-n}$
K_P =	5.0 x 10^{-2} mol/kg-DW		
n_S =	2.88	μ_{Pm} =	0.70 h^{-1}
Constants			
D =	1.0 x 10^{-3} m	L_G =	5.0 x 10^{-4} m
V =	8.0 x 10^{-5} m^3 (flask),	1.0 x 10^{-3} m^3 (TBR)	
W_C =	0.92	Y_P =	37 kg-DW/mol
ρ =	1.005 x 10^{3} kg-fresh cells/m^3		

and parameters listed in **Table 3.3**. As a results, the values of μ_{Pm} and K_P were evaluated as 0.70 h^{-1} and 5.0 x 10^{-2} mol/kg-DW, respectively (Table 3.3).

3.3.2 Kinetic expression of pigment formation

A) Modeling of pigment formation in red beet hairy root It is necessary to construct a model of growth and pigment formation of hairy roots, by considering the elongation at GP and the positional distribution in cellular age along one hairy root. As shown in **Fig.3.8**, a GP (L_G in length) is postulated to exist at the tip part of a cylindrical single hairy root (D in diameter). One hairy root is divided into two sections; growth section within GP ($0 \le l \le L_G$) where the root elongation occurs through cell division, and pigment formation section except GP ($L_G < l \le L$) where no cell division occurs and pigment is synthesized *via* the secondary metabolism in each cell. Particular cells existing in the range from l to $l + dl$ have their own cellular ages of θ to $\theta + d\theta$, being matured with increasing value of l. A gradient of

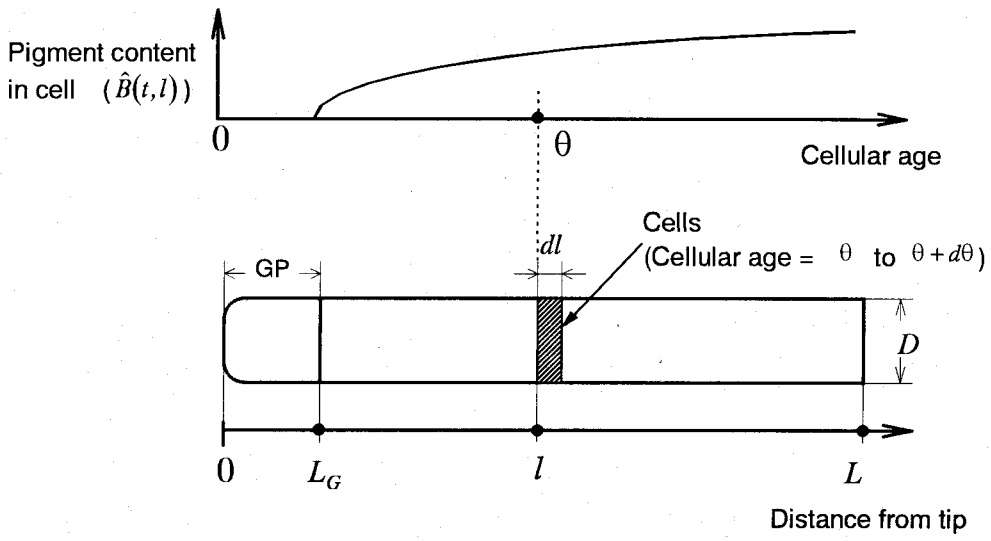


Fig.3.8 Conceptual drawing of single hairy root with distribution of pigment content

pigment content ($\hat{B}(t,l)$), consequently, appears depending on cellular age along a hairy root.

In tuberous roots of red beet, it is known that pigment is stored in vacuoles (denoted as VA) existing in cells (Zhang *et al.*, 1992). Moreover, intracellular phosphorus exerts a profound influence on pigment formation of red beet hairy root as described in Chapter 2. Taking these facts into account, the following assumptions are made in a pigment formation model of red beet hairy roots (**Fig.3.9**).

1) Pigment is formed only in a cell existing in the root section except GP (no pigmentation within GP).
2) Process of pigment formation in a cell consists of three steps; synthesis at a local point in cytoplasm (denoted as CY) close to tonoplast (denoted as TO), permeation through TO and accumulation in VA.

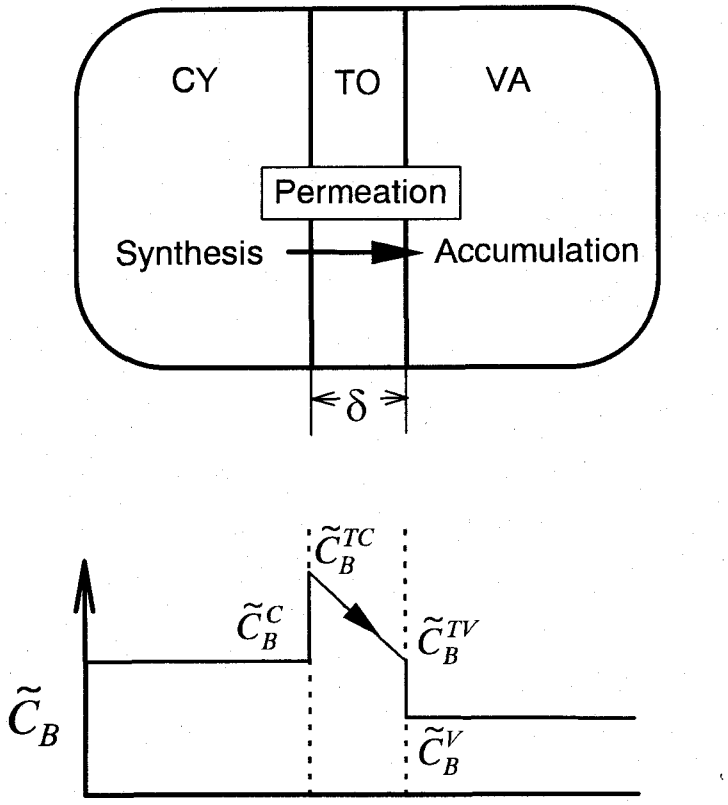


Fig.3.9 Conceptual drawing of pigment localization in single cell

3) Pigment concentration at a local point in CY ($\tilde{C}_B^C$) is held in equilibrium owing to the rapid synthesis of pigment and is expressed as a function of intracellular phosphorus content.
4) Permeation of synthesized pigment is a rate-determining step in a series of pigment formation process and thus a difference in pigment concentrations between both sides of TO occurs (CY side: $\tilde{C}_B^{TC}$ and VA side: $\tilde{C}_B^{TV}$).
5) The size of VA in a single cell remains unchanged during pigment accumulation and decomposition of pigment accumulated in VA does not occur.
6) The CYs and VAs of cells existing in a differential length (dl) have the

average pigment concentrations in CY ($\hat{C}_B^C$) and VA ($\hat{C}_B^V$), which are the same values as those in cytoplasm ($\tilde{C}_B^C$) and vacuole ($\tilde{C}_B^V$) of a single cell at a position of l, respectively.

According to assumptions 1) and 3), $\tilde{C}_B^C$ for a cell within $0 \le l \le L_G$ is regarded as zero, and an equation correlating metabolite concentration with intracellular phosphorus content proposed by Toda and Yabe (1979) is employed to express $\tilde{C}_B^C$ for a cell within $L_G < l \le L$.

$$\tilde{C}_B^C = \begin{cases} 0 & (0 \le l \le L_G) \quad (3.16) \\ \dfrac{1+K(P_f)^n}{1+\left(\tilde{C}_{Bm}^C / \tilde{C}_{Bb}^C\right)K(P_f)^n}\tilde{C}_{Bm}^C & (L_G < l \le L) \quad (3.17) \end{cases}$$

where $\tilde{C}_{Bm}^C$ and $\tilde{C}_{Bb}^C$ are the maximum and minimum values of $\tilde{C}_B^C$, respectively.

From assumption 4), the pigment flux through TO at steady state is expressed as follows.

$$j_s = \frac{D_s}{\delta}\left(\tilde{C}_B^{TC} - \tilde{C}_B^{TV}\right) \quad (3.18)$$

where the following relations hold due to the symmetrical structure of TO.

$$\tilde{C}_B^{TC} = K_T \tilde{C}_B^C \quad \text{and} \quad \tilde{C}_B^{TV} = K_T \tilde{C}_B^V \quad (3.19)$$

From assumption 6) concerning the cells in a differential length (dl) shown in Fig.3.8, the following equations hold.

$$\hat{C}_B^C = \tilde{C}_B^C \text{ and } \hat{C}_B^V = \tilde{C}_B^V \quad (3.20)$$

Eq.(3.18) can be rewritten as a partial differential equation.

$$\partial \hat{C}_B^V(t,l)/\partial t = k\left(\hat{C}_B^C - \hat{C}_B^V(t,l)\right) \quad (3.21)$$

where $k = AD_sK_T/\delta$.

The amount of pigment in a single cell ($\hat{M}(t,l)$) is expressed as follows.

$$\hat{M}(t,l) = \frac{\pi\alpha D^2 \hat{C}_B^V(t,l)}{4} dl \qquad (3.22)$$

where α is constant (refer to assumption 5)).

Integration of Eq.(3.22) gives the overall amount of pigment in a single hairy root (M).

$$M = \frac{\pi\alpha D^2}{4} \int_0^L \hat{C}_B^V(t,l) dl \qquad (3.23)$$

B) Kinetics of pigment formation in culture of red beet hairy roots

To elucidate the pattern of the pigment formation in red beet hairy roots, the flask cultures of the hairy roots with phosphate-free MS medium were conducted. At given culture times, the hairy roots were cut at the various positions (l = 1, 4, 8, 12 and 16 mm) and divided into four sections (Section a; l = 1-4 mm, b; l = 4-8 mm, c; l = 8-12 mm and d; l = 12-16 mm). **Figure 3.10** shows the relationship between the sectional pigment content ($\hat{B}(t,l)$) and the mean distance from the tip with respect to each section. It was found that $\hat{B}(t,l)$ values at given culture times increased with increasing l values, accompanying with the asymptotic properties of approaching saturated values.

For the mathematical consideration of experimental measurements, $\hat{C}_B^V(t,l)$ and $\hat{C}_B^C$ in Eq.(3.21) were revised as $\hat{B}(t,l)$ and $\hat{B}_S$ by following equation, respectively.

$$\hat{B}(t,l) = \frac{\alpha \hat{C}_B^V(t,l)}{\rho(1-W_C)} \text{ and } \hat{B}_S = \frac{\alpha \hat{C}_B^C}{\rho(1-W_C)} \qquad (3.24)$$

The experimental data shown in Fig.3.10 was matched with Eqs.(3.1)-(3.9) and (3.13)-(3.15), (3.21) and (3.24) by means of a nonlinear least-squares method. Thus, the parameter value of k was evaluated and $\hat{B}_S$ values at given culture times were also determined. The calculated values are indicated as the solid lines in Fig.3.10 and give reasonable expression to distribution of pigment content along the roots.

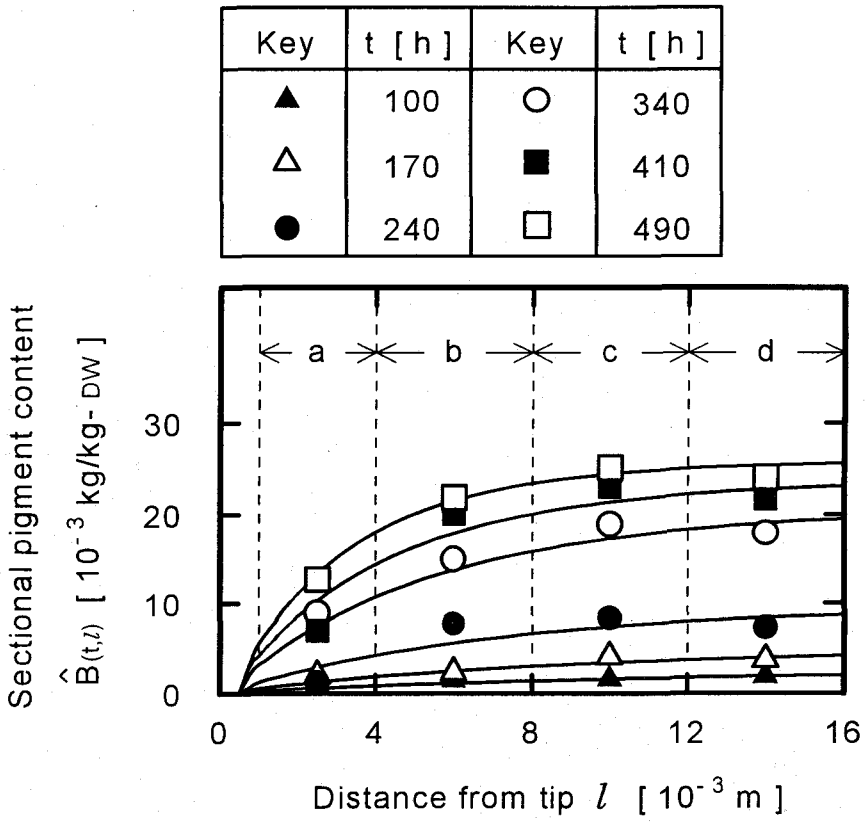


Fig.3.10 Relationship between $\hat{B}(t,l)$ and l values obtained from flask cultures of red beet hairy roots

Lines show the fitted lines.

To clarify the effect of intracellular phosphorus on pigment formation, the relationship between values of $\hat{B}_S$ and P_f calculated at various culture times was illustrated in **Fig.3.11**. The $\hat{B}_S$ values was remarkably enhanced with decreasing values of P_f and the maximum and minimum values of calculated $\hat{B}_S$ were 26.0 x 10^{-3} and 2.5 x 10^{-3} kg/kg-DW, respectively.

For numerical expression of the relation between $\hat{B}_S$ and P_f, $\tilde{C}^C_{Bb}$ and $\tilde{C}^C_{Bm}$ in Eq.(3.17) were converted into $\hat{B}_b$ and $\hat{B}_m$, respectively, by Eqs.(3.20) and (3.24). Using $\hat{B}_b$ =2.5 x 10^{-3} kg/kg-DW and $\hat{B}_m$ =26.0 x 10^{-3} kg/kg-DW, the

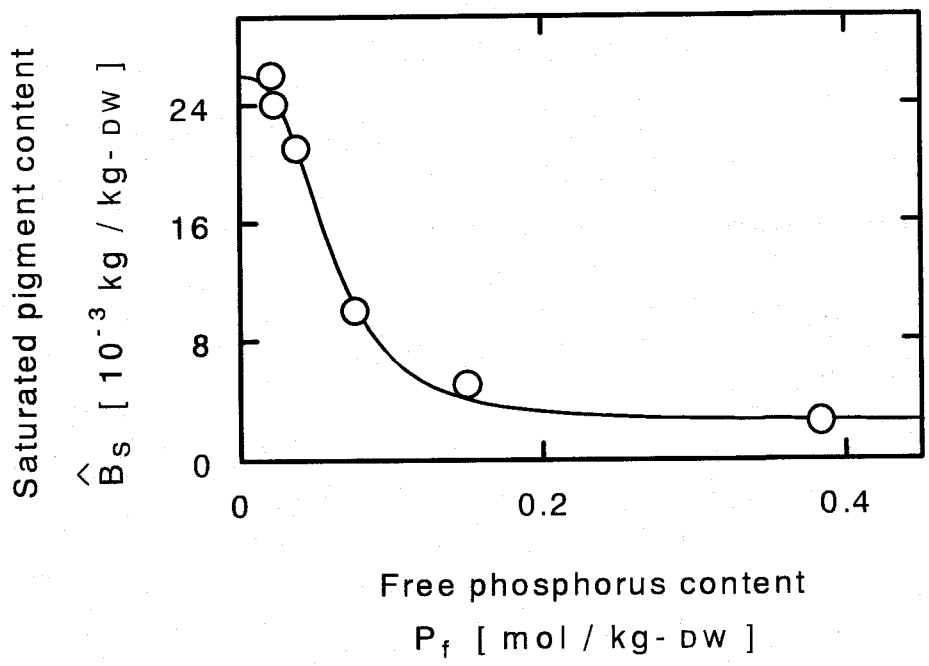


Fig.3.11 Relationship between $\hat{B}_S$ and P_f values obtained from flask cultures of red beet hairy roots
Line shows the fitted line.

parameters of K and n in Eq.(3.17) could be obtained by matching the calculated values with the data shown in Fig.3.11 by a nonlinear least-squares method. The estimated values of these parameters are shown in Table 3.3. The stimulated formation of secondary metabolites at a lower phosphorus level in cells was also recognized in other plant cells (Fischer *et al.*, 1993).

3.3.3 Comparison between calculated and experimental results with hairy root culture using fermentor

As the kinetic models of hairy root growth and pigment formation in the culture of red beet hairy roots were constructed, the model was evaluated by the comparison between calculated and experimental results during the culture using TBR. The calculated values of X and B were obtained from the following procedure. As shown in **Fig.3.12**, starting with initial values of

L_0 and $\hat{B}(0,l)$ in a hairy root, the values of L and X were first calculated by Eqs.(3.1)-(3.9) and (3.13)-(3.15). Next, $\hat{B}(t,l)$ value in a single cell at position of l was calculated by Eqs.(3.16), (3.17), (3.20), (3.21) and (3.24), and then using M value given by Eq.(3.21), the average content of pigment in hairy roots (B) was calculated by the following equation.

$$B = M/VX \tag{3.24}$$

To describe the kinetic behavior of growth and pigment formation of red

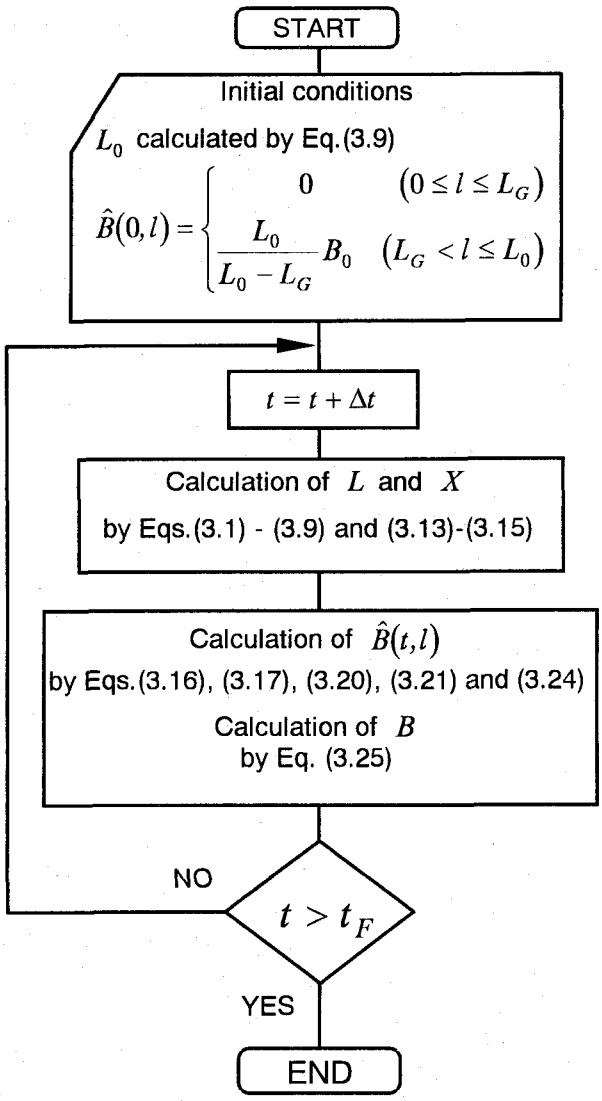


Fig.3.12 Calculation procedure for cell growth and pigment formation

beet hairy roots, cultures of the roots with TBR were conducted four times and each culture was terminated at t = 190, 240, 320 and 410 h, as shown in **Fig.3.13**. In the cultures for 320 and 410 h, the whole culture broth was exchanged with fresh phosphate-free MS medium at t = 240 h to avoid the starvation of nutrients other than phosphorus. The calculated values for X, B and P shown by the solid lines agreed closely with experimental data.

Figure 3.14 summarizes the relationships between the calculated and experimental values of X and B. In this figure, the data in the flask

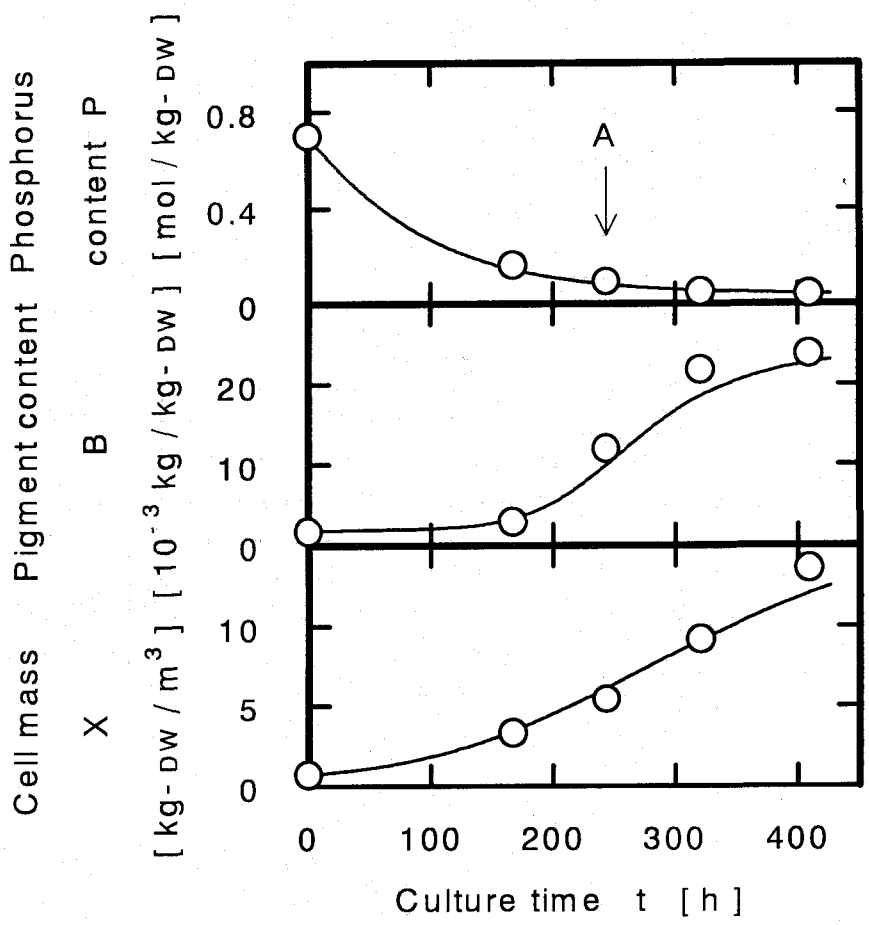


Fig.3.13 Time course of culture of red beet hairy roots in TBR
The lines and arrow A show the calculated values and the culture time of medium exchange, respectively.

cultures shown in Figs.3.7 and 3.10 are also included. The correlation coefficient among these results was 0.988. It was thus found that the kinetic model presented in this study was valid to express the behaviors of the growth and pigment formation in the culture of red beet hairy roots.

3.4 Summary

A kinetic model for hairy root proliferation, based upon the linear extension and lateral branching of the GP at root tip, was proposed. The model made it possible to simulate and characterize the hairy root growth of carrot, horseradish, pak-bung and senna hairy roots successfully.

In cultures of carrot hairy roots with various fermentors, growth behavior were changed due to varying extent of physical damage and these

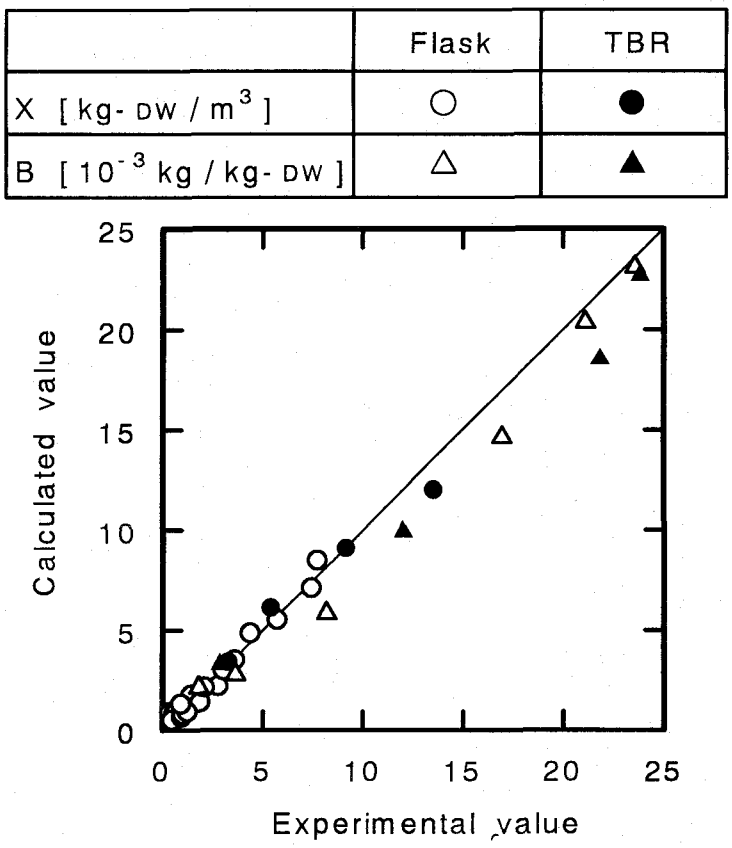


Fig.3.14 Relationship between the calculated and experimental values of X and B obtained from fermentor and flask cultures of red beet hairy roots

kinetics could be expressed by using the parameter of decay rate of growing points.

Furthermore, a kinetic model was proposed to represent the profile of pigment production associated with the growth of red beet hairy roots. This model was constructed on the basis of a concept of cellular age distribution arising from a linear growth mode of the roots. The kinetics of root proliferation was formulated using a Monod-type rate equation of root elongation including intracellular phosphorus as a limiting substrate. Positional dependence of pigment content was observed along the hairy roots; namely, the content increased with increasing distance from root tips and gradually approached a saturated value correlated with intracellular phosphorus content.

The kinetics of pigment formation of hairy roots was presented by considering the variation in pigment content along the roots and the intracellular phosphorus effect. It was demonstrated that the model made it possible to describe the kinetic behavior of growth and pigmentation during hairy root culture in TBR.

Chapter 4 Chemical Production in the Culture of Hairy Roots Associated with Repeated Processes of Growth and Release

4.1 Introduction

For the last decade, submerged culture of plant cells has been exploited for the production of plant-derived metabolites on the laboratory and industry scales (Curtin, 1983; Fowler,1981). Many plant materials have been so far employed extensively ranging in the morphology from dedifferentiated cells (callus or suspended cells) to differentiated cells (adventitious shoot and root, hairy root, organ or immature plant).

In terms of culture operations, the growth rates of plant cells are slower than those of microorganisms. This leads to technological importance to sustain high cell mass density in a bioreactor and to utilize the cell mass over a desirable period of culture time in the plant cell cultures.

In general, most of the secondary metabolites produced by plant cells are accumulated in vacuoles in cells, and are hardly released into the extracellular environment. So far, many workers have developed culture systems of plant cells for the *in situ* production of metabolites into a medium. Brodelius *et al.* (1988 a, b) studied the permeabilization of plant cells (*Catharanthus roseus* etc.) in order to release intracellular products by treatment with organic solvents (dimethylsulfoxide *etc.*) or by electroporation. Nakajima *et al.* (1986) reported that the presence of 0.2-0.3 mM L-cysteine was required for *Lavandula vera* cells to release blue pigments. In the culture of horseradish hairy roots, it was recognized that enhanced ionic strength of the medium stimulated the leakage of an intracellular enzyme, peroxidase (Uozumi *et al.*, 1992). Knorr *et al.* (1985) showed that intracellular substances including proteins were effectively released by

amaranth and common milkweed cells entrapped in gel matrix with chitosan. Recently, Kilby and Hunter (1990 a, b) reported that sonic exposure resulted in pigment release from beet callus.

In these methods, however, it seems likely that the products are contaminated with the additives and that heterogeneity of cell suspension retards the effective release of the products. In the previous paper (Taya *et al.*, 1992), A hairy root clone of red beet was established. The hairy roots possess the outstanding properties of genetic stability, active propagation and high content of red pigment (mainly betanin). It was found that a significant amount of the pigment was released into medium by intermitting oxygen supply to the hairy root cells in a shake culture. However, it is known that the beetroot pigment is labile in aqueous solution containing metal ions and the pigment degradation is promoted by the exposure to air. So it is desired that the pigment is removed from medium just after release of the pigment.

In the term of enhancement of pigment formation in the hairy roots, it was reported that the phosphate-free MS medium was established as the optimized medium for pigment formation considering effect of phosphate in medium on hairy root growth and pigment formation as described in Chapter 2. To realize the effective operation of cell growth associated with product release, it is necessary to express the kinetics of cell growth, product formation and product release. The kinetics of hairy root growth and pigment formation in the red beet hairy roots were reported in Chapter 3.

The aim of this chapter is to perform a submerged culture with repeated processes of cell growth and pigment release, using red beet and madder hairy roots. Moreover, based on the kinetic analysis of declination of cell viability during pigment release by the treatment of oxygen starvation, the evaluation of pigment release and growth ability are conducted. This kinetic models are applied to the culture with repeated processes of growth and

pigment release.

4.2 Experimental

A) Hairy roots and medium The hairy roots of red beet and madder were used and the hairy root cultures for maintenance and inoculum preparation were carried out as described in sections 1.2 and 3.1. For experiments, MS or phosphate-free MS liquid medium was used with 20 kg/m^3 fructose and 0.05 kg/m^3 antifoam (Silicone KM-72, Shin-etsu Chemical Industries Co., Tokyo).

B) Culture conditions The configuration of bioreactor systems is depicted in **Fig. 4.1**. The system consisted of two units for cell culture and pigment recovery. As a cell culture unit, a turbine-blade fermentor (Model TBR-2, Sakura Seiki Co., Tokyo) was used. The culture process of red beet

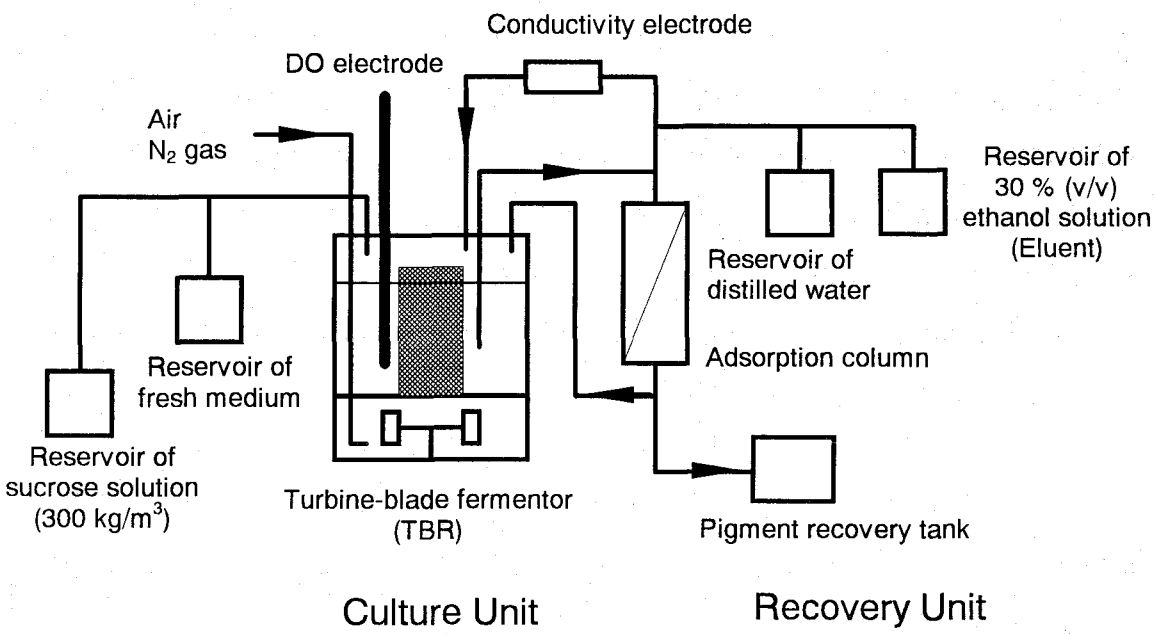


Fig.4.1 Schematic diagram of bioreactor system for culture of red beet hairy roots

hairy roots contained the growth and the pigment release. Initially, the culture started with an inoculum of about 1.0 kg-dry cells/m^3 at 25 °C. In the growth process, the hairy roots were grown with forming pigment in the fermentor. Dissolved oxygen (DO) concentration was measured with a DO meter (Model DO-1, Sakura Seiki Co., Tokyo), and controlled at 5 ppm by changing the agitation speed (150-200 rpm) and air flow rate (1.5-9.0 dm^3/h). To prevent the lack of nutrients, sucrose solution (300 kg/m^3) was adequately added to the culture, and the culture broth was exchanged with fresh medium.

In the pigment release process including the treatment of oxygen starvation, the operation for pigment release was carried out as follows. The air supply was first stopped and then pure N_2 gas was introduced into the fermentor for an hour to decrease the DO level substantially to zero. After the pigment release period for given time, the hairy root growth was resumed by supplying air.

To recover the released pigment, the culture broth was circulated through an adsorbent column (3 cm ϕ by 20 cm) packed with 25 g resin. Two columns were connected to the fermentor and each one was used alternately for the *in situ* adsorption and desorption of pigment. The columns were washed with sterilized water after the desorption of pigment.

C) Pigment adsorption and desorption with resins As adsorbents of red beet pigment, Diaion HP 20, and Sepabeads SP 207 and SP 850 resins (styrene-divinylbenzen copolymers supplied by Mitsubishi Kasei Co., Tokyo) were examined. These resins have the specific surface areas of 605, 627 and 995 x10^{-3} m^2/kg-resin, respectively. Sepabeads SP 207 is a modified type with enhanced hydrophobicity by introducing a substituent. Prior to use, these resins were soaked in methanol and washed with several volumes of water. A part of the resins was dried at 60 °C for 48 h to determine water content and to correct the resin amount on the dry basis.

As an authentic pigment, Sanbeet F (pigment preparation of beetroot procured from San-Ei Gen F. F. I. Inc., Osaka) in 30 mol/m^3 phosphate buffer (pH=6.0) was used, unless otherwise noted. A 0.2-dm^3 Erlenmeyer flask containing 8 g resins and 0.1 dm^3 of the pigment solution was shaken and allowed to equilibrate at 25 °C for 4 h. After the removal of resins by decantation, absorbance of supernatant was measured to determine pigment concentration. Pigment quantity adsorbed on the resins was calculated by Eq.(4.1).

$$q = \left\{A_{M,0}V - A_{M,eq}\left(V_r + V\right)\right\}/W_A \qquad (4.1)$$

In another experiment of pigment desorption with some solvents, 30%(v/v) aqueous ethanol solution was found to be available as an eluent (data not shown).

D) Analyses For the determination of dry cells (DW), the hairy roots were rinsed with water and dried at 70 °C for 48 h. In the culture with the bioreactor, the dry weight of red beet hairy roots was estimated on the basis of conductometric measurement of medium (Eq.(1.7), Taya *et al.*, 1989b). The empirical equation was *a priori* determined as follows.

$$X = -30(\Delta\kappa) + X_0 \qquad (4.2)$$

Eq.(4.2) was valid in the range of κ = 0.2-0.6 S/m.

Enzymatic reduction of triphenyl tetrazolium chloride (TTC) were used in order to evaluate the cell viability of hairy roots. The TTC reduction activity was measured according to the method described by Steponkus and Lanphear (1967). Here, the cell viability (f_T) was defined as the fraction of the extent of enzymatic reduction in the hairy roots (a_{T3}) at a given period of the pigment release process on the basis of that (a_{T1}) at the initial.

$$f_T = a_{T3}/a_{T1} \qquad (4.3)$$

Sugar concentrations were determined with HPLC system as described in section 2.2. Pigment amounts in cells and in medium (or buffer) were

spectrophotometrically analyzed as described in section 2.2, and expressed as those of betanin (main pigment in red beet).

4.3 Results and Discussion

4.3.1 Phenomenon of pigment release with treatment of oxygen starvation

A) Pigment release In a previous paper (Taya *et al.*, 1992), it was qualitatively demonstrated that the decrease of DO level in medium caused the pigment leakage from the cells of red beet hairy roots in a shake culture. Then, the effect of DO concentration on the pigment leakage was investigated in further detail using the TBR. The hairy roots were cultivated in advance for 192 h at C_O = 5 ppm to obtain active growing-cells (X = ca. 5 kg/m^3, B = ca. 4 x10^{-3} kg/kg-DW) , and then the DO concentration was controlled at 0, 1, 2, 4 or 5 x10^{-3} kg/m^3 by changing the air flow rate and introducing N_2 gas into the fermentor, on occasion.

Figure 4.2 shows the changes of pigment concentration in medium after the onset of DO control at various levels. When the DO concentration was maintained at 0 ppm, the pigment was sufficiently released into medium. At C_O = 1-5 x10^{-3} kg/m^3, whereas, the pigment concentration in medium was negligible even after 50 h. The pigment amount in medium increased with the time of DO control at 0 kg/m^3 and it reached the maximum of 6.68 x10^{-3} kg/m^3 at 26 h, which corresponded to 50 % of pigment release ratio from the cells (= 100 (amount of pigment in medium)/{(amount of pigment in medium) + (amount of pigment in cells)}). The lowering of pigment release rate at 26 h may be attributable to the decrease in the pigment content in the cells.

Thus, the pigment leakage out of the cells was caused only when the hairy roots were subjected to the culture condition of C_O = 0 kg/m^3 (namely oxygen starvation).

It is known that the pigment release from field-grown red beet roots

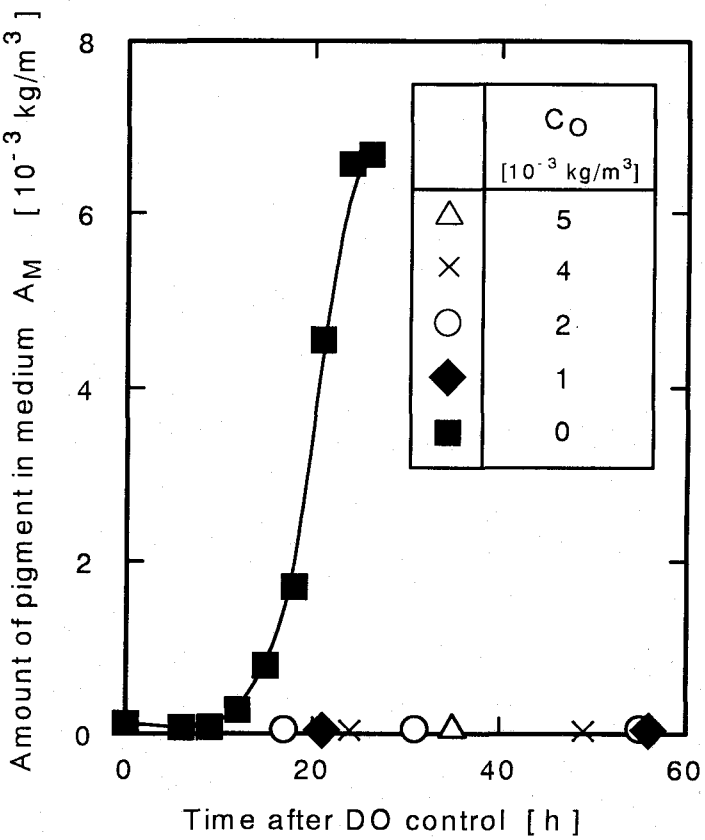


Fig.4.2 Change of pigment concentration in medium after DO control at various level

under anoxia condition occurred due to loss of integrity of the tonoplast and the cell membrane, accompanying the irreversible decay of cells (Zhang *et al.*, 1992). In the red beet hairy roots under anoxia condition, the pigment was released from the cells which were decayed, accompanying with declination of cell viability as shown in **Fig.4.3**.

B) Properties of released pigments Similarly to the characterization of pigment described in section 1.3, HPLC profiles of the pigments in the culture broth was investigated and the chromatographic patterns of the culture broth pigments were substantially identical to those extracted from the hairy and original roots, although minor peaks were observed for the broth pigments as shown in Fig.1.4. Moreover, the extracellular pigments

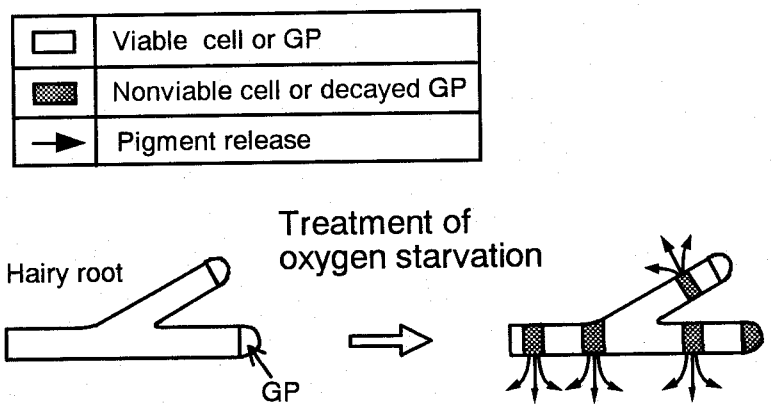


Fig.4.3 Conceptual drawing of pigment release from hairy roots by treatment of oxygen starvation

were also confirmed to consist of identical components to the intracellular ones from the hairy and original roots based on TLC analysis.

Further investigation of the Hunter color properties of released pigments using a color difference meter, appreciable differences were not found among the pigments in the culture broth and in the extracts from the cells (hairy and original roots) in terms of L_H, a_H, b_H, and $\tan^{-1}(b_H/a_H)$ values (pigment in culture broth; L_H= 61.7, a_H= 30.2, b_H= 11.2, and $\tan^{-1}(b_H/a_H)$ = 20.3, the values of extracts from the cells were shown in Table 1.2). From these analyses, the pigments recovered from the broth were regarded to be substantially the same as the original ones.

4.3.2 Culture of red beet hairy roots associated with repeated processes of growth and pigment release

A) Selection of pigment adsorbent For the recovery of pigment released into medium, the adsorption operation was adopted because the operation is advantageous to separating and concentrating the product of interest from a dilute aqueous mixture.

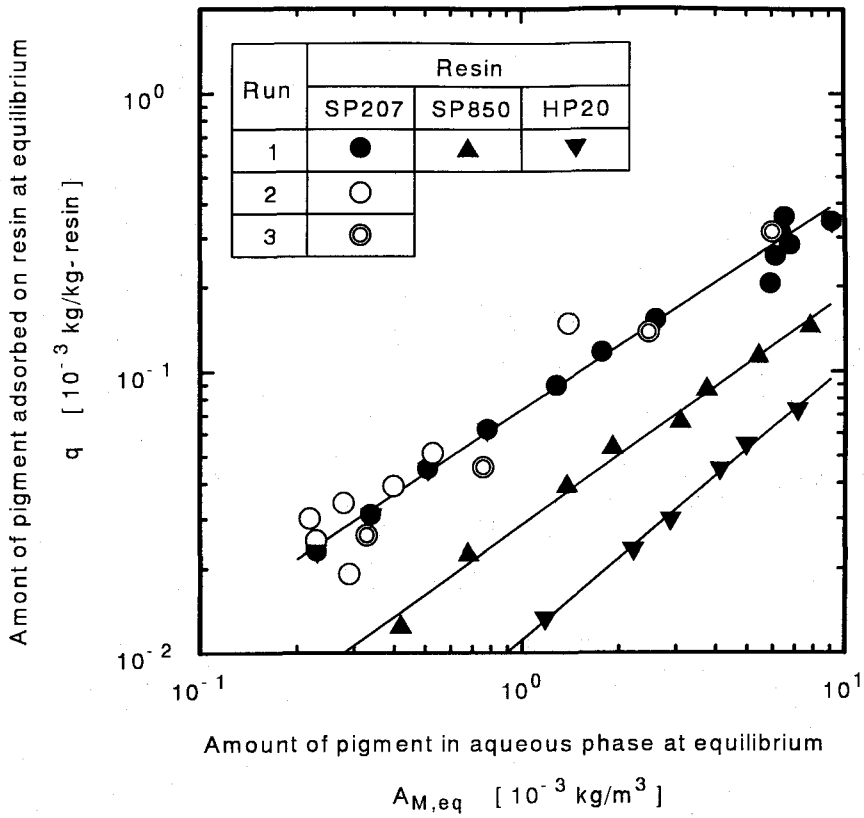


Fig.4.4 Isotherms of pigment adsorption with Sepabeads SP 207 and SP 850, and Diaion HP 20

Run 1: adsorption of authentic pigment; Run 2: adsorption of pigment released by hairy roots; Run 3: adsorption of pigment using resin regenerated by eluting adsorbed pigment with 30% (v/v) ethanol.

The lines show the values calculated by Freundlich adsorption isotherm ($q = a\left(A_{M,eq}\right)^{1/n_q}$); a = 7.31x10^{-2} (m^3/kg)$^{1/nq}$, n_q = 1.32 for SP 207; a = 2.85x10^{-2} (m^3/kg)$^{1/nq}$, n_q = 1.22 for SP 850; a = 1.11x10^{-2} (m^3/kg)$^{1/nq}$, n_q = 1.03 for HP 20.

The three types of synthetic polymer resins (Diaion HP 20, and Sepabeads SP 207 and SP 850) were tested using the authentic pigment preparation of beetroot. **Figure 4.4** shows the adsorption isotherms of the resins. Sepabeads SP 207 had the largest capacity to adsorb the pigment. The affinity of this resin to the pigment may result from the enhanced hydrophobicity interacting with the indole and pyridine residues of pigment. Their isotherms were explained by Freundlich adsorption isotherm. Concerning Sepabeads SP 207, an adsorption experiment was also carried out with the medium containing the pigment released from the hairy roots. No significant difference was observed between the adsorption data with the

authentic and hairy root pigments (symbols ● and ○ respectively).

In another experiment, the pigment adsorption was conducted using Sepabeads 207 which underwent the treatment of regeneration by eluting adsorbed pigment from the resin with 30%(v/v) ethanol. As shown in Fig.4.3, the data (symbol ◎) closely agree with those using the untreated resin (symbol ●).

Several researchers also applied the adsorption operation with polymeric resins to the production of plant metabolites including anthraquinone (Robins and Rhodes, 1986), indole alkaloids(Payne and Shuler, 1988) and peroxidase (Kato *et al.*, 1991). However, few data were presented with respect to the interactions between the resins and medium nutrients. As shown in **Table 4.1**, the concentrations of major nutrients (sucrose, ammonium, potassium, nitrate and phosphate) were compared between the MS media treated and untreated with Sepabeads 207. It was recognized that the sugar and ions were hardly lost by the adsorption treatment with the resin. Moreover, the hairy roots could grow normally in

Table 4.1 Comparison of major nutrient concentrations and cell growth between MS media with and without adsorption treatment with resin (Sepabeads SP 207)

	Concentration [kg/m^3]	
	without treatment	with treatment
Sucrose	24	22
K^+	0.77	0.74
NH^+	0.34	0.34
NO_3^-	1.9	1.9
PO_4^{3-}	0.12	0.11
Dry cells*	5.7	5.2

* The hairy roots were cultivated in each medium for 192 h with an Erlenmeyer flask.

the medium with the resin treatment, indicating that the trace components (vitamins, metals etc.) remained at enough levels to sustain the cell growth after the treatment.

B) Culture performance Sepabeads SP 207 was selected as an adsorbent to be incorporated into the bioreactor system (see Fig.4.1). The culture of red beet hairy roots was performed with repeated processes of growth and pigment release. Here, in preliminary experiment, it was found that the efficiency of regrowth after oxygen starvation was decreased with increasing the period of pigment release process (t_r) values and the t_r value in the range from 12 to 16 h was adequate for the culture.

From these results, the time course of hairy root culture was conducted. As shown in **Fig.4.5**, the hairy roots were cultivated normally for 172 h, and

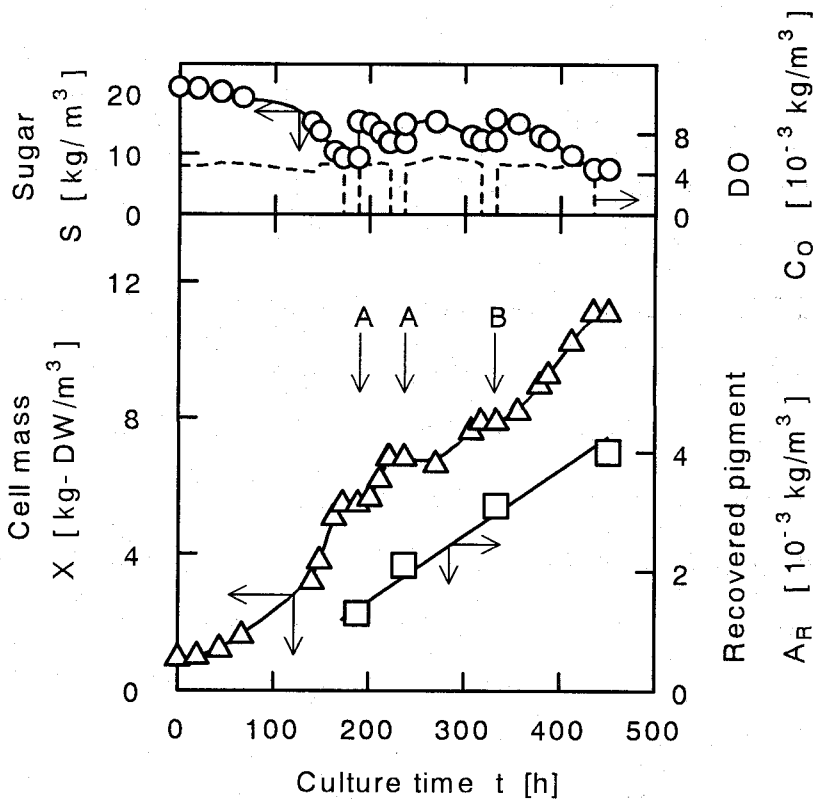


Fig.4.5 Culture of hairy roots in bioreactor systems

Arrows A and B show the culture time of sucrose addition and medium exchange, respectively.

Table 4.2 Summary of culture results of hairy roots with bioreactor system

		Run 1	Run 2
t_F	[h]	450	480
t_r	[h]	16	12
n_r	[-]	4	4
X_F	[kg-DW/m^3]	11.1	13.0
A_R	[10^{-3} kg/m^3]	4.0	1.9
P_B	[10^{-6} kg/(m^3•h)]	11.3	6.7

afterwards the operation of pigment recovery was repeated 4 times (n_r =4) with the pigment release by the oxygen starvation of t_r = 16 h. The amount of pigment produced in each operation was 0.8-1.3 x10^{-3} kg/m^3. At the end of culture (t_F = 450 h), the pigment content in cells (B_F) was 1.05 x10^{-3} kg/kg-DW, suggesting that the extracellular production of pigment becomes feasible afterwards.

Table 4.2 summarizes the results of hairy root cultures which were performed under the conditions of the oxygen starvation time; t_r = 16 h in Run 1 and t_r = 12 h in Run 2. In both cultures, the final concentrations of dry cells (X_F) and the amounts of pigment produced out of bioreactor (A_R) were 11.1 kg-DW/m^3 and 4.0 x10^{-3} kg/m^3-broth (Run 1), and 13.0 kg-DW/m^3 and 1.9 x10^{-3}kg/m^3 (Run 2), respectively. The extracellular production rate of pigment (P_B) in the culture of Run 1 was 11.3 x10^{-6} kg/(m^3•h) on the average and this production rate was about 1.7 times as high as that in the culture of Run 2 (P_B = 6.7 x10^{-6} kg/(m^3•h)), although the cell growth was somewhat better in the latter culture. The sum of total amount of recovered pigment and retained in cells was almost the same in these cultures, namely 15.7

$x10^{-3}$ kg/m^3 in the culture of Run 1 and 16.6 $x10^{-3}$kg/m^3 in the culture of Run 2. Therefore, the increase in the extracellular production rate in the culture of Run 1 was due to the enhancement of pigment amount released from the hairy root cells during the oxygen starvation treatment. Thus, the oxygen starvation time of 16 h was preferred for the extracellular pigment production by the hairy root culture.

4.3.3 Application of metabolite release by treatment of oxygen starvation to cultures of madder hairy roots

In submerged bioreactor cultures of various hairy roots, it is also desirable that metabolites of interest in the cells should be extracellularly produced. And then oxygen starvation treatment to a submerged culture of madder hairy roots for the release of anthraquinone pigments into the medium was applied.

When pigment release was carried out in submerged culture in the fermentor, the air supply was first stopped and then pure N_2 gas was sparged to reduce the medium DO level to zero, keeping the pH at a given value by the addition of 0.1 kmol/m^3 KOH. After pigment release for a given period, the culture broth containing released pigment was exchanged for fresh medium and the hairy roots were grown again by supplying air and adjusting the pH to 5.8 with 0.1 kmol/m^3 HNO_3. Medium used in the culture was NI medium (KNO_3: 6.07 kg/m^3, and the others: the same as those of MS medium) with 20 kg/m^3 fructose which was improved for the pigment production as described in Chapter 2.

The cell mass concentration on a working volume basis was determined by measuring the electrical conductivity of the medium (Eq.(1.7)). The empirical equation for madder hairy roots was predetermined as follows:

$$X = -25(\Delta\kappa) + X_0 \qquad (4.4)$$

In the present study, The trial to obtain the release of anthraquinone pigments from madder hairy roots on the basis of the same culture strategy

as that in the culture of red beet hairy roots was conducted. The conditions for pigment release from madder hairy roots were determined in advance as follows. Period of oxygen starvation by maintaining C_O = 0 kg/m^3: 24 h; pH value during oxygen starvation: 7.5; culture period among pigment release by oxygen starvation treatment: 192 to 240 h, corresponding to the active growth phase.

For the successive production of pigments, repeated-batch culture of madder hairy roots was performed with a turbine-blade fermentor, accompanied by root growth and pigment release treatments. The time course of the hairy root culture is presented in **Fig.4.6**. The culture broth was

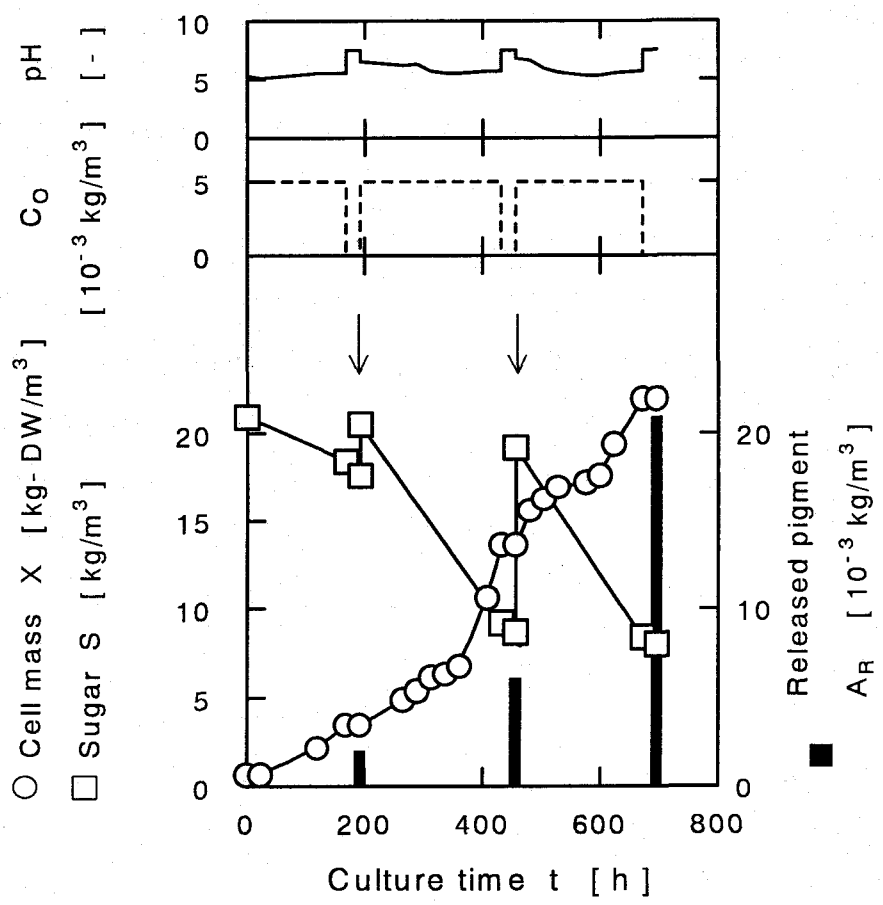


Fig.4.6 Time course of repeated-batch culture of madder hairy roots with pigment release treatment

Arrows show the culture time of medium exchange.

exchanged for fresh medium to recover the released pigments after the oxygen starvation treatment. The concentration of pigments released during each operation increased with increasing cell mass concentration. In three pigment-release operations, the value of A_R was 21x10^{-3} kg/m^3, giving P_B value of 30.0x10^6 kg/(m^3•h).

At the end of the culture (t_F = 700 h), the value of X_F was 22 kg-DW/m^3 and the amount of pigment retained in the roots was 0.42 kg/m^3. The ratio of pigment recovery during culture for 900 h was 5%, where the ratio of pigment recovery was estimated as [amount of released pigments] / {[amount of released pigments] + [amount of pigments retained in roots]}. In the culture of red beet hairy roots in a fermentor, the ratio of pigment recovery was 11-25 % and the average production rate of released pigments was 6.67-11.3 x10^{-6} kg/(m^3•h). Thus, the value of P_B was relatively high in the culture of madder hairy roots, which is considered to be attributable to the enhancement of the hairy root growth and pigment formation by modifying the medium compositions.

In HPLC analysis, peak was mainly found on a chromatogram for culture broth with the released pigments, while no significant difference was recognized between the chromatographic patterns of the pigments retained in the roots with and without oxygen starvation as shown in Fig.1.6.

4.3.4 Kinetic expression in culture with repeated processes of growth and pigment release

A) Model development *General statement of culture with pigment release by oxygen starvation* Taking into account the phenomena in the culture of red beet hairy roots with repeated processes of growth and pigment release which was described in section 4.3.1, the assumptions for the modeling of the extracellular production of pigment are made (see **Fig.4.7**).

1) Hairy root cells (X) consist of viable cells (X_V) and the nonviable

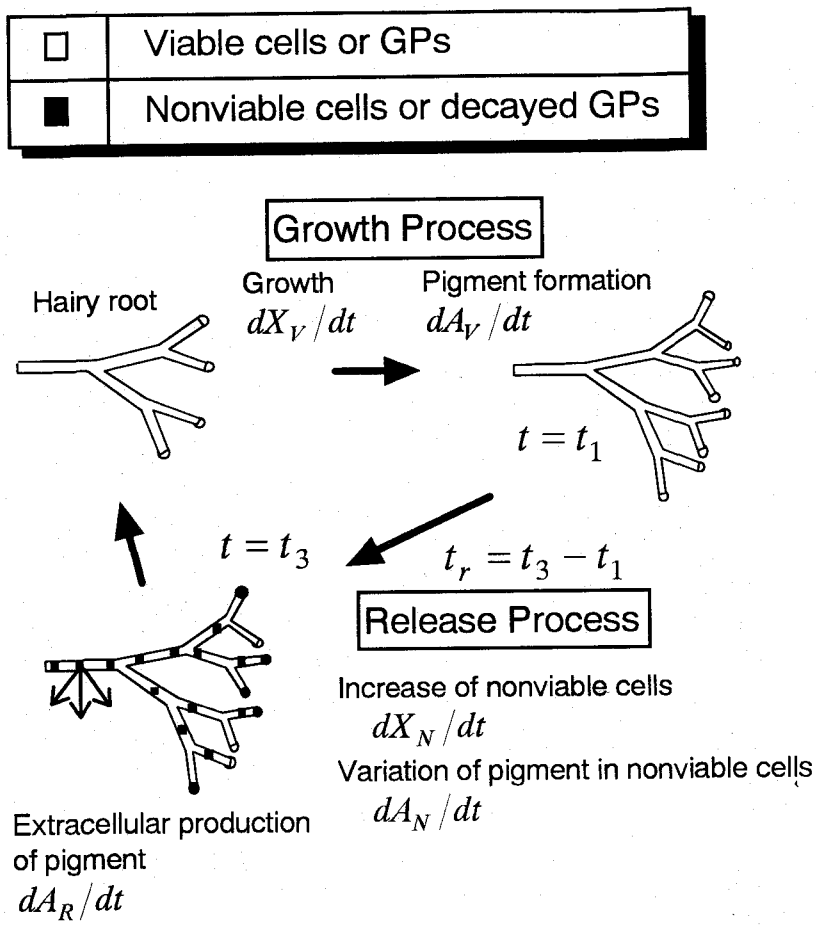


Fig.4.7 Conceptual drawing of hairy root growth and pigment release in culture accompanied with repeated processes

cells (X_N).

2) During the growth process (in the range of culture time from t_0 to t_1), the viable cells increase with hairy root growth associated with the elongation and the division at their tip parts (*i.e.* GPs), and pigment formation is conducted in viable cells.

3) During the release process (in the range of culture time from t_1 to t_3), the nonviable cells gradually increase with decaying the viable cells. The pigment in nonviable cells is released into a medium and the released pigment is increased with increasing the period (t_r) of release process. The number of GPs which affects growth rate after

pigment release process is also decreased with increasing t_r.

According to assumption 1), throughout the culture containing the processes of the growth and the pigment release, the total concentration of cells (X) and the total amount of the pigment (A_T) is are represented as follows.

$$dX/dt = dX_V/dt + dX_N/dt \tag{4.5}$$

$$dA_T/dt = dA_V/dt + dA_N/dt + dA_R/dt \tag{4.6}$$

where subscripts of V, N, and R are denoted as the values of viable cells, nonviable cells and extracellular production, respectively.

Model of growth and pigment formation in growth process During the growth process, the decay of viable cells does not occur, and Eqs.(4.5) and (4.6) can be rewritten as,

$$dX/dt = dX_V/dt \tag{4.7}$$

$$dA_T/dt = dA_V/dt \tag{4.8}$$

According to the kinetic model of hairy root growth and pigment formation described in Chapter 3, the concentration of hairy root cells (X) and amount of pigment in hairy roots (M) are calculated.

A_V are expressed as follows.

$$A_V = M/V \tag{4.9}$$

Model of pigment release considering decay of viable cells in pigment release process During the release process, the hairy root growth and pigment formation do not occur, and Eqs.(4.5) and (4.6) can be rewritten as follows.

$$dX_N/dt = -dX_V/dt \tag{4.10}$$

$$dA_R/dt = -\left(dA_V/dt + dA_N/dt\right) \tag{4.11}$$

Cell activity (f_T) is considered to be equal to the fraction of remaining viable cell mass presented as X_V/X_{V1}. Consequently the concentrations of nonviable cells (X_N) are expressed as,

$$X_N = X_{V1}(1 - f_T) \tag{4.12}$$

where X_{V1} is the viable cell mass at initial of pigment release process ($t = t_1$) and the variation in f_T is expressed as the function of t_r.

$$f_T = \begin{cases} 1 & (0 \le t_r \le k_L) \\ 1 - \exp(-(t_r - k_L)/\tau) & (k_L < t_r) \end{cases} \tag{4.13, 4.14}$$

Concerning the cells which are decayed at a given time ($t = t_2$), the variations in pigment concentrations ($C_B(t, X_{N2})$) in the cells are expressed as follows.

$$\frac{\partial C_B(t, X_{N2})}{\partial t} = \begin{cases} 0 & (t < t_2) \\ -k_R(C_B(t, X_{N2}) - A_M) & (t_2 < t) \end{cases} \tag{4.15, 4.16}$$

Here, the pigment concentration in viable cells at the initial of the pigment release process ($C_B(t_1, X_{N2})$) is calculated as follows.

$$C_B(t_1, X_{N2}) = \rho(1 - W_C)A_{V1}/X_{V1} \tag{4.17}$$

Thus, A_R is described by the integration of Eqs.(4.15) and (4.16).

$$dA_R/dt = \int_{X_{N1}}^{X_N} k_B(C_B(t, X_{N2}) - A_M)dX_{N2} \tag{4.18}$$

where A_R is equal to A_M during the pigment release process without removing pigment from medium.

Using the number of GPs at $t_r = 0$ (N_1), the variation in number of GPs is expressed as,

$$N = N_1 f_{GP} \tag{4.19}$$

where the variation in the fraction of remaining number of GPs (f_{GP}) is considered to be equal to that in f_T.

<u>B) Response of oxygen starvation to pigment release and cell viability in red beet hairy root culture using phosphate-free medium</u> To realize the effective operation of cell growth associated with product release, it is necessary to enhance pigment production in the hairy roots and express the kinetics of cell growth, product formation and product release. By

considering the enhancement of pigment formation in the hairy roots, phosphate-free MS medium was established as the optimized medium as described in Chapter 2. Thus, the first trial to estimate the kinetics of pigment release after the culture of red beet hairy roots using phosphate-free MS medium was conducted.

In **Fig.4.8**, the variations in the amounts of released pigment were investigated using the hairy roots which have different pigment contents. In Run 1 the red beet hairy roots after 240 h-cultivation cultivation in phosphate-free MS medium started to release pigment into a medium after t_r = 6 h. Amount of released pigment in a medium (A_M) at t_r = 24 h reached 20.6 x10^{-3} kg/m^3 which was 3.1 times higher than that in Run 2 whose data was shown in Fig.4.2, using the hairy roots after 192 h-cultivation in MS

Run	Run 1	Run 2
Key	○	△
X_1 [kg-DW/m^3]	7.8	4.8
B_1 [10^{-3} kg/kg-DW]	11.4	4.0

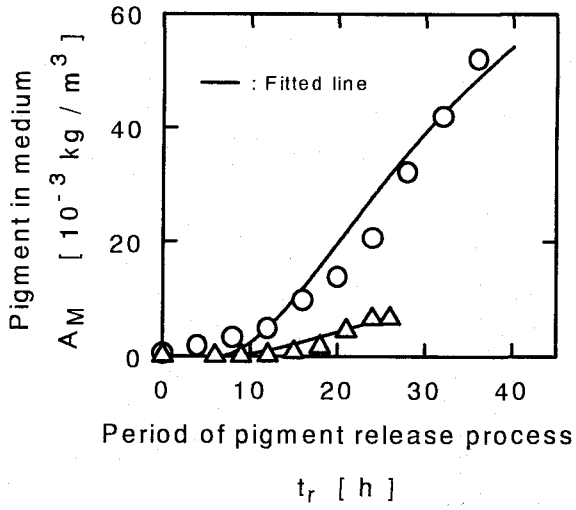


Fig.4.8 Time courses of pigment release from red beet hairy roots during pigment release process

Lines show the fitted lines.

medium. The enhancement of the pigment content in the hairy roots was considered to cause the increase in A_M values during the pigment release process.

As shown in **Fig.4.9**, using the hairy roots after the various values of t_r the cell viability was obtained by measuring the extent of enzymatic reduction of TTC. f_T values were decreased with increasing t_r values after t_r = 6 h and approached zero. The variation in f_T was indicated as the time response of first-order lag plus dead time. For the determination of k_L and τ values in Eq.(4.14) the calculated values of f_T were fitted to the experimental ones shown in Fig.4.9 by using nonlinear least-squares method. The fitted values were indicated by the solid lines. Here, the evaluated values of k_L and τ were listed in **Table 4.3**.

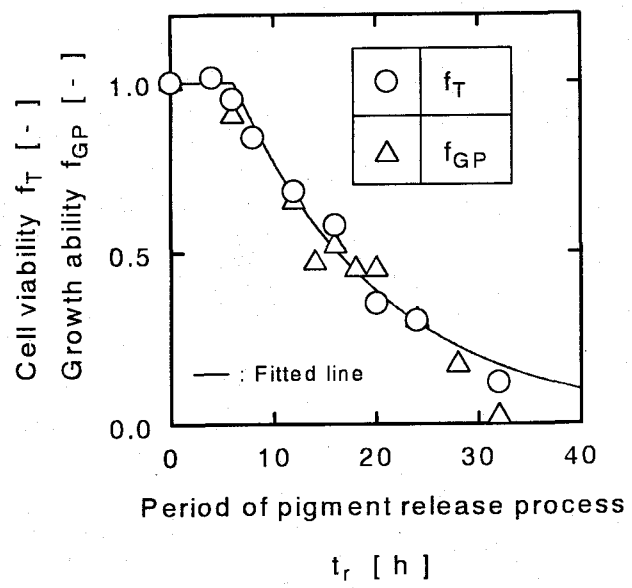


Fig.4.9 Behavior of cell viability and growth ability during pigment release process

Line shows the fitted line.

Table 4.3 Parameter values used for calculation

k_R = 6.0 x 10^{-2} h^{-1}	k_L = 6.0 h	τ = 14.7 h

Moreover, the profile of A_R in Fig.4.8 was also evaluated using Eqs.(4.12)-(4.14), (4.17) and (4.18). Here, the constant values of ρ and W_C were used as shown in Table 3.3. By fitting the experimental data shown in Fig.4.8, the parameter of k_R in Eq.(4.18) was estimated with nonlinear least-squares method. The fitted values were indicated as the solid lines in Fig.4.8 and gave reasonable explanation to pigment release.

C) Effect of oxygen starvation on hairy root growth after pigment release processAs shown in **Fig.4.10**, red beet hairy roots were cultured for 240 h in phosphate-free MS medium with the fermentor and the pigment release process of t_r = 0, 12 or 24h was conducted (see arrow in Fig.4.10). Thereafter the growth process started again after the culture medium was

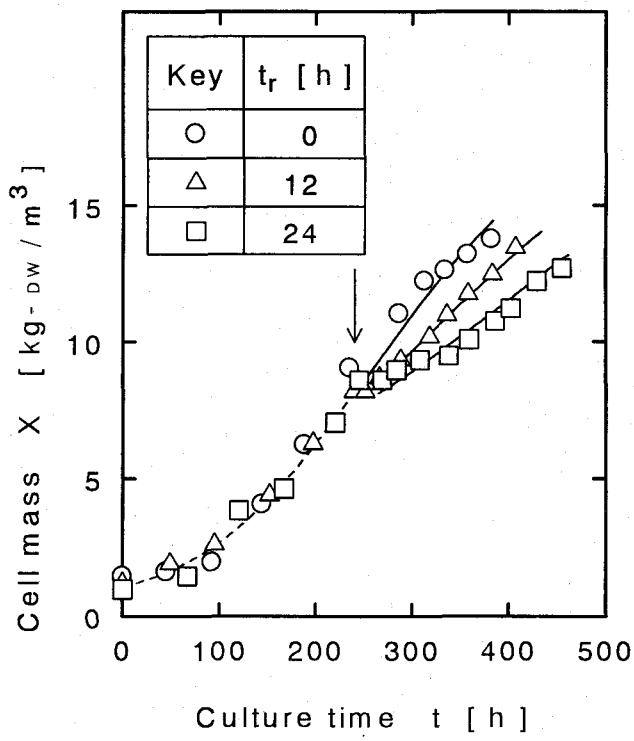


Fig.4.10 Growth of red beet hairy roots in culture with release process

Arrow, solid lines and broken line show the starting time of release process, the fitted values and the calculated values, respectively.

exchanged for fresh medium to avoid the influence on growth and pigment formation due to the diminishment of nutrients for the hairy root growth. With increasing t_r values, the growth rates after the pigment release processes were decreased. In the culture with t_r = 24 h, the time-average growth rate during the growth process (t = 264 - 364 h) became 1.4 x10^{-2} kg-DW/(m^3•h) and was about one third against that in the culture with t_r = 0 h (t = 240 - 340 h).

The courses of cell mass after the pigment release processes was tried to evaluate with various t_r values. The decrease in the growth rate was considered to occur due to the increase in numbers of decayed GPs during the pigment release process. Thus, the growth ability after pigment release was denoted as the fraction of remaining number of GPs equal to N_3/N_1.

The numbers of GPs at the initial of the pigment release processes (N_1) were calculated by simulating the growth kinetics described in Chapter 3 (see the broken line in Fig.4.10). While the numbers of GPs at the end of the pigment release processes (N_3) were determined by matching the data shown in Fig.4.9 during the growth processes after the pigment release processes. The lines fitted by using the growth kinetics described in Chapter 3 were indicated as the solid lines in Fig.4.10.

f_{GP} values in the range of t_r = 0-32 h including the data of the cultures in Fig.4.10 were estimated in Fig.4.9. the variation in f_{GP} was well agreed with that in f_T. Thus, the decay of GPs with increasing t_r values was seen to occur in the same frequency as that of the viable cells in the hairy roots.

$$f_T = f_{GP} \tag{4.20}$$

From these results, t_r governed the amount of released pigment during pigment release process as well as the growth rate after pigment release process due to controlling the extent of decay of both viable cells and GPs. To accomplish the extracellular pigment production from hairy roots effectively

it is required to set an appropriate value of t_r.

C) Extracellular pigment production in the culture of red beet hairy roots considering the effect of period of pigment release Based on the kinetics of pigment release during pigment release process and growth after pigment release process, which were affected by t_r, the extracellular pigment production from red beet hairy roots accompanied with repeated pigment release was conducted. The released pigment in medium was *in situ* recovered by means of adsorption with resin. As shown in **Fig.4.11** during the growth process for 240 h the hairy root were grown in the bioreactor system incorporated with the adsorption columns. Afterwards the pigment

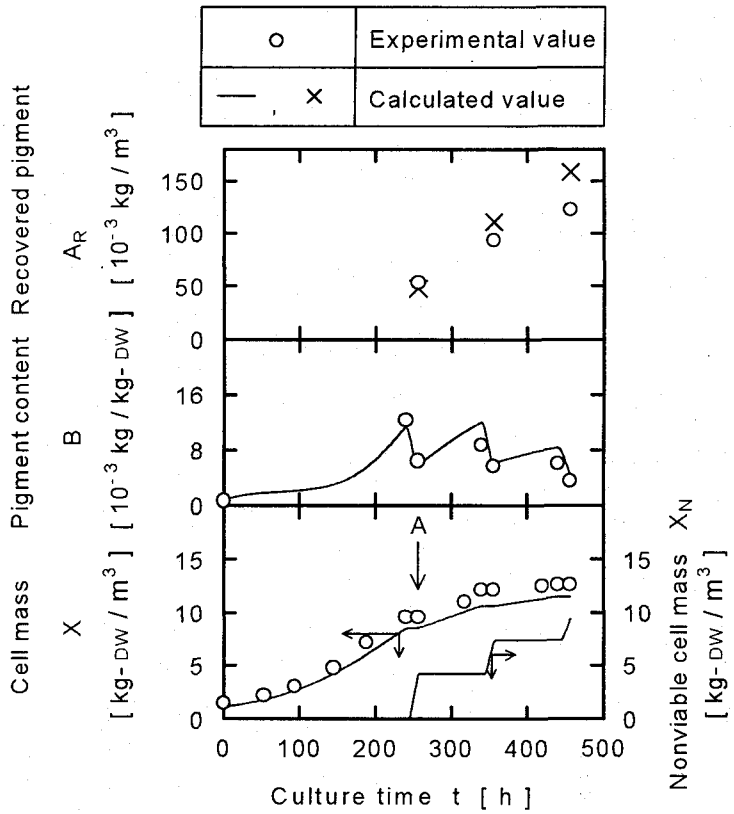


Fig.4.11 Culture of red beet hairy roots associated with repeated processes of growth and pigment release

Arrow A shows the culture time of medium exchange.

release process was carried out 3 times every 100 h. The value of t_r was set as 16 h which was obtained from the simulation to give the maximum production of extracellular pigment. Here, the pigment released from the hairy roots was *in situ* recovered by means of adsorption with resin and the amount of extracellular pigment (A_R) was indicated by using total amount of recovered pigment.

According to increasing number of the pigment release processes, A_R values were increased but the increments of X and B during each growth process were decreased. The average production rate of extracellular pigment was 2.70 x10^{-4} kg/(m^3•h).

The calculated values of X, B and A_R were obtained from the procedure shown in **Fig.4.12** where N at the initial of growth process was calculated by Eqs.(4.13), (4.14), (4.19) and (4.20). The pigment content in hairy roots (B) consisting of viable and nonviable cells is denoted as,

$$B = A_V / (X_V + X_N) \tag{4.21}$$

The values of X, B and A_R were indicated by the solid lines and open bars in Fig.4.11. The calculated value of A_R at the first pigment release process was well agreement with the experimental one, but the calculated value at the second or third process was evaluated excessively in comparison with the experimental one, respectively. Moreover, it was found that the calculated values of X_N were increased during the release processes and at the final of the culture (t_F = 456 h) the calculated values of X_{NF} was up to 9.4 kg/m^3 which meant 80 % of the calculated value of X_F. From these results, by setting the appropriate period of pigment release process, it made be possible to maintain the growth ability and to repeat the pigment release.

In the present study, a release system for pigments from red beet hairy roots, with preserved cell viability, by limitation of the oxygen supply to the cells was reported. This system offers a promising strategy for the successive

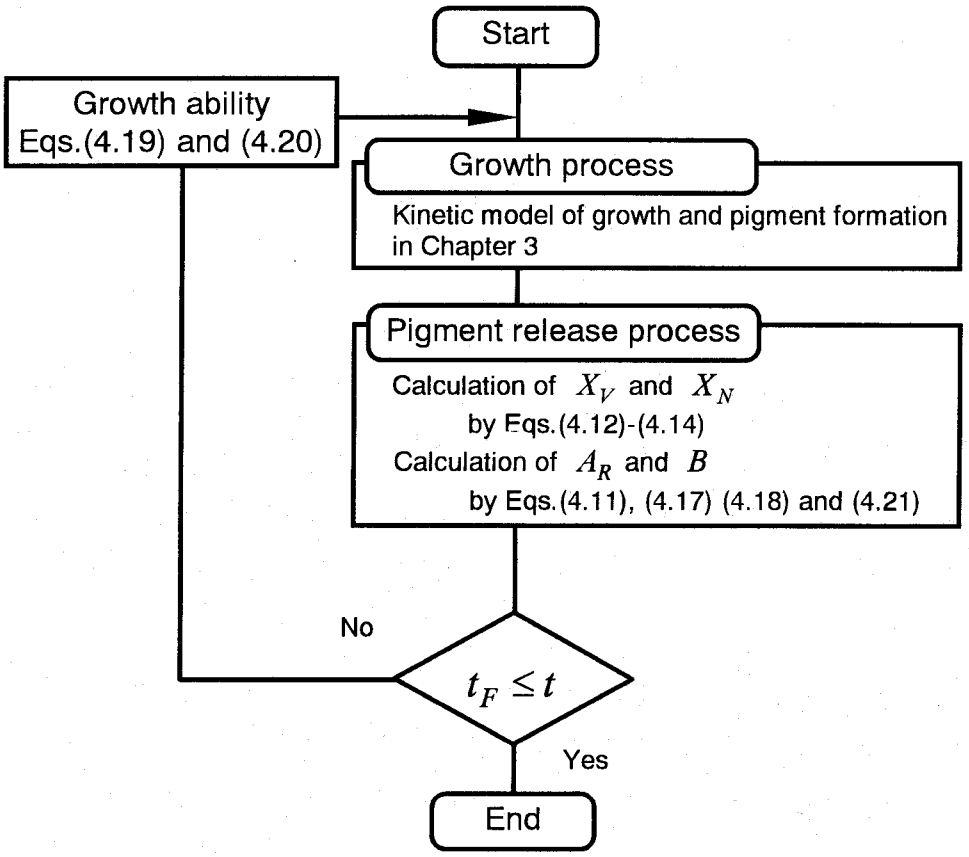


Fig.4.11 Calculation procedure of pigment production in culture with repeated processes of growth and pigment release

production and harvesting of pigments, because the DO level is easily controlled in a bioreactor and the products are not contaminated with impurities since no additives are introduced into the culture.

4.4 Summary

In the culture of red beet hairy roots, the red pigment (mainly betanin) was released from the cells into medium when the cells were subjected to the culture condition under oxygen starvation by keeping the DO concentration in medium at 0 ppm. The amount of released pigment was increased with increasing time length of oxygen starvation up to 26 h. The adsorption column with a hydrophobic resin, Sepabeads SP 207 (styrene-divinylbenzen

copolymer), was incorporated in a bioreactor system and the long-term culture of the hairy roots was carried out with the repeated processes of growth and pigment release. In the culture with the oxygen starvation time of 16 h, the extracellular production rate of pigment was obtained at 11.3 $\times 10^{-6}$ kg/(m^3•h) on the average.

The culture of madder hairy roots with repeated processes of growth and pigment release was also performed and an average production rate of released pigments of 30.0×10^{-6} kg/(m^3•h) was achieved.

Moreover, the kinetic analysis in the culture of red beet hairy roots associated with repeated processes of growth and pigment release were conducted.

The phenomenon of pigment release into medium by oxygen starvation was found that a part of hairy root cells associated with declination of cell viability was decayed and the pigment release arose from the cells. The kinetics of pigment release was expressed by the declination of cell viability during oxygen starvation as the time response of first-order lag plus dead time. Moreover, the variation in cell viability affected the growth rate after oxygen starvation and the growth rate was evaluated by the extent of the decay of growing points in hairy roots. According to the kinetics, the culture accompanied with repeated pigment release was performed at the period of oxygen starvation to achieve the maximum extracellular production. From experimental data, it showed that the extracellular production rate of 2.70×10^{-4} kg/(m^3•h) attained when oxygen starvation whose period was 16 h was repeated three times. It was demonstrated that the model made it possible to describe the kinetic behaviors of growth and pigment recovery during the culture.

Chapter 5 Development of a Bioreactor Considering Effect of Liquid Flow and Optimization of Culture Operation in Integrated System

5.1 Introduction

Bioreactors for cell suspension cultures can not be applied to hairy root cultures, because hairy roots grow with a highly branched morphology. Thus it becomes necessary to develop the bioreactor suitable to the hairy roots. Several workers reported that hairy roots could be satisfactorily cultivated with fermentors in which hairy roots were anchored to supporters and nutrients were supplied to hairy roots by means of medium flowing (Kondo *et al.*, 1989; Hilton and Rhodes, 1990; Rodriguez-Mendiola *et al.*, 1992; McKelvey *et al.*, 1993; Curtis,1993; Ramakrishnan and Curtis, 1994).

For bioreactor design, many researchers have been investigating oxygen transfer. Film resistance to oxygen transfer in the fermentor exists at the interfaces among gas-liquid-solid. Majority of researches was about oxygen transfer at the interface between gas and liquid because the main stream of culture method so far had suspension culture in which oxygen transfer between liquid and solid was much larger than that between gas and liquid. However, in the culture of hairy roots, oxygen transfer between liquid and solid as well as gas and liquid will become an important factor since hairy roots can not be suspended in the fermentor.

In this chapter, the culture of red beet hairy roots was conducted in a single column reactor (SCR) where the efficiency of oxygen supply to hairy roots could be varied, and the influence of liquid flow on hairy root growth in SCR was investigated by means of the analysis of the oxygen transfer. From a view point of oxygen transfer a bioreactor for hairy root culture was constructed. Moreover the pigment productivity was optimized by calculation

in the integrated culture system which includes the results described in Chapters 2 - 4.

5.2 Experimental

A) Hairy roots, media and conditions Hairy roots of red beet were used. Unless otherwise noted, the culture media and conditions were the same as described in section 2.2.

B) Culture in bioreactor To analyze the effect of liquid flow rate on cell growth, red beet hairy roots were grown at 25℃ in an SCR with 1dm^3 of MS medium containing 20 kg/m^3 fructose. The configuration of SCR is shown in **Fig.5.1**. This bioreactor consisted of two units; single growth column for

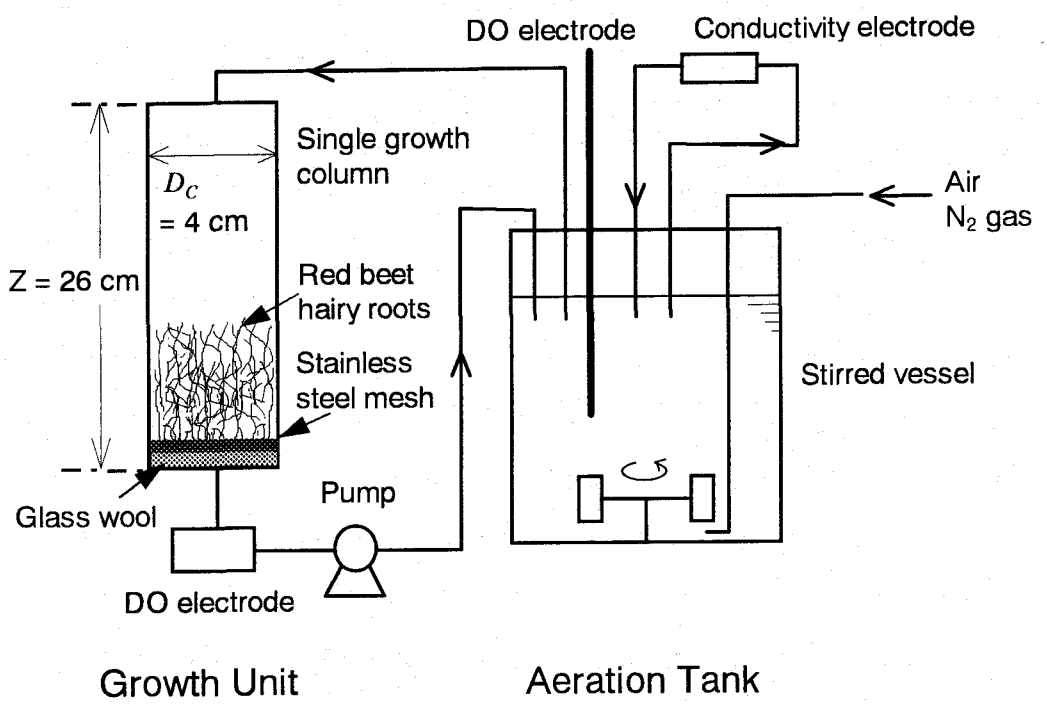


Fig.5.1 Schematic diagram of single column reactor (SCR) for hairy root culture

hairy root growth (growth unit) and stirred vessel (Model TBR-2 fermentor, Sakura Seiki Co., Tokyo) for oxygen enrichment (aeration tank). Inoculated hairy roots of *ca.* 20 g fresh mass (*ca.* 1.0 g of a dry weight) were anchored to a stainless steel mesh equipped at the bottom of the column, and the medium subjected to O_2 enrichment in the aeration vessel was made to flow from top to bottom of the column. The flow rate of medium was kept in the range from 0.1 to 0.5 dm^3/min. The total volume of the medium in this culture system was 1.0 dm^3 (0.3 dm^3 in the growth unit and 0.7 dm^3 in the aeration tank). Dissolved oxygen (DO) concentration in the bulk liquid at the inlet (C_{O1}) or the outlet (C_{O2}) of the column was measured with a DO meter. C_{O1} was controlled at 7.5 x10^{-3} kg/m^3 by changing the agitation speed (150-450 r.p.m.) and air flow rate (0.1-0.4 dm^3/min) in the aeration tank.

Similarly to the configuration of SCR, the radial flow reactor (RFR) was also constructed. As shown in **Fig.5.2A**, RFR consists of the radial-flow reactor as the growth unit and the bubble column as the aeration tank. And between these units the medium was circulated using a tube pump. The total volume of medium in this culture system was 1.5 dm^3. In growth unit

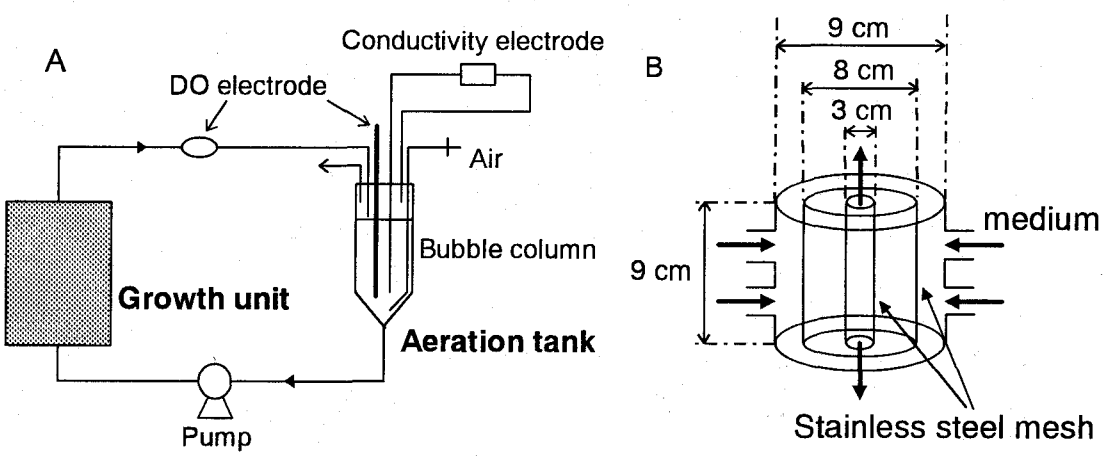


Fig.5.2 Schematic diagram of radial flow reactor (RFR) for hairy root culture
A: Total system of RFR, B: Growth unit in RFR

(Fig.5.2B), the volume of medium was 0.6 dm^3 and the hairy roots were incubated in the compartment with partitions of two cylindrical meshes. To minimize the variation in fluid flow rate through the compartment, the medium flowed from four ports on the side of outer cylinder to its center through the compartment. The superficial velocity was defined as the value of liquid flow rate per average arithmetic lateral area between inner and outer cylindrical stainless steel meshes (mesh size: 200 mesh (inner), 100 mesh (outer)). On the other hand, during the hairy root growth DO concentration in aeration tank was controlled at 7.5 $\times 10^{-3}$ kg/m^3 by changing the air flow rate (1.5-9.0 dm^3/h).

C) Analyses For the determination of dry cells (DW) were measured as described in section 1.2 and in the culture using the bioreactor, the concentration of dry cells (X) was estimated on the basis of conductometric measurement of medium in Eq.(4.2).

The specific oxygen uptake rate of red beet hairy roots at different DO concentrations was measured by a dynamic method (Shirai *et al.*, 1988). First, for realizing suspension of the hairy roots, they were cut into about 5mm segments and introduced into a closed vessel (volume: 34cm^3) containing a medium saturated in advance with air and a magnetic stirrer bar coated by glass. The change in DO concentration was measured using a DO electrode and the specific oxygen uptake rate versus the treatment time was obtained. Here, the film resistance to oxygen transfer between medium and cells was considered to be negligible by vigorous stirring in the vessel (500 r.p.m.), that is, the measured DO concentration was regarded as the DO concentrations at the surface of cells (C_{OS}).

The growth unit of SCR was modified as shown in **Fig.5.3** for the measurement of various factors affected by liquid flow such as the oxygen transfer between medium and hairy roots, the elongation rate of hairy roots, and the pressure drop.

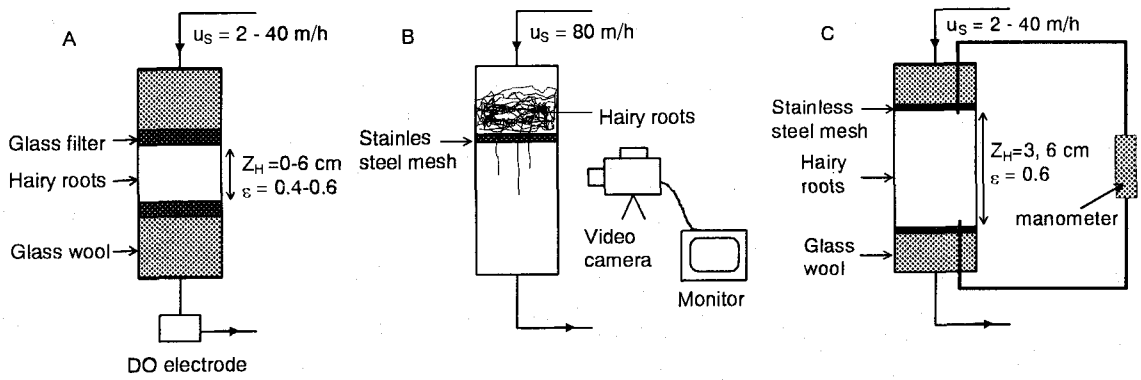


Fig.5.3 Schematic diagram of experimental apparatus of growth unit in SCR
A: measurement of oxygen transfer rate, B: measurement of elongation rate,
C: measurement for pressure drop

In the experiment for the estimation of oxygen transfer rate between medium and hairy roots in SCR, the modified growth unit (Fig.5.3A) was used. Red beet hairy roots, which were cultivated in advance for 168 h in flasks, were packed into the compartment between two glass filters to realize the laminar flow in the column. DO concentrations in the bulk liquid at inlet (C_{O1}) and outlet (C_{O2}) of this column were measured and the variation in DO concentrations in the bulk liquid in the column (C_O) was obtained by measuring C_{O2} at various heights (Z_H) of hairy roots packed in the column. The hold-up of hairy roots in the compartment (ε) and its height were varied. Here, the hold-up was measured by a simple method as follows. After measurements of C_{O1} and C_{O2} under various conditions, the volume of hairy roots (V_H) was measured by a graduated cylinder, and ε was calculated by following equation.

$$\varepsilon = \frac{\left(\pi D_C^2 Z_H/4 - V_H\right)}{\pi D_C^2 Z_H/4} \tag{5.1}$$

where D_C is the diameter of single growth column.

Culture conditions were changed as follows to investigate the relationship between DO concentration at cell surface and elongation rate. The superficial velocity (u_S) was set to as high as 80 m/h and DO concentration in bulk liquid was regarded as equal to that at cell surface. Here, to measure the elongation rate, growth unit was also modified (Fig.5.3B). In the column, stainless steel mesh (mesh size: 5 mesh) was attached at the middle of the column, and the hairy roots were put on it. After several days, hairy roots were elongated through the mesh and the length of hairy roots was monitored by video image processor.

In the experiment for the measurements of the pressure drop against various superficial velocities in the growth unit, the pressure drop between the inlet and outlet of the column was measured by a manometer as shown in Fig.5.3C.

5.3 Results and Discussion

5.3.1 Investigation of effect of liquid flow in bioreactor on hairy root growth

A) Culture of red beet hairy roots in single column reactor In the culture of red beet hairy roots with SCR, the relationship between X and C_{O2} was first examined under the conditions of u_S = 2.2 and 8.9 m/h, and C_{O1} = 7.5x10^{-3} kg/m^3.

As shown in **Fig.5.4**, C_{O2} values gradually decreased with increasing X values, and at u_S = 2.2 m/h the C_{O2} value became approximately zero when X value was about 3.6 kg-DW/m^3. When O_2-enriched medium (C_{O1} = 7.5 x 10^{-3} kg/m^3) was introduced into the column at u_S = 8.9 m/h, the more active growth of the hairy roots was ensured due to sufficient supply of medium saturated with O_2.

For the further investigation of influence of oxygen supply on the cell

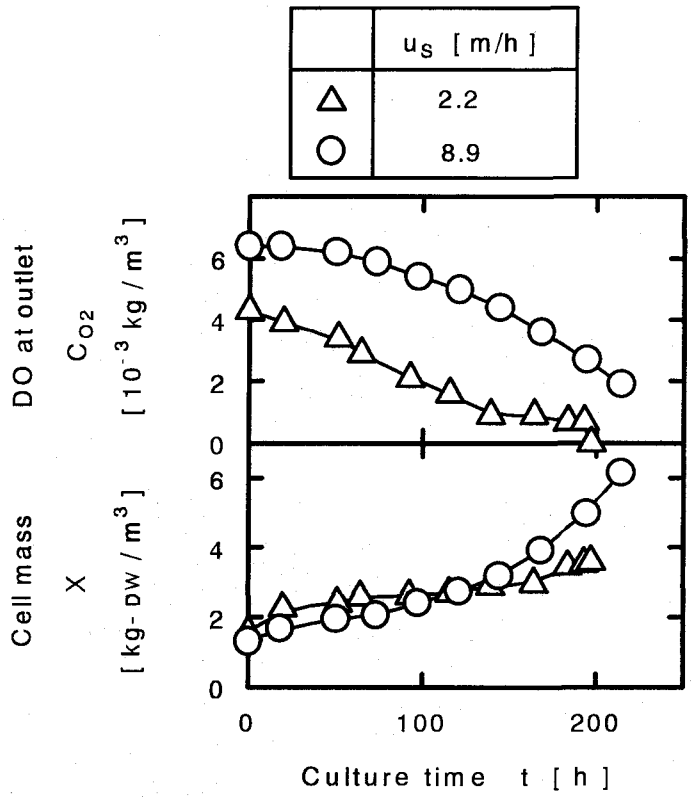


Fig.5.4 Time courses of cultures of red beet hairy roots in SCR

growth, the hairy roots were grown in the SCR at various u_S values under the condition of C_{O1} = 7.5 x10^{-3} kg/m^3.

As shown in **Fig.5.5**, at t = 190 h the maximum ΔX value of 5.7 kg-DW/m^3 was obtained in the culture of u_S = 15.3 m/h. This value was 3 times larger than that in the culture of u_S = 2.2 m/h. Moreover the apparent specific oxygen uptake rate in the bioreactor (Q^{app}) was estimated from the difference between DO concentrations in bulk liquid at the inlet and outlet of the growth column.

$$Q^{app} = \frac{C_{O1} - C_{O2}}{V \cdot X}\left(\pi D_C^2 u_S / 4\right) \qquad (5.2)$$

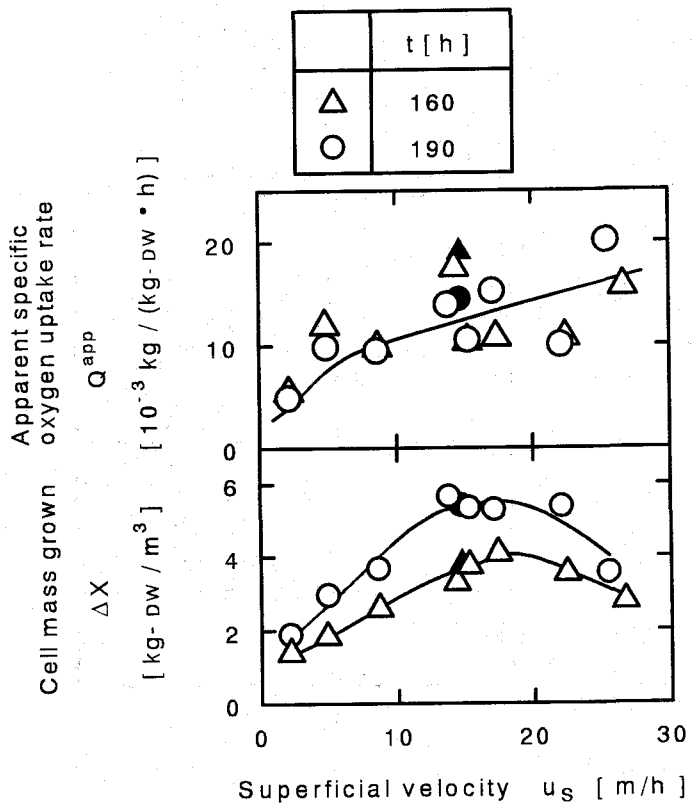


Fig.5.5 Cell mass grown and apparent specific oxygen uptake rates at various superficial velocities

The value of X_0 was 1.0 kg-DW/m^3. The closed symbols show the values of RFR.

It was found that Q^{app} value was increased with increasing u_S values. On the other hand, the declined cell growth was observed at t = 190 h in the range of higher u_S values.

In another experiment, the relationship between apparent specific oxygen uptake rates and superficial velocities was investigated varying DO concentration at the inlet of single growth column in SCR. As shown in **Fig.5.6**, Q^{app} values increased with increasing the values of u_S and C_{O1}. These results suggest that the film resistance lies at the hairy root surface

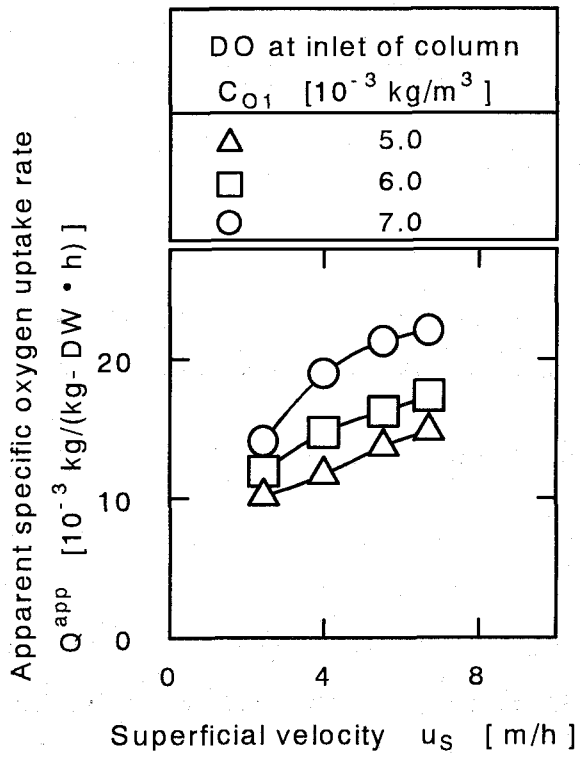


Fig.5.6 Relationship between apparent specific oxygen uptake rates and superficial velocities at various DO concentrations at inlet of single growth column

The experiment was conducted at X = 3.4 kg-DW/m^3.

and the change in Q^{app} to the hairy roots is controlling the growth rate of the hairy roots.

From these phenomena, the influence of liquid flow on hairy root growth is considered to result from following relationships (**Fig.5.7**).

With increasing the superficial velocity, the efficiency of oxygen transfer increases. Therefore DO concentration at cell surface increases. Thus growth rate of hairy roots increases with increasing superficial velocity. On the other hand, With the extensive increase of superficial velocity, the

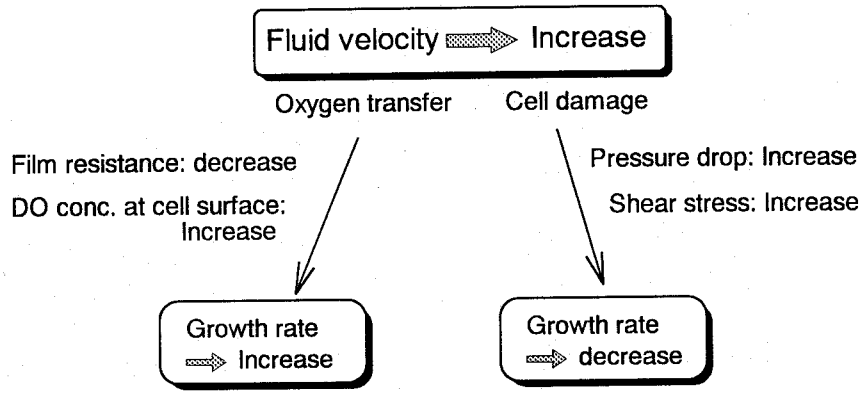


Fig.5.7 Factors affecting hairy root growth in growth unit

shear stress to the hairy roots increases, accompanying the increase of pressure drop. Thus hairy root growth decreases with increasing superficial velocity. And optimal superficial velocity in growth column seems to be 15 m/h.

To ensure these influences on the growth, these relationships of oxygen transfer against superficial velocity, elongation rate of hairy root against DO concentration at cell surface, and pressure drop against superficial velocity were investigated as follows.

B) Distribution in DO concentration in SCR The variation in DO concentrations (C_O) in the single column in the range of Z_H = 0.0 - 6.0 x10^{-2} m was shown in **Fig.5.8**. With increasing Z_H at u_S = 3.6 m/h, C_O decreased. The value of C_O at Z_H = 6.0 x10^{-2} m reached one-third value at the inlet of column. At u_S = 18.5 m/h, C_O values in the range of Z_H = 0.0 - 6.0 x10^{-2} m were maintained over 6 x10^{-3} kg/m^3 due to the efficient oxygen supply.

The apparent specific oxygen uptake rates in the column at Z_H = 6.0 x10^{-3} m which were obtained from Eq.(5.2), were 8.1 x10^{-3} kg/(kg-DW•h) and 12.3 x10^{-3} kg/(kg-DW•h) at u_S = 3.6 m/h and 18.5 m/h, respectively.

On the other hand, the specific oxygen uptake rate at cell surface was

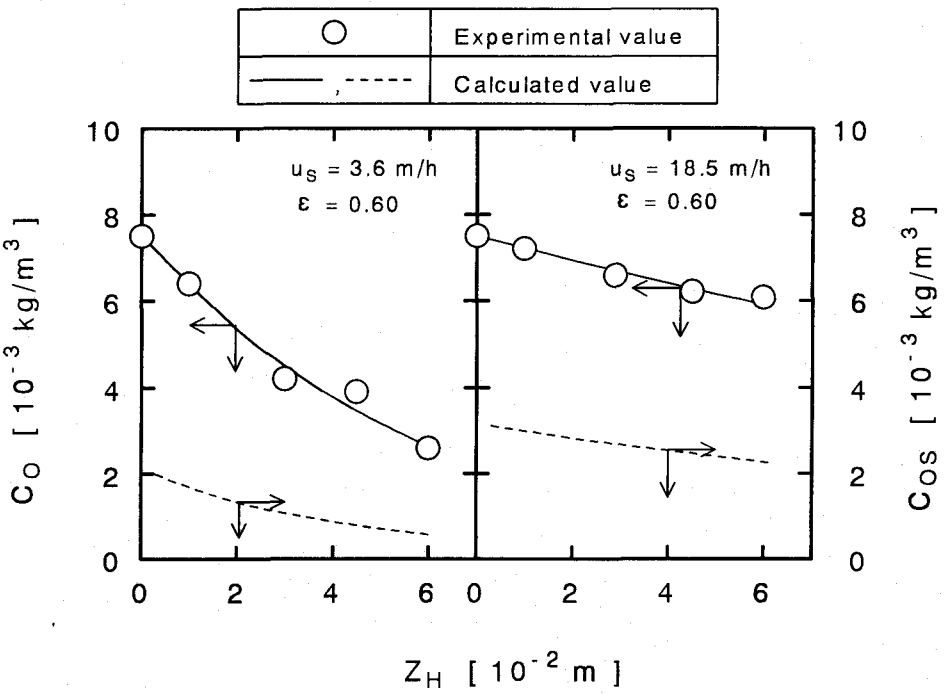


Fig.5.8 Distribution in DO concentration in single growth column

expressed as the function of DO concentration at surface of hairy root as,

$$Q = -\frac{1}{X}\frac{dC_{OS}}{dt} = Q_m \cdot C_{OS}/(K_O + C_{OS}) \qquad (5.3)$$

The values of Q_m and K_O in Eq.(5.3) were evaluated by measuring the specific oxygen uptake rate of the hairy roots in the closed vessel. **Figure 5.9** shows the typical behavior of DO concentration in closed vessel. The kinetics of DO concentration were fitted to Eq.(5.3) by a nonlinear least-squares method. Thus, Q_m = 32.6x10^{-3} kg/(kg-DW•h) and K_O = 3.11 x10^{-3} kg/m^3 were determined by averaging these values which were obtained after several measurements.

Curtis *et al.* (1995) suggested the existence of large film resistance between the bulk liquid and the cell surface in the culture of *Hyoscyamus muticus* hairy roots and proposed the necessity of the analysis of the oxygen

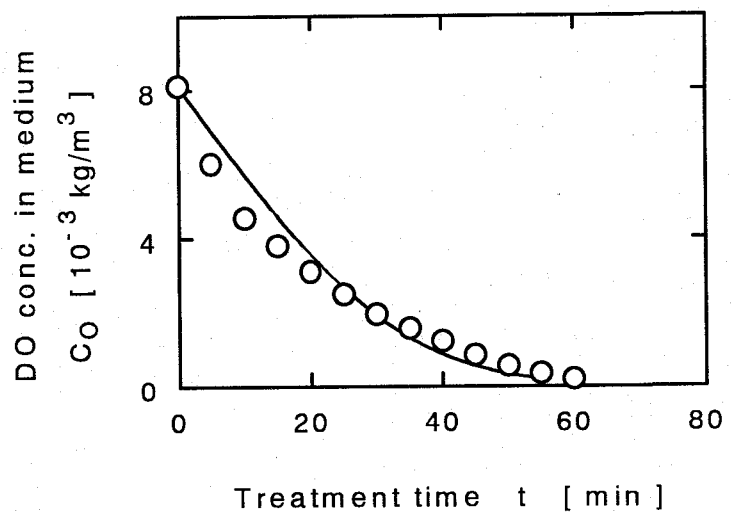


Fig.5.9 Change in DO concentration in closed vessel

Inoculum size: 0.6 kg-DW/m^3. Solid line shows the calculated values (Q_m = 32.6 x10^{-3} kg/(kg-DW•h), K_O =3.11 x10^{-3} kg/m^3).

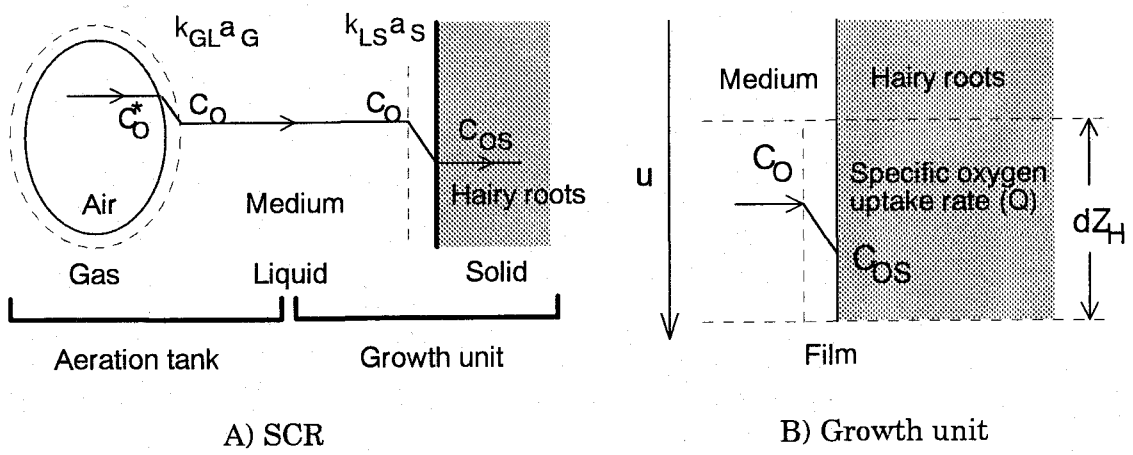


Fig.5.10 Schematic drawing of oxygen transfer in SCR

transfer in the fermentor on the basis of the oxygen uptake rate at cell surface and not on the apparent oxygen uptake rate in the bulk liquid.

C) Analysis of oxygen transfer between medium and hairy roots

The illustration of oxygen transfer in the SCR which has two film resistances

between gas (air) and liquid (medium), and between liquid and solid (hairy roots) is shown in **Fig.5.10**A. Especially, oxygen transfer between medium and hairy roots is an important factor for growth in SCR, and then oxygen transfer in growth unit is estimated (Fig. 5.10B).

Oxygen transfer rate on a reactor volume basis (N_{LS}) is expressed as,

$$N_{LS} = k_{LS} a_s (C_O - C_{OS}) \tag{5.4}$$

When the medium moves along a vertical direction of the single column in a plug flow manner and the oxygen diffusion to the horizontal direction is negligible, one obtains a mass balance yielded by Eq.(5.5) in the vertical direction.

$$u \cdot dC_O / dZ_H = -N_{LS} / \varepsilon \tag{5.5}$$

And hence,

$$u = u_S / \varepsilon \tag{5.6}$$

N_{LS} and differential dry weight (ΔX) in differential height (ΔZ) are expressed by the following equations, respectively.

$$N_{LS} = \rho(1 - W_C)(1 - \varepsilon)Q \tag{5.7}$$

$$\Delta X = \rho(1 - W_C)(1 - \varepsilon)A_C \cdot \Delta Z_H / V \tag{5.8}$$

Using Eqs.(5.3) - (5.8), $k_{LS}a_S$ values were obtained at various values of u_S, Z_H and ε, according to the procedure in **Fig.5.11**. Here, the relative error of C_{O2} between the calculated (C_{O2}^{calc}) and experimental (C_{O2}^{exp}) values was used as the condition for convergence.

$$\frac{\left| C_{O2}^{calc} - C_{O2}^{exp} \right|}{C_{O2}^{exp}} \leq 10^{-3} \tag{5.9}$$

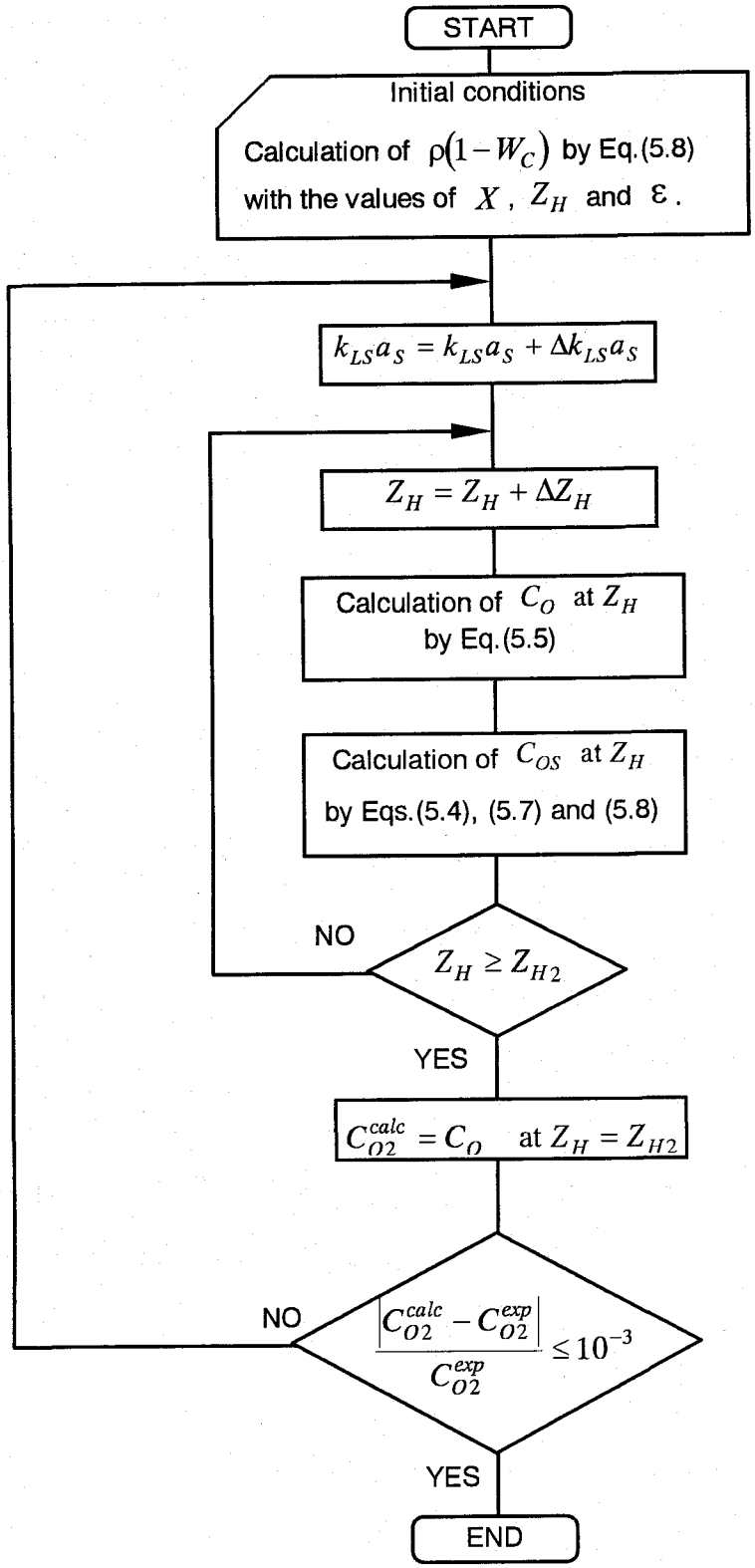


Fig.5.11 Procedure of determination of $k_{LS}a_S$ value

Furthermore, to see the effect of liquid flow on oxygen transfer in the single column, $k_{LS}a_S \cdot \varepsilon/(1-\varepsilon)$ was plotted against u_S/ε as shown in **Fig.5.12**. $k_{LS}a_S$ increased with increasing u_S and the following experimental equation was obtained.

$$k_{LS}a_S \cdot \varepsilon/(1-\varepsilon) = 75.5(u_S/\varepsilon)^{0.27} \qquad (5.10)$$

The average error in estimating $k_{LS}a_S$ by Eq.(5.10) was 26 % for 41 data in the experimental ranges of 1.5 m/h $\leq u_S \leq$ 47 m/h, 1.0x10^{-2} m $\leq Z_H \leq$ 6.0 x10^{-2} m and $0.4 \leq \varepsilon \leq 0.6$.

As shown in Fig.5.8, the variation in values of C_O and C_{OS} were calculated by Eqs.(5.3)-(5.8). Here, the values of $k_{LS}a_S$ were calculated in

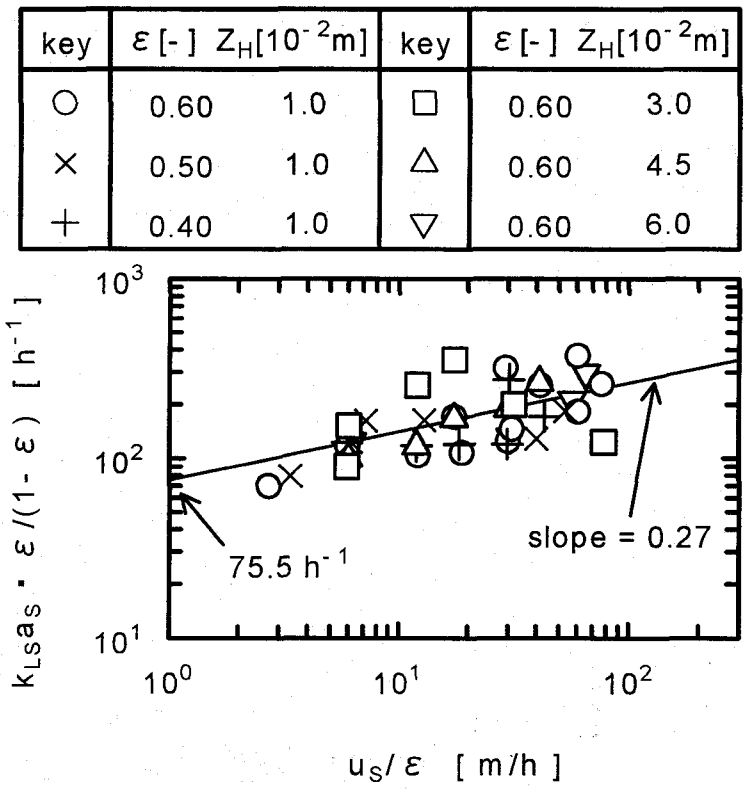


Fig.5.12 Relationships between oxygen transfer rate and superficial velocity

Eq.(5.10) as 81.7 h^{-1} and 127 h^{-1} at u_S = 3.6 and 18.5 m/h, respectively. C_O values agreed well with those observed experimentally. At u_S = 3.6 m/h C_{OS} was much lower than C_O and increased with increasing u_s due to the improvement of the oxygen transfer between medium and hairy roots.

From these results, the oxygen transfer between medium and hairy roots is significantly influenced by the film resistance whose extent is affected by the liquid flow rate, and can not be negligible, compared to that between air and medium. Thus, the liquid-solid interfacial oxygen transfer is important factor for the design of fermentors suitable to hairy root cultures.

From these results, the oxygen transfer between medium and hairy roots is significantly influenced by the film resistance and the hairy root cultures in the bioreactor should be performed, taking into account the volumetric oxygen transfer coefficient between medium and hairy roots.

D) Effect of dissolved oxygen concentration and shear stress on hairy root growth For further investigation of the relationship between DO concentration at cell surface and elongation rate, red beet hairy roots were cultured in SCR at u_S = 80 m/h. The elongation rates in the culture at various DO concentrations in the bulk liquid are shown in **Fig.5.13**. With increasing C_O values, elongation rates of hairy roots were increased, and the elongation rate at C_O = 7.3 x 10^{-3} kg/m^3 was about 2 times higher than that at C_O = 3.0 x 10^{-3} kg/m^3.

While, the liquid flow in the cell growth column could be regarded as laminar flow at the given range of u_S. To investigate the shear stress in the culture at higher value of u_S, the pressure drop against various superficial velocities ($\Delta P/X$) were measured. The value of $\Delta P/X$ was considered to be proportional to the shear stress (McCabe and Smith, 1967). The pressure drop between the inlet and outlet of a single growth column (ΔP) was manometrically measured (Fig.5.3C). In this experiment, the value of ε was

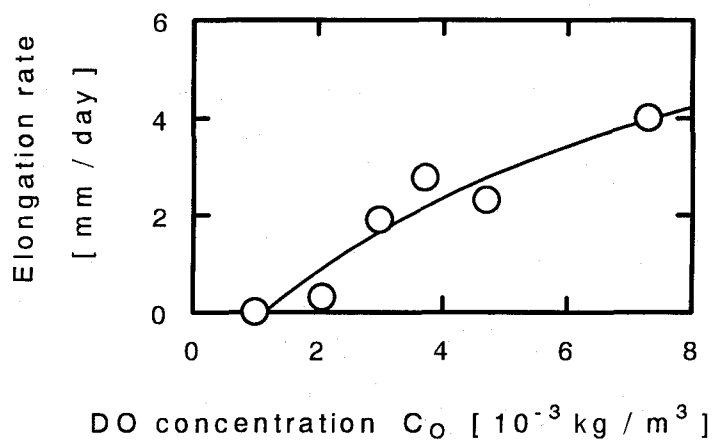


Fig.5.13 Relationship between elongation rate and DO concentration in bulk liquid

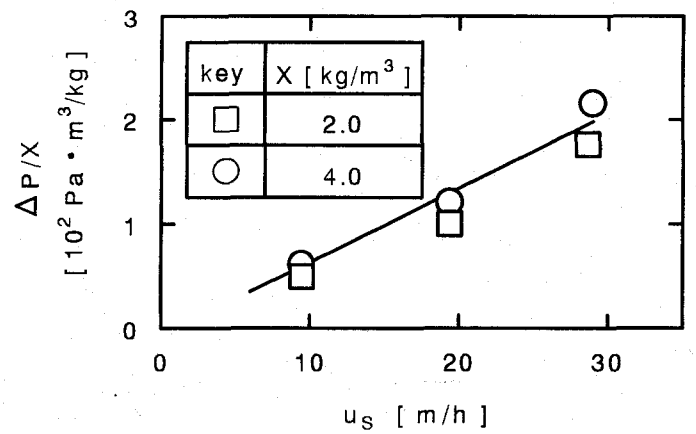


Fig.5.14 Relationship between pressure drop and superficial velocity

kept at 0.6. As shown in **Fig.5.14**, the value of $\Delta P/X$ was proportional to the u_S value. Thus, the hairy roots in growth column were subject to shear stress which was increased with increasing u_S value.

As shown in **Fig.5.15**, hairy roots have growing points which are located at the tip part of roots and roots can elongate only at the growing points. The number of growing points can also increase by branching roots. Thus, the growth rate of hairy root is governed by two factors. One is the elongation rate at the growing points and the other is the change in the

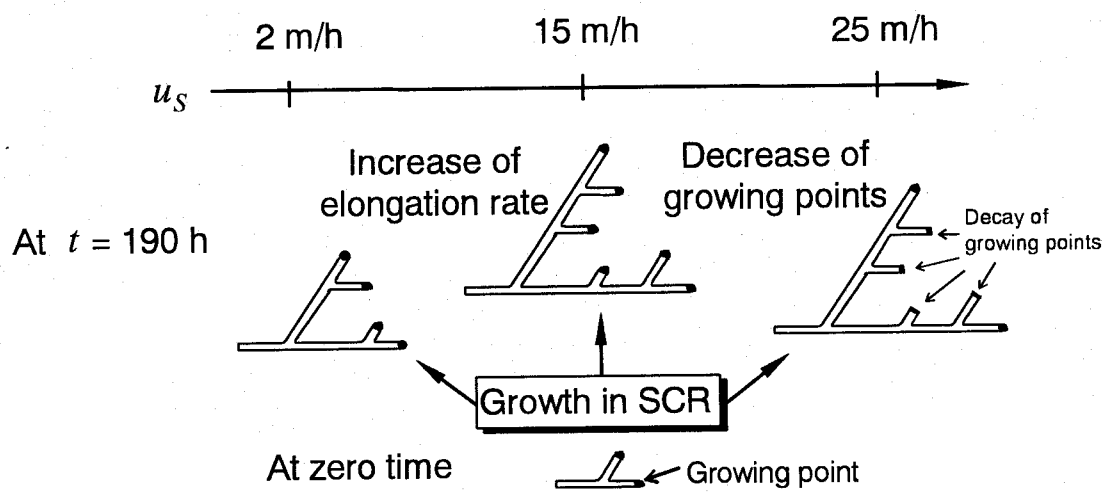


Fig.5.15 Conceptual drawing of effect of fluid velocity on hairy root growth

number of growing points.

Concerning the results shown in Fig.5.5, with increasing flow rate in the SCR, DO concentration at cell surface is increased, accompanying that the film resistance between hairy roots and medium is decreased. The growth of hairy roots was strongly affects by DO concentration.

When u_S value is in the range from 2 to 15 m/h, as shown in Fig.5.15, the enhancement of hairy root growth in this study (Fig.5.5) is caused by the increase in elongation rate. On the other hand, in the range from 15 to 25 m/h, the value of Q is almost constant and the elongation rates may be kept at higher level than those in the range from 2 to 15 m/h. However, the number of growing points might be decreased with increasing u_S, due to shear stress. From these results, the fluid velocity is a very important factor in attaining appreciate growth rate in the culture of hairy roots.

5.2.2 Construction of a bioreactor for hairy root culture and the optimization of pigment production in culture system

A) Culture performance in radial flow reactor For the construction of a bioreactor suitable to hairy root cultures, it is important to consider the

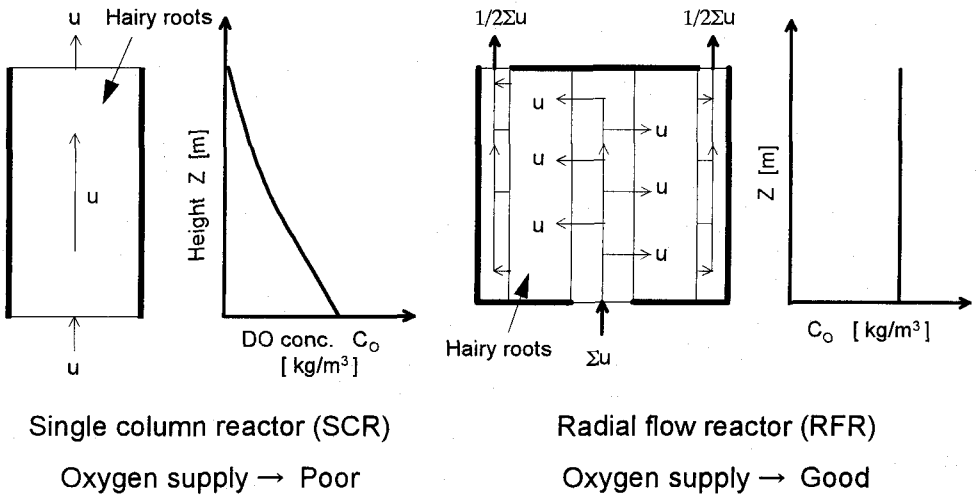


Fig.5.16 Design of bioreactor suitable to hairy root cultures

Table 5.1 Comparison of DO concentration in bulk liquid at outlet of growth unit between cultures with SCR and RFR

X	C_{O2} [10^{-3} kg/m^3]	
[kg-DW/m^3]	SCR	RFR
2.0	3.6	7.5
4.0	2.6	7.2
6.0	1.9	6.1

u_S = 15.3 (SCR), 14.7 m/h (RFR), C_{O1} = 7.5 x10^{-3} kg/m^3

effect of liquid flow on elongation rate by improving oxygen transfer and decay of number of growing points. It was found in the culture with the SCR that the successful growth was achieved in the range of u_S values of 15 m/h.

In term of scale-up of reactor, it is important to maintain high DO concentration in the reactor. As shown in **Fig.5.16**, in growth unit of SCR, there is a distribution in DO concentration in bulk liquid along its height. Thus it is difficult to maintain high DO concentration in the whole growth unit and growth rate becomes low. As shown in **Table 5.1**, however, C_{O2}

value was significantly reduced from C_{O1} value of 7.5 g/m^3 when X value was 6.0 kg-DW/m^3. In the RFR, the oxygen supply is improved and scaling-up is also easy by increasing the height of this unit. Thus, the trial relating to the construction of RFR was conducted and the cultures of red beet hairy roots with MS medium containing 20 kg/m^3 fructose at various u_S values were performed. Here, in each culture when X value was achieved at ca. 6 kg-DW/m^3, the culture broth was exchanged with fresh medium.

Figure 5.17 shows the culture of red beet hairy roots with the RFR at u_S values of 3.6 and 14.7 m/h. In each culture, active growth occurred in a manner of linear growth after ca. t = 150 h and growth rates were not decreased in the high density culture over 10 kg-DW/m^3. At t = 280 h, X

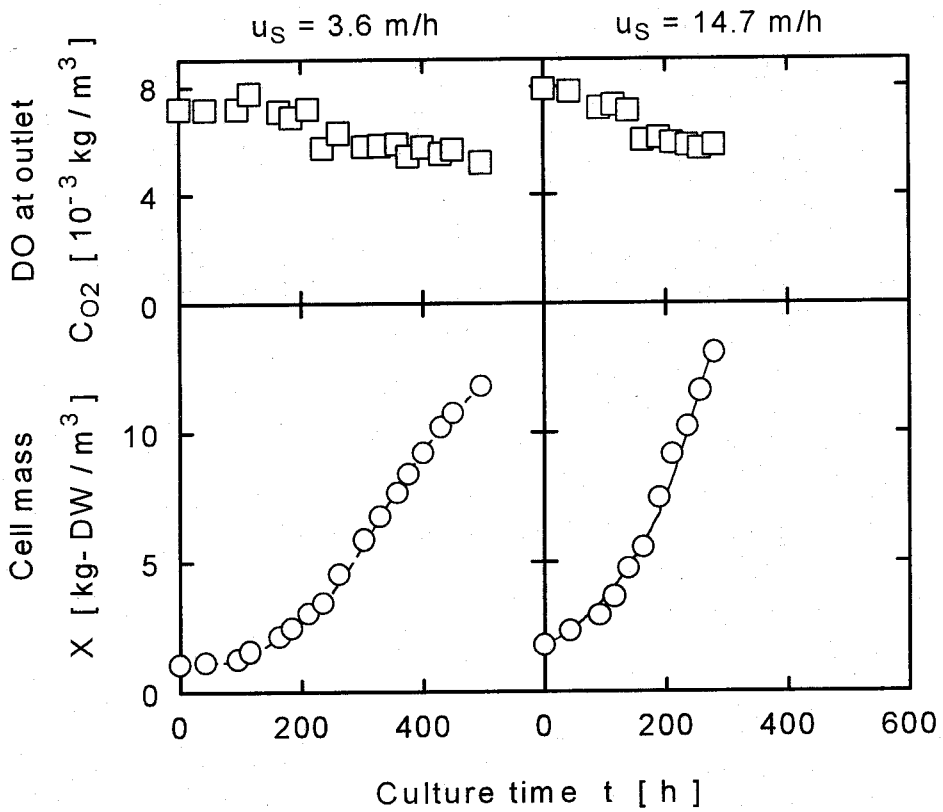


Fig.5.17 Time courses of cultures of red beet hairy roots in RFR
Solid line of X values at u_S = 14.7 m/h shows the calculated values.

value of 13.1 kg-DW/m^3 in the culture at u_S = 14.7 m/h was achieved and this value was 2.4 times larger than that in the culture at u_S = 3.6 m/h. The difference between growth rates of these cultures is likely to be attributed to the efficiency of oxygen supply which can be varied by controlling superficial velocity. The values of C_{O2} in culture with the RFR at u_S = 14.7 m/h were constantly higher than those in the SCR (Table 5.1) and the oxygen supply was improved. The cell mass grown at t = 190 h in the culture using RFR, as shown in Fig.5.5, was almost equal to that of SCR.

Moreover, **Fig.5.18** shows the cell mass grown at t = 210 h in the culture using RFR at various values of u_S. The behavior of ΔX value against u_S value was similar to that in the culture using SCR and each value of C_{O2} was over 5.9 x 10^{-3} kg/m^3. Considering the efficiency of oxygen supply, the RFR was superior to SCR in the high density culture of hairy roots.

The data obtained in RFR were applied to the growth model described in section 3.3. The growth of hairy roots was affected by the elongation and

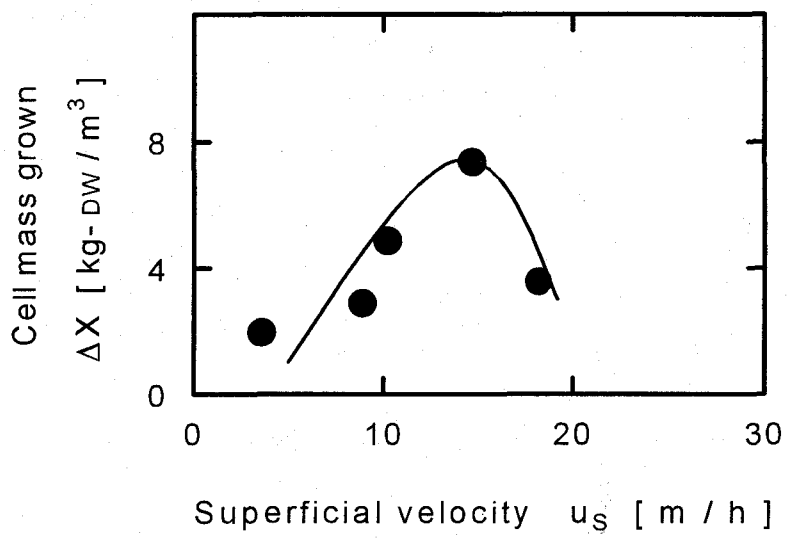


Fig.5.18 Cell mass grown in the culture of red beet hairy roots using RFR at various u_S values

Culture time :210 h.

branching of growing points, and the growing point was decayed by the damage such as shear stress. The growing process was expressed by Eq.(3.6) using the parameter of k_d. By supplying Eqs.(3.1)-(3.10) to the data of u_S = 14.7 m/h in Fig.5.17, the value of k_d was evaluated as 2.6 x10^{-3} h^{-1}.

This value is almost equal to that in the culture with TBR (Table 3.1), indicating that cultivation in this reactor is conducted under good condition. Moreover, it is considered that this reactor can be scaled up easily by means of increasing the height of the growth unit and the effective production of pigment by red beet hairy roots can be realized.

b) Optimization of culture operation in integrated system with radial flow reactor The validity of the kinetic models for the culture of red beet hairy roots associated with repeated processes of growth and pigment release was indicated as described in Chapter 4. Furthermore, the pigment production system was constructed by integrating the results described in Chapters 1-5 (**Table 5.2**). Using this system, the optimization of culture

Table 5.2 Culture conditions of pigment production in integrated system

Plant cells	Red beet hairy roots (Chapter 1)
Medium	Phosphate-free MS medium (Chapter 2)
Culture operation	Culture with repeated processes of growth and pigment release (Chapter 4)
Reactor	Radial flow reactor (Chapter 5)
Calculation	Growth and pigment formation models (Chapter 3) Boundary conditions after pigment release (Chapter 4)

Initial values for calculation

X_0 = 1.0 kg-DW/m^3, P_0 = 0.70 mol/kg-DW, B_0 = 0.5x10^{-3} kg/kg-DW

Periodic interval = 100 h

Parameter values for calculation

Table 3.3, Table 4.3, k_d =2.6 x10^{-3} h^{-1} (Radial flow reactor)

operation for the enhancement of pigment production with RFR was tried by performing the simulation with the models described in section 4.3.3 (see Fig.4.11). The initial culture conditions used in the calculations were shown in Table 5.2 and a periodic interval was constant value of 100 h. The values of t_r and the number of release process (n_r) as the manipulated variables were adopted. **Figure 5.19** shows the simulation results regarding the pigment productivity (P_B) calculated by Eq.(5.11) as the performance index.

$$P_B = A_R / t_F \tag{5.11}$$

The variation in t_r had the large influence on P_B value and in the range

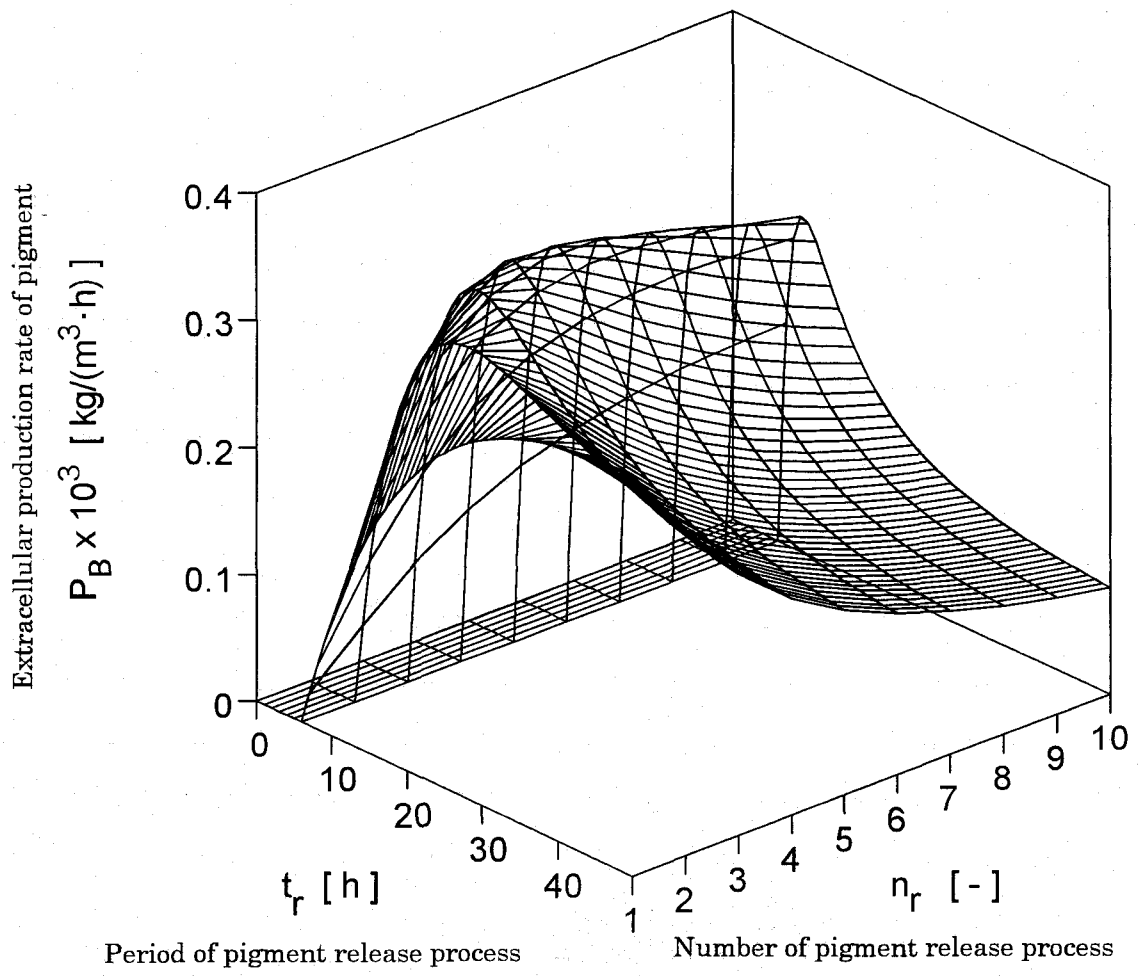


Fig.5.19 Simulation results of pigment productivity in culture of red beet hairy roots with repeated processes of growth and pigment release in RFR

of t_r = 10-20 h at each value of n_r, the curves passed through a maximum value of P_B. The value at t_r =13 h and n_r = 4 attained a maximum of 3.35 x 10^{-4} kg/(m^3•h) which was 29 times higher than the productivity in the repeated culture with MS medium performed in section 4.3.2 (P_B =1.13x10^{-5} kg/(m^3•h)).

The other manipulated variables such as periodic interval and amount of limiting substrate still exist and much more research is needed to realize pigment production by optimized periodic operations.

5.4 Summary

To construct a bioreactor for hairy root culture, the effect of liquid flow on the growth of red beet hairy roots was investigated with single column reactor where superficial velocity of liquid flow was changed. The optimal growth was performed at the superficial velocity of 15 m/h. By considering the morphological property of hairy roots, in the range of lower superficial velocity, the oxygen transfer rate between medium and hairy roots governed the growth because the elongation rate was decreased due to the declination of DO concentration at cell surface in comparison with that in bulk liquid. On the other hand, in the range of higher superficial velocity the hairy roots received shear stress and the declination in the number of growing points at tip part of hairy root occurred.

Based on these results, a novel bioreactor of radial flow reactor (RFR) was constructed and the culture of red beet hairy roots was performed. The declination of DO concentration in bulk liquid could be prevented in the high density culture with radial flow reactor and the high efficiency of oxygen supply in comparison with SCR was realized and the radial flow reactor was ensured to be suitable for hairy root culture.

Furthermore, using the results obtained from Chapter 1 to Chapter 5, including the selection of the cell line, the medium modification, the kinetic

analysis, the development of culture operation and the design of a novel bioreactor, the integration of the system for the red pigment production in the culture of red beet hairy roots was tried. The culture of red beet hairy roots with repeated processes of growth and pigment release in radial flow reactor was simulated using the kinetic models at the periodic interval of 100 h by varying the value of oxygen starvation time and periodic number. The maximum productivity of 3.35×10^{-4} kg/($m^3 \cdot h$) was calculated at 13 h-oxygen starvation and 4 times release process. This integrated system was valid for the pigment production.

General Conclusions

This theses deals with constructing a culture system suitable to the production of plant-derived chemicals by using plant hairy roots. By taking the bioprocess engineering approach, selection of cell lines, improvement of medium, development of culture operation for high density culture and construction of a bioreactor were conducted. To resolve some key problems related with engineering aspects in hairy root culture and to pursue a development of production process, various kinds of hairy roots were examined. Especially, in the case of utilization of red beet hairy roots, as a model species which produce betanin (red pigment), a consistent research was performed.

In Chapter 1, the preponderance of hairy roots used as materials for the chemical production was described as the basic study. Especially, pigment content in red beet hairy roots is comparable to those in various parts (root, stem and leaf), and the high efficiency of pigment production was revealed. While, the amount of main inorganic nutrients were estimated by measurement of electrical conductivity and pH in medium, assuming that the medium conductivity decrease is exclusively attributable to the consumption of potassium, ammonium and nitrate ions, and that plant cells do not secrete ionic products into medium. The calculated amounts of the ions were in fair agreement with the experimentally analyzed data during the cultures of various plant cells. The measurements of medium conductivity and pH made it possible to do the real-time monitoring of the amount of the main inorganic nutrients in these plant cell cultures.

In Chapter 2, for the improvement of culture conditions, the strategy for enhancement of productivity of desired metabolite was described by finding important medium components and modifying medium compositions in the cultures of various kinds of hairy roots. In particular, the effect of medium

modification on pigment productivity was shown in the culture of red beet hairy roots and phosphate was identified as an important constituent which stimulated pigment formation by red beet hairy roots. In the culture of madder hairy roots, carbon and nitrogen sources were important factors and the productivity of pigment was drastically enhanced in the culture using ammonium-free medium containing fructose as a carbon source. Moreover, the influence of ammonium ion concentration was also investigated in the hairy root cultures of red beet, carrot, horseradish and pak-bung and the growth of each hairy roots was inhibited by ammonium ion. While, the superoxide dismutase activity (desired product) of pak-bung hairy roots was enhanced by large ammonium addition to medium.

In Chapter 3, the kinetic descriptions of growth and pigment formation in various kinds of the hairy roots were described. In the kinetics of hairy root proliferation, the model based upon the linear extension and lateral branching of the GP at root tip made it possible to simulate and characterize the growth of various hairy roots successfully. Especially, in cultures of carrot hairy roots using various fermentors, growth behaviors were changed due to the extent of physical damage and these kinetics could be expressed by using the parameter of decay rate of growing points. Moreover, in the culture of red beet hairy roots, the kinetics of root proliferation was formulated using a Monod-type rate equation of root elongation, which includes intracellular phosphorus as a limiting substrate. A positional dependence of pigment content was observed along with the hairy roots; namely, the pigment content increased with increasing distance from root tips and gradually approached a saturated value correlated with intracellular phosphorus content. Based on these phenomena the kinetics of pigment formation of hairy roots was presented by considering the variation in pigment content along the roots and the intracellular phosphorus effect. And the resultant model also made it possible to describe the kinetic

behaviors of growth and pigmentation during hairy root culture in fermentor.

In Chapter 4, for the development of the culture operation for the effective production of pigment by red beet hairy roots, repeated cultures associated with operations of growth and pigment release were described. In the culture of red beet hairy roots, the red pigment was released from the cells into medium when the cells were subjected to the culture condition under oxygen starvation. The adsorption column with a hydrophobic resin, Sepabeads SP 207 (styrene-divinylbenzen copolymer), was incorporated in a bioreactor system and the long-term culture of the hairy root was carried out with the repeated operations of cell growth, and pigment release and recovery. In the culture with the oxygen starvation time of 16 h, the extracellular production rate of pigment was obtained at 1.1 $\times 10^{-5}$ kg/($m^3 \cdot h$) on the average. The repeated culture of madder hairy roots with root growth and pigment release treatment were also performed and an average production rate of released pigments of 3.0×10^{-5} kg/($m^3 \cdot h$) was achieved. The kinetic analysis in the culture of red beet hairy roots with repeated processes of the growth and pigment release was also conducted. The pigment release was caused by partial decay of hairy root cells due to declination of cell viability under anoxia condition. The kinetics of pigment release was evaluated by representing the kinetics of declination of cell viability as time response of first-order lag plus dead time. On the other hand, the regrowth kinetics after the treatment of oxygen starvation was represented by the decay of growing points of hairy roots. Thus, the culture with repeated processes of the growth and the pigment release was performed and the kinetic models was verified.

In Chapter 5, taking into account oxygen transfer between medium and hairy roots, development of a bioreactor and optimization of culture condition in integrated system were described. To construct a bioreactor for hairy root

culture, the effect of liquid flow on hairy root growth was investigated by conducting cultures of red beet hairy roots with single column reactor where superficial velocity of liquid flow could changed. The optimal growth was performed at superficial velocity of 15 m/h and the morphological property of hairy root growth could explain the variation in growth rate affected by liquid flows. Based on these results, the radial flow reactor (RFR) was constructed and the culture of red beet hairy roots was performed. The declination of DO concentration could be prevented in the high density culture with RFR and the high efficiency of oxygen supply was realized. The RFR was ensured to be suitable for hairy root culture. While, by integrating the results obtained from Chapter 1 to Chapter 5, the culture of red beet hairy roots with repeated processes of the growth and the pigment release in RFR was simulated using the kinetic models at the periodic interval of 100 h by varying the value of oxygen starvation time and periodic number. The maximum productivity of 3.35×10^{-5} kg/(m^3•h) was calculated at 13 h-oxygen starvation and 4 times release process and this integrated system was valid for the pigment production.

Suggestions for Future Work

To extend the findings obtained in this work, the following studies are recommended.

(1) Biological study

One of the important factors which will limit the possibility of economical production of chemicals from plant cell culture is the degree of achievement of high yield of products. In shikonin production by *Lithospermum erythrorhizon* its yield was attained at 0.15 kg/kg-DW. However, maximum yield of red pigment in red beet hairy roots was much lower than that of shikonin and the value was 0.03 kg/kg-DW. Therefore, improvements of activities in metabolic path ways and cell lines are required by progressing the technique in the aspect of genetic engineering, enzymatic engineering or pathway engineering.

(2) Bioprocess control

The real-time sensing in fermentor is important to control the culture conditions. Thus far the measurement methods of dissolved oxygen concentration, pH, pressure, electrical conductivity etc. have been established. In this work, the measurement method of concentration of medium compositions was also developed. However, there are many factors to be monitored. Especially, in the culture described in Chapter 4, the treatment of the oxygen starvation deactivated cells and enhanced the product release. In view of long-term culture, controlling the cell activity becomes very important factor and the technique for the measurement of cell activity is required.

(3) Scale-up and design of fermentor using computer simulation

The strategy for scale-up and design of fermentor suitable to hairy root culture have not been established because growth kinetics of hairy root is not perfectly expressed in view of following points. The growth of hairy roots has

the morphological property and hairy roots can not be applied to suspension culture. This means heterogeneous growth occurring in fermentor. Thus in view of growth kinetic analysis, the expression of behavior of cell mass concentration is not adequate and the hold-up of hairy roots in fermentor should be considered. Especially, in high density culture the space where hairy roots can grow will become growth factor because the excess pack of hairy roots will cause the growth inhibition such as steric hindrance. Moreover, the distribution of hold-up of hairy roots exists in fermentor because homogeneous inoculation can not be realized. Hairy root growth occurs in many parts where hairy roots are inoculated. This means that there are localized conditions in fermentor and the fluid condition become complicate.

Taking into account these phenomena, the analyses of growth kinetics should be established. If steric visualization (3D visualization) of hairy root growth in fermentor is carried out, the direction of scale-up and design of fermentor will be made easily by considering influence of liquid flow and capacity of nutrient supplement under heterogeneous condition.

Nomenclature

a	=	parameter of Freundilich adsorption isotherm	$[(m^3/kg)^{1/n_q}]$
a_H	=	parameter of redness in Hunter color	[-]
a_T	=	extent of enzymatic reduction in hairy roots	[mol/kg-DW]
A	=	tonoplast surface area per unit volume of vacuole	$[m^{-1}]$
A_B	=	amount of pigment in red beet hairy roots	$[kg/m^3]$
A_C	=	sectional area of growth column	$[m^2]$
A_M	=	amount of pigment in medium	$[kg/m^3]$
$A_{M,eq}$	=	concentration of pigment in medium at equilibrium	$[kg/m^3]$
$A_{M,0}$	=	concentration of pigment in medium at initial time	$[kg/m^3]$
A_N	=	amount of pigment in nonviable cell	$[kg/m^3]$
A_R	=	amount of extracellular pigment	$[kg/m^3]$
A_T	=	total amount of pigment	$[kg/m^3]$
A_V	=	amount of pigment in viable cell	$[kg/m^3]$
b_H	=	parameter of yellowness in Hunter color	[-]
B	=	average content of pigment in hairy roots	[kg/kg-DW]
$\hat{B}$	=	content of pigment in single cell	[kg/kg-DW]
C_B	=	average concentration of pigment in hairy root	$[kg/m^3]$
$\hat{C}_B$	=	average concentration of pigment in cells existing in differential length	$[kg/m^3]$

$\widetilde{C}_B$	=	local concentration of pigment in single cell	[kg/m^3]
C_N	=	nitrogen content of cell mass	[kg/kg-DW]
C_O	=	DO concentration in bulk liquid	[kg/m^3]
C_O^*	=	Equilibrium oxygen concentration in bubble	[kg/m^3]
C_{OS}	=	DO concentration at surface of hairy roots	[kg/m^3]
C_{O1}	=	DO concentration in bulk liquid at inlet of growth unit	[kg/m^3]
C_{O2}	=	DO concentration in bulk liquid at outlet of growth unit	[kg/m^3]
C_{O2}^{calc}	=	calculated DO concentration in bulk liquid at outlet of growth unit	[kg/m^3]
C_{O2}^{exp}	=	experimental DO concentration in bulk liquid at outlet of growth unit	[kg/m^3]
C_P	=	phosphorus concentration in medium	[mol/m^3]
D	=	diameter of hairy root	[m]
D_C	=	diameter of growth column	[m]
D_s	=	pigment diffusivity in tonoplast	[m^2/h]
f_{GP}	=	fraction of growth ability evaluated by number of GPs	[-]
f_T	=	cell viability	[-]
F	=	concentration of fructose in medium	[kg/m^3]
I	=	a given ion species	
j_s	=	pigment flux through tonoplast	[kg/(m^2•h)]

k	=	apparent permeation rate constant	[h^{-1}]
k_d	=	decay rate constant of growing point	[h^{-1}]
$k_{GL}a_G$	=	volumetric mass transfer coefficient of oxygen between gas and liquid on reactor volume basis	[h^{-1}]
k_L	=	dead time	[h]
$k_{LS}a_S$	=	volumetric mass transfer coefficient of oxygen between liquid and solid on reactor volume basis	[h^{-1}]
k_R	=	rate constant of pigment release	[h^{-1}]
K	=	experimental constant	[$(mol/kg\text{-}DW)^{-n}$]
K_A	=	dissociation constant for ammonium	[$kmol/m^3$]
K_O	=	oxygen saturation constant	[kg/m^3]
K_P	=	phosphorus saturation constant	[mol/kg-DW]
K_{P2}	=	second dissociation constant for phosphate	[$kmol/m^3$]
K_T	=	partition coefficient	[-]
K_W	=	ionization constant for water	[$kmol^2/m^6$]
l	=	distance from tip of hairy root	[m]
L	=	length of hairy root	[m]
L_B	=	average length between branches	[m]
L_G	=	length of growing point	[m]
L_H	=	parameter of lightness in Hunter color	[-]
m	=	number of decayed GPs	[-]

M	=	amount of pigment in hairy root	[kg]
$\hat{M}$	=	amount of pigment in single cell	[kg]
n	=	number of branching	[-]
n_q	=	parameter in Freundlich adsorption isotherm	[-]
n_r	=	number of pigment release process	[-]
n_S	=	experimental constant	[-]
N	=	number of GPs	[-]
N_{LS}	=	oxygen transfer rate between liquid and solid on reactor volume basis	[kg/(m^3•h)]
P	=	average content of phosphorus in hairy roots	[mol/kg-DW]
P_b	=	content of minimum phosphorus in hairy roots	[mol/kg-DW]
P_B	=	productivity of pigment	[kg/(m^3•h)]
P_f	=	average content of free phosphorus in hairy roots	[mol/kg-DW]
q	=	amount of pigment adsorbed on resin at equilibrium	[kg/kg-resin]
Q	=	oxygen uptake rate of hairy roots	[kg/(kg-DW•h)]
Q^{app}	=	apparent oxygen uptake rate in SCR	[kg/(kg-DW•h)]
Q_m	=	maximum oxygen uptake rate of hairy roots	[kg/(kg-DW•h)]
S	=	sugar concentration in medium	[kg/m^3]
t	=	culture time	[h]
t_r	=	treatment time of pigment release process	[h]

u	=	velocity of liquid fluid in growth unit	[m/h]
u_S	=	superficial velocity of liquid fluid in growth unit	[m/h]
V	=	culture volume	[m^3]
V_H	=	volume of hairy roots in growth column	[m^3]
V_r	=	volume of water in resin	[m^3]
W_A	=	weight of adsorbent	[kg]
W_C	=	water content of hairy roots	[-]
X	=	concentration of hairy roots	[kg-DW/m^3]
X_N	=	concentration of nonviable cells	[kg-DW/m^3]
X_V	=	concentration of viable cells	[kg-DW/m^3]
Y_P	=	root mass yield based on intracellular phosphorus	[kg-DW/mol]
Y_S	=	root mass yield based on sugar	[kg-DW/kg]
Z	=	height	[m]
Z_H	=	height of hairy roots packed in column	[m]
[ ]	=	concentration of species in brackets	[mol/m^3]
α	=	volume of vacuole per unit volume of single cell	[m^3-vacuole/m^3-cell]
β	=	empirical coefficient	[kg-DW/(S•m^2)]
γ_0	=	parameter value	[mol/m^3]
δ	=	thickness of tonoplast	[m]
ΔP	=	pressure drop in cell growth column	[Pa]

ΔX	=	differential concentration of hairy roots	[kg-DW/m^3]
ΔZ	=	differential height of hairy roots in cell growth column	[m]
$\Delta\kappa$	=	differential electrical conductivity of medium	[S/m]
ε	=	voidage in growth unit	[-]
φ	=	empirical coefficient	[-]
μ	=	specific elongation rate	[h^{-1}]
μ_{Pm}	=	maximum specific elongation rate in case of sugar	[h^{-1}]
μ_{Sm}	=	maximum specific elongation rate in case of phosphorus	[h^{-1}]
ρ	=	density of hairy root on fresh weight basis	[kg-fresh cells/m^3]
θ	=	cellular age	[h]
τ	=	time constant	[h]
κ	=	electrical conductivity of medium	[S/m]
λ	=	molar ionic conductivity	[S•m^2/mol]
λ'	=	modified molar ionic conductivity	[S•m^2/mol]

<subscripts>

AM	=	ammonium ion
b	=	minimum value
F	=	value at final time
H	=	hydrogen ion
i	=	a given ion species
I	=	value at initial time
m	=	maximum value

n = value at n-th branching

NI = nitrate ion

OH = hydroxide ion

PO = potassium ion

$P1$ = dihydrogenphosphate ion

$P2$ = hydrogenphosphate ion

S = saturated value

0 = value at zero time

1 = value at the initial of pigment release process

2 = value during pigment release process

3 = value at the end of pigment release process

∞ = infinite dilution

<superscript>

C = value in cytoplasm

TC = value at cytoplasm side on tonoplast

TV = value at vacuole side on tonoplast

V = value in vacuole

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