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Continuous production of scopolamine by a culture of *Duboisia leichhardtii* hairy root clone in a bioreactor system

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Abstract. The scopolamine-releasing hairy root clone DL47-1 of *Duboisia leichhardtii* was cultured in an Amberlite XAD-2 column-combined bioreactor system for continuous production of scopolamine. The medium used was continuously exchanged during culture to maintain the electrical conductivity of the medium constant. After culturing the hairy roots in the system for 11 weeks, 0.5 g/l of scopolamine was obtained in the column. When the roots were cultured in the reactor system containing polyurethane foam or stainless-steel mesh to support the hairy roots, scopolamine recovery was increased. Thereafter, a two-stage culture, the first stage in the medium for hairy root growth and the second stage in the medium for scopolamine release, was carried out in this system by using a turbine-blade reactor with stainless-steel mesh as a support. Under these conditions, 1.3 g/l of scopolamine was recovered during 11 weeks of culture in the medium for scopolamine release. This bioreactor system seems applicable for the production of various plant metabolites by cultures of hairy roots.

Introduction

Culturing of *Agrobacterium rhizogenes*-induced hairy roots has been carried out for the production of secondary metabolites such as alkaloids, steroids and terpenoids (reviewed by Signs and Flores 1990; Hashimoto and Yamada 1991). So far, several workers have proposed bioreactor configurations suitable for hairy-root culture. Hilton et al. (1988) reported that when the hairy roots of *Datura stramonium* were cultured in a stirred-tank reactor, the root tissues were damaged by the impeller. Thereafter, a turbine-blade reactor and a modified stirred reactor were used for growth of the hairy roots of *Daucus carota* (Kondo et al. 1989) and *Datura stramonium* (Hilton and Rhodes 1990). In addition, Taya et al. (1989) found that an air-lift column reactor with polyurethane foam to support the root tissues was suitable for the culture of the hairy roots of *Armoracia rusticana* (horseradish). Also, Taya et al. (1989) reported that there was a linear relationship between the biomass of the hairy roots and the amount of decrease in the medium conductivity. So, the measurement of medium electrical conductivity seemed to be useful for on-line monitoring of growth of the root tissues in a reactor.

One approach to the production of secondary metabolites by hairy root culture in a bioreactor system is to recover the products released from the hairy roots into culture media. Wilson et al. (1987) reported that constant removal of nicotine from the culture medium in a 1.5-l column reactor led to a fourfold increase in the total production of nicotine by a culture of *Nicotiana rustica* hairy roots. Hilton and Rhodes (1990) reported that when the hairy roots of *D. stramonium* were cultured in a 14-l modified stirred tank reactor, hyoscyamine was released into the medium to the extent of 3.6% of the total amount. Recently, several attempts have been made to recover continuously compounds released from hairy roots into media. Peroxidase produced by a culture of horseradish hairy roots was efficiently adsorbed onto a hydrophobic adsorbent resin (Kato et al. 1991). Shikonin released from hairy roots of *Lithospermum erythrorhizon* (Shimomura et al. 1991), and betanin released from hairy roots of *Beta vulgaris* (red beet) (Kino-oka et al. 1992) were also recovered in the same way.

As reported previously (Muranaka et al. 1992), we have established scopolamine-releasing hairy root clones of *Duboisia leichhardtii*. Scopolamine was released into a medium by the culture of a scopolamine-releasing clone in a reactor system, and the scopolamine in the medium was recovered by using an Amberlite XAD-2 column. In this paper, we describe the culture of the scopolamine-releasing hairy root clone of *D. leichhardtii* in a bioreactor system under medium exchange conditions with and without a support for the

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hairy root growth. A two-stage culture, the first stage for growth of the hairy roots and the second stage for release of scopolamine, was also examiend in this system.

Materials and methods

Hairy root cultures

The hairy root clone DL47-1 of *D. leichhardtii* used was cultured as reported previously (Muranaka et al. 1992). The roots were subcultured in a liquid HF medium (Mano et al. 1989) every 4 weeks.

Approximately 300 mg fresh weight of the roots were inoculated into 30 ml liquid medium in a 125-ml polycarbonate flask, and then incubated at 25° C in the dark on a model G10 gyratory shaker (New Brunswick Scientific, N.J., USA) at 85 rpm. Roots grown for 3 weeks were transferred aseptically into a reactor.

Culture system

A bioreactor system consisting of a reactor (1 or 3 l) and a column (50 or 100 ml) packed with Amberlite XAD-2 was constructed for continuous production of scopolamine by a culture of the hairy root clone DL47-1 as shown in Fig. 1.

Reactors and culture media. Two types of reactor were used; a 3-l air-lift reactor (Shibata Hario, Tokyo, Japan) and a 1-l turbine-blade reactor (Model TBR-2, Sakura Seiki, Tokyo, Japan). The air-lift reactor was filled with 3 l of HF-3 medium (Muranaka et al. 1992) and inoculated with 30 g fresh weight of the hairy roots. Influent air was passed through a 0.2-μm hydrophobic membrane filter (Nihon Millipore, Tokyo, Japan) and blown into the medium at a rate of 1.5 l/min by means of a glass filter at the bottom of the vessel. The turbine-blade reactor was filled with 1 l of HF medium and inoculated with 10 g fresh weight of the hairy roots. An impeller with eight turbine blades was controlled at 50 rpm. The reactor is separated into the part for growing hairy roots and that of the turbine blades. Influent air was passed through a 0.2-μm hydrophobic membrane filter and introduced into an agitation space. During culture in HF medium, cell density was estimated by decrease in the electrical conductivity of the culture medium as described by Taya et al. (1989). With both reactors, the temperature was maintained at 25° C and pH was monitored every 1 to 3 days. After cultivation, the hairy roots were harvested and measured for fresh weight gravimetrically.

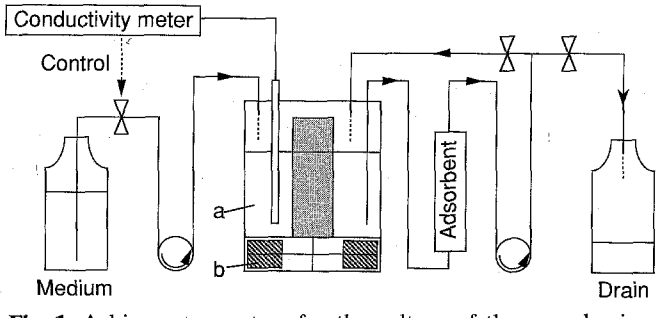

Fig. 1. A bioreactor system for the culture of the scopolamine-releasing hairy root clone. The system was constructed by the combination of a 3-l air-lift or a 1-l turbine-blade reactor (this figure) and a column packed with Amberlite XAD-2. A reactor was connected with a column by means of a silicone tube. A portion of culture medium was passed through a column and eluent was returned into the reactor continuously using a low pressure pump. Medium conductivity was measured by using a dip-type conductivity cell, and then the medium was exchanged to hold the medium conductivity constant. With the turbine-blade reactor, the part for growing hairy roots (*a*) and that of the turbine blades (*b*) was separate

Scopolamine production. Scopolamine produced from the hairy roots into the medium was continuously adsorbed onto a column containing 50 ml or 100 ml of Amberlite XAD-2 by passing through the culture medium at flow volume SV 5 with a low pressure pump. The column was removed every week and eluted with a mixture of methanol and 28% NH_4OH to recover scopolamine. The column was washed with distilled water, autoclaved and then reconnected to the reactor.

Controlled medium exchange (CME). Medium electrical conductivity was measured by using a dip-type conductivity cell (Shibata Hario, Tokyo, Japan) and monitored with a digital conductivity meter (Toa Electronics, Tokyo, Japan). A portion of medium was removed every week, and the same volume of fresh fivefold concentrated HF-3 medium [(x5)HF-3] was supplied into the vessel to maintain the initial conductivity of HF-3 medium.

Use of a support for hairy root growth. A polyurethane foam (diameter, 20 cm; length, 10 cm) was put into the air-lift reactor prior to culture. Also, a stainless-steel mesh (diameter, 3 cm; length, 15 cm) was attached to the centre of the turbine-blade reactor.

Two-stage culture. The hairy roots were cultured in HF medium for rapid growth in the first stage, and then cultured in HF-3 medium for scopolamine release in the second stage.

Analysis of scopolamine

Scopolamine recovered from an Amberlite XAD-2 column was analysed by HPLC as reported previously (Muranaka et al. 1992).

Results and discussion

Hairy root culture under CME conditions

The hairy root clone DL47-1 of *D. leichhardtii* (30 g fresh weight) was inoculated into the air-lift reactor with 3 l of HF-3 medium and cultured for 11 weeks under CME conditions to maintain the initial medium conductivity (Fig. 2). During the first 10 weeks, the medium conductivity was held at the initial value of 7.9 mS/cm. The biomass of the roots increased eight times during culture. The scopolamine produced increased linearly during weeks 2 to 7: 0.5 g/l of scopolamine was recovered during 11 weeks of culture.

During culture the root tissues floated in the vessel following air flow and aggregated tightly. After 8 weeks of culture, the aggregated root tissue seemed to become autolysed because of O_2 deficiency, and reduced the rate of scopolamine production (Fig. 2).

Use of a support for hairy-root growth

For uniform growth of the hairy roots, polyurethane foam was put into the vessel. The roots were cultured

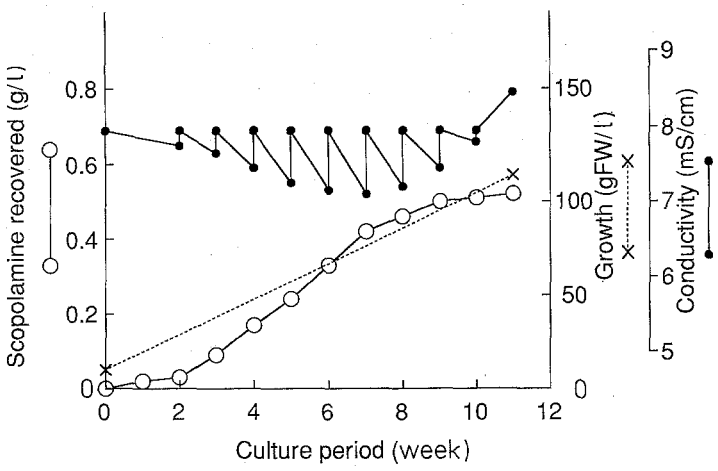

Fig. 2. Scopolamine production by a culture of the scopolamine-releasing hairy root clone under controlled medium exchange (CME) conditions in an air-lift reactor. A sample of 30-g fresh weight (FW) of hairy roots was inoculated into a 3-l air-lift reactor

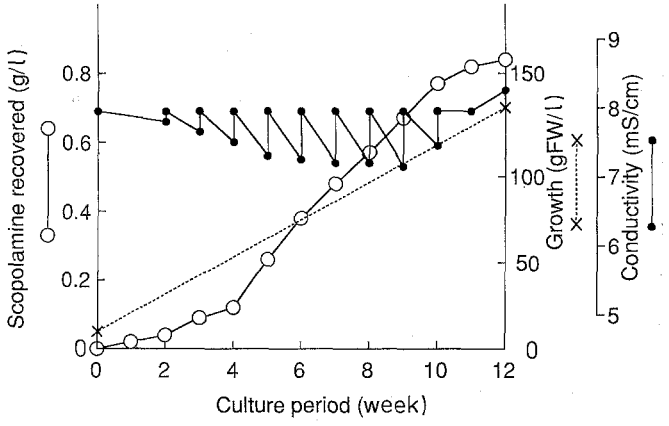

Fig. 3. Scopolamine production by a culture of the scopolamine-releasing hairy root clone under CME conditions in an air-lift reactor with polyurethane foam as a support for hairy root growth. A sample of 30-g fresh weight (FW) of the hairy roots was inoculated in a 3-l air-lift reactor

for 12 weeks under CME conditions to maintain the initial medium conductivity (Fig. 3). The root tissue grew well and spread uniformly onto the polyurethane foam in the vessel. The biomass of the roots increased 14 times and 0.8 g/l of scopolamine was recovered during culture.

Two-stage culture

Although HF-3 medium was suited for scopolamine production and release into the medium, HF medium was suited for root growth (Mano et al. 1989). So, a two-stage culture consisting of the first stage for hairy root growth and the second stage for scopolamine release was carried out in the turbine-blade reactor with a stainless-steel mesh as a support for uniform growth

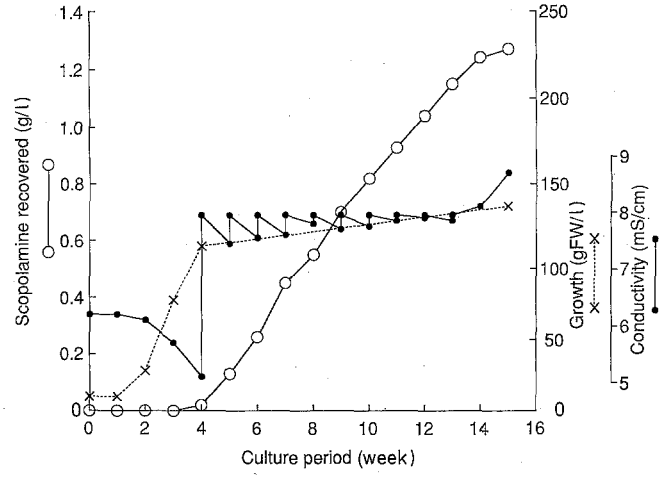

Fig. 4. Scopolamine production by a culture of the scopolamine-releasing hairy root clone in a turbine-blade reactor with stainless-steel mesh as a support for the hairy root growth in two-stage culture. A 10 g fresh weight (FW) sample of the hairy roots was inoculated into a 1-l turbine-blade reactor. The hairy roots cultured in HF medium for 4 weeks, then changed to HF-3 medium and cultured for an additional 11 weeks under CME conditions

of the root tissues. Clone DL47-1 was cultured in HF medium for the first 4 weeks and then in HF-3 medium for the additional 11 weeks under CME conditions to maintain the conductivity constant (Fig. 4).

The hairy roots increased 12-fold during 4 weeks culture in HF medium and were then cultured in HF-3 medium. The medium conductivity was maintained at the initial value of 7.9. The root tissues increased 1.2-fold during the additional 11 weeks in HF-3 medium. The density of the hairy roots in the vessel was 2.4 g fresh weight/l (the yield of the hairy roots was 1.4 g fresh weight, and the cultivation space of the vessel was 600 ml). This value was comparable to those reported previously (Kondo et al. 1989; Hilton and Rhodes 1990; Uozumi et al. 1992). Although scopolamine was hardly released into HF medium, scopolamine released into the HF-3 medium increased linearly for about 2 months: 1.3 g/l of scopolamine was recovered during 11 weeks of culture in HF-3 medium.

Table 1 summarizes the above results. Each fermentation was finished when the medium conductivity was out of control (Figs. 2–4). At that time, growth of the hairy roots almost stopped (data not shown), perhaps because the O_2 supply was insufficient for further root growth. To compare the scopolamine production of each experiment, the value of scopolamine recovered from the medium during the 11 weeks of culture in HF-3 medium was used. When the hairy root clone DL47-1 was cultured in the air-lift reactor under CME conditions, the scopolamine production was 0.5 g/l and the culture period was 11 weeks. The culture period was twofold longer, and both growth index and scopolamine production were 2.5-fold higher than those of the culture under conditions without medium exchange (Muranaka et al. 1992). When polyurethane foam was used as a support for hairy root growth, the amount of

Table 1. Continuous production of scopolamine by cultures of *Duboisia leichhardtii* hairy root clone in a column-combined reactor system

Reactor	Condition	Culture scale (l)	Medium (weeks)	Growth index[a]	Scopolamine recovered[b] (g/l)
Air-lift	CME	3	HF-3 (11)	11	0.5
Air-lift	CME+S	3	HF-3 (12)	14	0.8
Turbine-blade	CME+S/ 2-stage	1	HF (4)/ HF-3 (11)	12/ 1.2	1.3

CME, controlled medium exchange; S, use of support; 2-stage, two-stage culture

[a] The harvest fresh weight per inoculum fresh weight

[b] Scopolamine recovered from the medium during the 11 weeks of culture in HF-3 medium

scopolamine produced was increased. The scopolamine recovered as 0.8 g/l during first 11 weeks of culture. When the hairy root was cultured in the turbine-blade reactor under the combined conditions (CME, use of stainless-steel mesh as a support and two-stage culture), the scopolamine recovered was 1.3 g/l during 11 weeks of culture in HF-3 medium. The scopolamine content in the hairy roots after each fermentation was less than 0.001% fresh weight, although the initial content was about 0.05% fresh weight (data not shown). Thus, more than 99%, by rough estimation, of the scopolamine produced by the hairy roots during culture was recovered from the medium.

The exchange of a portion of the culture medium to hold constant the medium ion concentration also continuously supplied nutrients such as nitrate, phosphate and potassium needed for root growth. In addition, the nitrate concentration seemed to be maintained at a suitable level for scopolamine release, as reported previously (Muranaka et al. 1992). Uozumi et al. (1992) also reported that replacement of culture medium with fresh medium containing 50 mM $CaCl_2$ increased the amount of peroxidase release into the medium by a culture of horseradish hairy roots.

The use of polyurethane foam in the air-lift reactor and stainless-steel mesh in the turbine-blade reactor increased the growth of the hairy roots and their uniform spread in the vessel. Figure 5 shows photographs of the hairy roots growth in a 1-l turbine-blade reactor for 15 weeks. Under the culture conditions, it appears that a high level of O_2 was maintained and that the biomass of hairy roots was increased, as reported by Kondo et al. (1989).

Fujita et al. (1981) reported that a two-stage culture was useful for production of shikonin by a culture of *L. erythrorhizon* cells. In the present study, the two-stage culture accompanied by the combination of CME and the use of a support for the hairy root growth was also suitable for the continuous production of scopolamine by a culture of hairy roots in a turbine-blade reactor. This bioreactor system seems useful for the production of various plant metabolites by cultures of hairy roots,

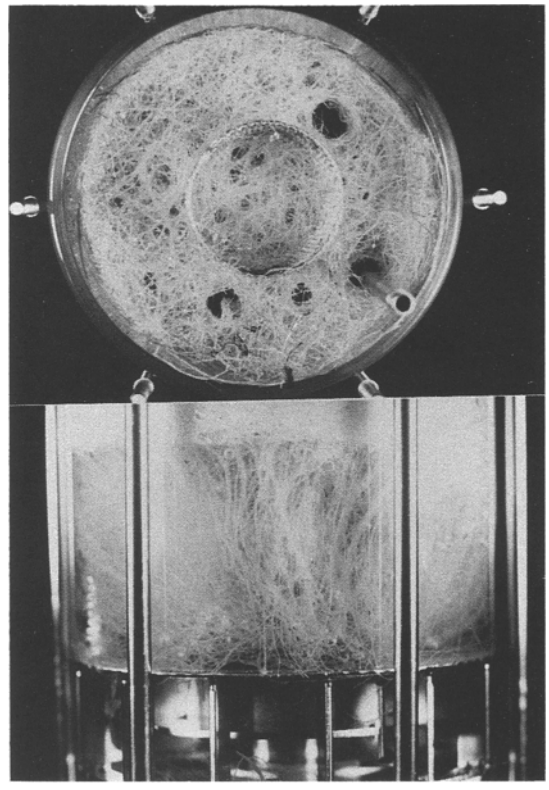

Fig. 5. Photographs of *Duboisia leichhardtii* hairy roots grown in a 1-l turbine-blade reactor for 15 weeks

and also to be applicable to bioconversion systems using hairy roots as a "catalyst".

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