Journal of Microbiology and Biotechnology
Vol. 2, No. 1, 41-45, 1992

Production of Shikonin by A Hairy Root Culture of *Lithospermum erythrorhizon*

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Received 17 January 1992 / Accepted 3 March 1992

Shikonin production was examined in a bubble column bioreactor system with the hairy roots of *Lithosphermum erythrorhizon*. The volumetric productivity was higher than those obtained from other reactor configurations with free or immobilized cells of the same cell line. The productivities of the bubble column reactor, with and without a product absorption trap, were 7.4 and 4.5 mg of shikonin/l/d, respectively. This indicated the importance of the product removal in the design and operation of the shikonin production system with hairy root culture.

Many attempts have been made to produce plant secondary metabolites by tissue or organ cultures of plants. Generally the cultures of undifferentiated cells have low yields, and are often unstable during the production of secondary metabolites (2, 3). In addition, mass cultivation is difficult because of their high sensitivity to the shear generated to facilitate the mass transfer in a bioreactor system (15). Hairy root culture could be considered as an alternative to obviate these problems in the production of the organ specific metabolites.

Hairy roots, induced by the infection of a plant pathogen, *Agrobacterium rhizogenes*, can be cultivated in phytohormone-free media, and grow much faster than normal roots (5). They also produce the same metabolites as their parent plants do. There have been many studies on the production of plant secondary metabolites including alkaloids (5, 7), and oligosaccharides (8) using hairy root cultures. They produced the same or higher levels of the secondary metabolites compared to the cultivated plant or suspension culture of the cells.

Shikonin has been reported as the most successful example of the mass production of plant secondary metabolites by cell suspension culture. A two-stage suspension culture system was developed on an industrial scale with a highly productive shikonin producing *Lithospermum erythrorhizon* cell line (14). Recently we have reported a successful use of polyurethane foam matrices as an immobilization support for *L. erythrorhizon* cells (11). As an effort to improve the system productivity, the possibility of using hairy roots of *L. erythrorhizon* was also tested.

In this paper, an establishment of a hairy root clone of *L. erythrorhizon*, medium optimization for shikonin production, and the use of bubble column type bioreactor with and without a product adsorption trap is reported.

MATERIALS AND METHODS

Bacterial Strain and Plant Materials

The bacterial strain used in the present study was *Agrobacterium rhizogenes* A4. The strain was cultivated on potato dextrose agar medium at 25℃. Seeds of *L. erythrorhizon* were sterilised with 1% sodium hypochlorite solution and aseptically germinated on Murashige and Skoog (MS) basal salt agar medium (10). They were then grown in a growth chamber for 4 weeks at 25℃, with photoperiods of 12 h and the light intensity of 2500 lux. The one month old plant was then used as a material for the induction of hairy roots.

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Key words: Shikonin production, *Lithospermum erythrorhizon* hairy root culture

Culture Media

Basal media of MS (10) and Schenk and Hildebrandt (SH) (13) were used for the induction of hairy root and the growth of hairy root clones, respectively. MS medium containing carbenicillin (500 mg/l) was designated as MSC medium. All media contained 30 g/l sucrose and pH was adjusted to 5.8 with 0.1 N NaOH before autoclaving. Hairy root clones were cultivated in a gyratory shaking incubator at 25℃ and 80 rpm in the dark.

Induction and Establishment of Hairy Root Clones

Stems of the one month old plant were cut into small sections of about one centimeter long and placed vertically on MS agar medium. They were then inoculated with *A. rhizogenes* A4 (10^8 cells/ml) using a loop at the cut end of the explant. The infected stem segments were incubated for hairy root induction in a growth chamber at 25℃, with photoperiod of 12 h and 1200 lux, for 4 to 6 weeks.

The adventitious root tips formed on the cut end of the stem were excised and placed on MSC agar medium to eliminate *Agrobacterium* contamination at 25℃ in the dark for a week. After four to five transfers, hairy root clones without the bacterial contamination were established and subcultured in MS liquid medium once a month.

Bioreactor Systems for Hairy Root Culture

Schematic diagrams of the bubble column type bioreactor, with and without a product adsorption trap, used in this study are described in Fig. 1. The adsorption trap for shikonin was constructed with a pyrex glass column (4×8 cm) filled with polyurethane foam cubes. The foam cubes were prepared as described in the previous paper (11).

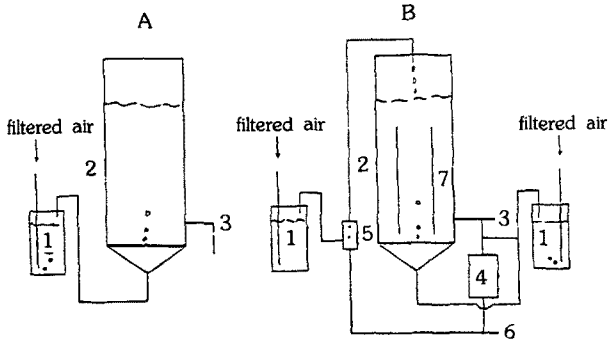

Fig. 1. Schematic diagrams of the bioreactor used in the present study.

A: bubble column, B: bubble column with a product adsorption trap, 1: humid chamber, 2: bubble column, 3: sampling port, 4: product adsorption column, 5: aerator, 6: product extraction port, 7: stainless steel mesh

A stainless steel mesh was installed to retain the hairy roots inside the reactor. A preliminary experiment showed that the existence of the mesh did not affect on hairy root growth and shikonin production. The culture broth was aerated by supplying filtered air at a fixed rate of 0.1 vvm through the sintered glass filter (pore size: about 40 μm). In the system(B) with an adsorption trap, the liquid medium was forced to circulate by an extra aeration in a range of 1~2 vvm. Temperature was maintained at 25℃ by circulating constant temperature water through the jacket.

Analytical Methods

After separating the hairy roots from the culture medium, fresh cell weight(FCW) was measured and then dry cell weight (DCW) was determined by drying the samples in an oven at 95℃ to a constant weight. Cell growth in the bioreactor was indirectly estimated by the conductivity measurement technique (4). Shikonin was extracted with chloroform and its concentration was determined by the method of Mizukami *et al.* (9). Sucrose concentration was determined by DNS (1) method after invertase treatment. Opine analysis was performed by the method of paper electrophoresis as described by Petit *et al.* (12).

RESULTS AND DISCUSSION

Establishment of Hairy Root Clones

Hairy roots of *L. erythrorhizon* were effectively induced by the infection with *A. rhizogenes* A4. Incubation for 7 d under the experimental conditions resulted in tumorous tissues on the cut end of the stem explant. Adventitious roots were formed from the tumorous tissue after 4 to 5 weeks (Fig. 2A).

The adventitious roots about one centimeter long were cut and placed on MSC agar medium to eliminate the possible bacterial contamination. After 4 to 5 transfers on fresh MSC medium, twenty hairy root clones were obtained. Among these, the hairy root clone No. 9, HR9, was selected because it showed fairly good growth in the MS medium.

HR9 showed a highly branching morphology and also exhibited agravitropism of the hairy root phenotype (6) (Fig. 2B). Since mannopine And agropine were detected in the opine analysis of HR9 (data not shown), it could be confirmed that HR9 was a transformant of root-inducing (Ri) plasmid of *A. rhizogenes* A4.

Hairy Root Growth and Shikonin Production in SH Medium

When the SH medium was used, it was necessary to adjust the salt concentrations for root growth and shikonin production. Since SH medium contained a va-

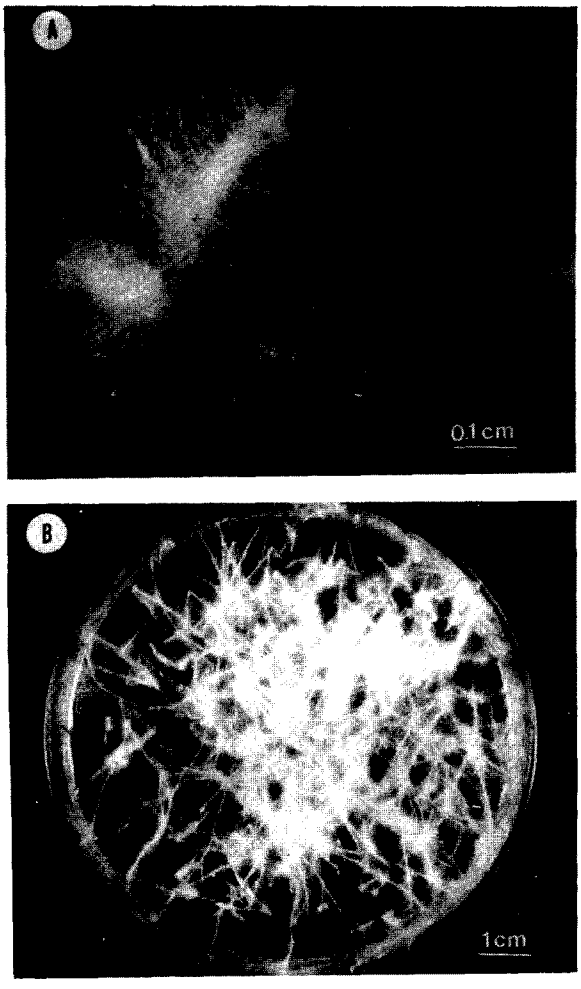

Fig. 2. Hairy root induction(A) and morphology of the hairy root clone, HR9, in SH agar medium(B).

riety of salts at different concentrations, the optimization of each salt concentration was rather laborious. To simplify the optimization process, therefore, we tested diluted concentration of the medium. SH medium was diluted to two and three-fold, and root cultures were carried out in the diluted media (Table 1). Although the root growth was favored at high salt concentration, shikonin production was significantly imporved at diluted salt concentration. Three-fold diluted SH medium (1/3 SH medium) was thus adopted as the medium for shikonin production although it might be considered suboptimal.

The 1/3 SH medium was further optimized in terms of other key nutrient components including surcrose, potassium nitrate, ammonium phosphate and cupric surlfate. The resulting modified SH medium contained 40 g/l sucrose, 25 g/l potassium nitrate, 100 mg/l ammonium phosphate and 0.25 mg/l cupric sulfate.

A series of flask culture experiments using the hairy roots were conducted with 250 ml Erlenmeyer flasks containing 40 ml of the modified SH medium. A typical root growth and final shikonin concentration reached were 57 g^{FCW}/l and 320 mg/l, respectively, in 24 d.

The growth of hairy roots could be well estimated indirectly in the whole growth phase by measuring the conductivity of the medium which showed a very good linearity up to the conductivity of 3 mmho to 0.6 mmho. In the modified SH medium a decrease of 1 mmho conductivity corresponded to an increase in the cell concentration of 47 g^{FCW}/l.

Shikonin Production in a Bubble Column Bioreactor

After inoculating the hairy roots, the bubble column type bioreactor (Fig. 1A) containing one liter of the modified SH medium was operated at 25℃ under the dark condition and at an aeration rate of 0.1 vvm. A typical operational result is shown in Fig. 3. The cell growth estimated on the basis of conductivity decrease and shikonin production were 54 g^{FCW}/l and and 109 mg/l, respectively. The final cell weight estimated was in good agreement with the actual measurement (57 g^{FCW}/l) after 24 d of the operation. It could be noted that the production rate of shikonin in bubble column bioreactor was lower than that obtained in the flask culture. It is not clear at the moment what kind of physical or chemical factors may affect the productivities in each system. Further investigation should be followed to elucidate the system behavior of hairy root cultures in more detail.

Enhanced Shikonin Production with an Adsorption Trap

A microscopic observation of the cultivated hairy roots

Table 1. Effects of concentrations of SH medium on the growth of hairy root and shikonin production

Medium conc**	Final FCW(Xf) g/40 ml	Final DCW(Xf) g/40 ml	Growth index* (Xf/Xi)	Final shikonin conc. (mg/l)		
				medium	root	total
1/1	1.201	0.091	45.6	1	2.5	3.5
1/2	1.441	0.108	54.2	11.9	6.9	18.8
1/3	0.906	0.067	33.6	19.9	11.7	31.6

*Initial FCW(Xi): 0.027 g/40 ml (DCW: 0.002 g/40 ml), 30 d culture
**The SH medium was diluted by addition of distilled water

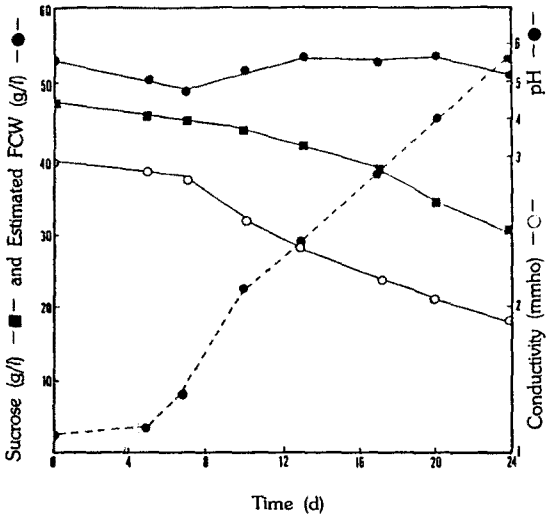

Fig. 3. Time course of hairy root growth in bubble column reactor.
final fresh cell mass: 56 g/l, final shikonin concentration: 109 mg/l after 24 d.

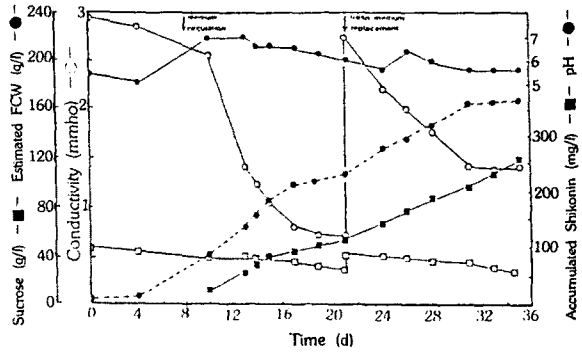

Fig. 4. Time course of hairy root growth and shikonin production in bubble column reactor with product adsorption trap/
final FCW: 167 g/l, accumulated shikonin: 258 mg/l after 35 d, FCW: estimated FCW, shikonin: accmulated shikonin.

reveals that shikonin is located in the outer layer of root and root hair. As the hairy root grows, a portion of shikonin produced is released into the medium: about 50% of total shikonin exists in the cell debris-attached form and also as shikonin droplets (unpublished data). This outer compartmentation of shikonin is considered to be related with the polarity of the root epidermis. Therefore, it is considered reasonable that the productivity can be improved by continuous removal of released shikonin from the medium, if it reduces the product inhibition.

An adsorption trap containing polyurethane foam cubes was therefore employed as shown in Fig. 1(B). In the reactor system, the medium was recirulated to the reactor bed after it passed through the adsorption trap. Shikonin in the medium was irreversibly adsorbed to polyurethane matrix in the adsorption trap, resulting in practically clear culture broth in the bubble column reactor. Shikonin was intermittently extracted from the adsorption trap by using chloroform as an extractant. Hydrophobic interaction is considered to be the main adsorption mechanism of shikonin onto polyurethane as discussed in the previous paper (11).

The result of operation in the bioreactor with one liter of the modified SH medium is shown in Fig. 4. The liquid medium was circulated through the aerator at a rate of 300 ml/min after 8 d of reactor operation. Conductivity of the medium decreased rapidly with time and levelled off to 0.7 mmho after 19 d. After 21 d of the operation the medium was replaced with a fresh one and the reactor operation was continued. After 35 d of the consecutive operation, a final harvested cell mass and total accumulated shikonin production were 167 g^{FCW}/l and 258 mg/l, respectively.

The result was compared to the other free or immobilized cell systems (Table 2). A significant improvement in shikonin production could be obtained by using the

Table 2. Comparisons of the hairy root culture with other production systems

	Cell suspension culture**	Immobilized cell culture with PU foam**	Hairy root culture w/o adsorption trap	Hairy root culture with adsorption trap
Final DCW (g/l)	14	12.48	2.8	8.35
Final shikonin cnc. (mg/l)	negligible	112	109	258
Culture time (d)	14	20	24	35
μmax (d^{-1})	0.068	0.085	0.112	0.180
Volumetric productivity (mg/l/d)*	negligible	5.6	4.5	7.4

*Productivity based on the reactor working volume
**Refer to the previous report (ref. 11).

external adsorption trap. The volumetric productivity was 7.4 mg/l/day, a 1.3-fold increase over that from the immobilized cell system (11). Another advantage of the present hairy root culture system over the immobilized cell system is that a continuous production of shikonin over a prolonged period is possible by replacing the used culture medium with fresh one; this is not possible in the immobilized cell system (11), where the cell growth and product adsorption simultaneously occur inside the polyurethane foam matrices. In the present system, hairy roots can be further propagated if the nutrients are supplied continuously and the product concentration in the bulk medium is kept low by using the external product adsorption trap. It was reported in a previous paper (11) that a single stage culture with immobilized cells could yield higher productivity than a two-stage suspension culture system. More interestingly, the present hairy root culture system can offter higher productivity than the immobilized cell system. It is believed that the enhanced product formation of the hairy roots is due to the alleviation of product inhibition effect by a *in situ* product removal.

In conclusion, it is possible to achieve a continuous production of shikonin for a prolonged period by hairy root culture in a bubble column bioreactor system. Further improvements should of course be possible if a hairy root cell line of higher productivity is employed.

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