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PRODUCTION OF CRYPTOTANSHINONE AND FERRUGINOL BY IMMOBILIZED CULTURED CELLS OF *SALVIA MILTIORRHIZA*

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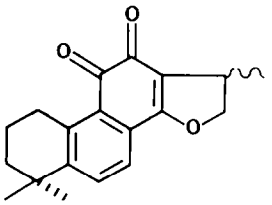
Key Word Index—*Salvia miltiorrhiza*; Labiatae; immobilized plant cells; diterpene; cryptotanshinone; ferruginol.

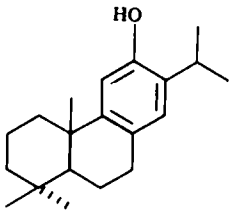
Abstract—The production of the diterpenes cryptotanshinone and ferruginol by immobilized cultured cells of *Salvia miltiorrhiza* was examined. Cryptotanshinone and ferruginol were produced continuously by the immobilized cells. Much of the cryptotanshinone was released into the medium, while most of the ferruginol was retained in the cells. The production of cryptotanshinone and ferruginol by the immobilized cells was about 39% and 61% of those by cell suspensions. Re-use of the immobilized cells for the production of these compounds was also examined.

INTRODUCTION

Recently there has been much interest in the use of immobilized cultured plant cells for the production or biotransformations of secondary metabolites [1–6]. Previously we reported [7] a two-stage culture method, with normal medium for growth and then medium without Fe-EDTA for the production of cryptotanshinone (**1**) and ferruginol (**2**). Since cell growth was significantly suppressed in the production medium, we thought that this culture method might be used for the production of these compounds by immobilized cells. In this study, we investigated the production of **1** and **2** by immobilized cultured cells of *S. miltiorrhiza*.



Cryptotanshinone (**1**)



Ferruginol (**2**)

RESULTS AND DISCUSSION

A cryptotanshinone (**1**) producing cell line (line A5) [7] was maintained by subculture [7] in MS medium containing 2,4-D(0.1 ppm) and kinetin (0.1 ppm) at 20 day intervals. Cells from these stock suspension cultures in the early stationary phase of growth were used for all experiments. The cells were grown in the growth medium as free cell suspensions, immobilized and then inoculated into the production medium [7], which was MS medium without Fe-EDTA but with kinetin (1 ppm).

The effect of the size of cell aggregates on the production of **1** and **2** was examined in free cell suspension culture. The cells in a stock suspension culture were sieved through nylon cloths (10, 20, 40 and 80 mesh) and the cell aggregates with size ranges of 0–420 μm, 420–840 μm and 840–1600 μm were transferred to the production medium. The cell aggregates of the different size ranges showed no significant differences in their production of **1** (Table 1), but since aggregates in the size range of 840–1600 μm showed a slightly higher production of **1**, a clinically active principle in this plant, and the cultures were mainly composed of cell aggregates in this size range, we immobilized aggregates of this size for production of **1** and **2**.

For immobilization, cells from stock suspension cultures were collected on nylon cloth (20 mesh), washed with sterile water and entrapped on calcium alginate beads (mean diameter 4 mm). About 0.6 mg (dry weight) of cells were entrapped per bead. We inoculated 300 beads into 60 ml of production medium (five beads/ml), and took samples of 25 beads and 5 ml of medium at 5 day intervals for analysis. Since cell division was almost completely suppressed in the production medium [7], the cells did not leak from the beads into the medium. We determined the contents (μg/bead) of **1** and **2** in the cells and the gel, and also calculated the amounts of **1** and **2** released into the medium per bead. Figure 1 shows the time-course of the production of **1** (Fig. 1a) and **2** (Fig. 1b) by the immobilized cells. As with free cells [7], the total content of **1** increased continuously until about day 20. About 74% of **1** was released into the medium and only 17% and 9% were retained in the cells and the gel, respectively, by day 25. The total content of **2** also increased continuously until about day 20, but unlike **1**, about 47% was retained in the cells and only 38% was

Table 1. Effect of the size range of cell aggregates of inoculum on the productions of cryptotanshinone and ferruginol by free suspension cultures of *S. miltiorrhiza*

Size range of cell aggregates of inoculum (μm)	Diterpene production (mg/g dry wt of inoculum)	
	Cryptotanshinone	Ferruginol
0–420	23.1	62.1
420–840	18.6	42.6
840–1600	24.8	51.3

Determinations were carried out 20 days after transfer of the cells to the production medium (medium without Fe-EDTA but with kinetin, 1 ppm). The initial dry wt of cells was 4.1 g/l.

Table 2. Production of cryptotanshinone and ferruginol by free and immobilized *S. miltiorrhiza* cells

		Diterpene production (mg/g dry wt of inoculum)	
		Free cells	Immobilized cells
Cryptotanshinone	In cells	17.3	1.5
	In gel	—	0.9
	In medium	5.9	6.6
	Total	23.2	9.0
Ferruginol	In cells	13.2	5.3
	In gel	—	1.3
	In medium	4.7	4.4
	Total	17.9	11.0

Determinations were carried out 20 days (free cells) and 25 days (immobilized cells) after transfer of the cells to production medium (medium without Fe-EDTA but with 1 ppm of kinetin).

released into the medium by day 25. We cultured part of the cells as a free cell suspension and compared their production of **1** and **2** (mg/g dry weight of inoculum) with those of immobilized cells (Table 2). The immobilized cells and the free cells were compared when they showed maximal yields (days 25 and 20, respectively). The total production of **1** and **2** by immobilized cells was about 39% and 61% of those by the free cells. But about 74% of **1** was released into the medium by the immobilized cells, whereas only 25% was released by free cells, indicating the possibility of re-use of the immobilized cells for the production of **1**. A similar 'release effect' was reported for alginate-entrapped cultured cells of *Catharanthus roseus* by Brodelius *et al.* [1], who ascribed this 'release effect' to the permeabilization of the cells by a trace amount of chloroform present in the reaction mixture. Increased release of L-DOPA by alginate-entrapped cultured cells of *Mucuna pruriens* was also reported [2]. However, the mechanism of this effect is unknown.

Next we examined the re-use of immobilized cells for the production of **1** and **2**. We inoculated 270 beads into 54 ml of production medium (5 beads/ml), renewed the medium at 10-day (Fig. 2a) or 15-day (Fig. 2b) intervals and took samples of 25 beads for determination of **1** and **2** at the end of each passage. The density of beads was kept at 5 beads/ml throughout the experiment. Of the total **1**, 90% in the samples taken at 10-day intervals and 95% in those taken at 15-day intervals was also released into the medium, whereas most of the **2** was retained in the cells and only a little was released. Figure 3 shows the 'total production' of **1** and **2** by the immobilized cells during each passage. 'Total production' means the amounts of **1** and **2** newly produced per bead during each passage, and is defined as follows. Total production (μg/bead) = [Final content in the cells, gel and medium] − [Initial content in the cells and gel].

When the medium was renewed at 10-day intervals (Fig. 3a), the 'total production' of **1** in the second passage was about 44% of that in the first passage and only 2.5% in the sixth passage. When the medium was renewed at 15-day intervals, the 'total production' of **1** was again reduced significantly in the second passage (Fig. 3b). The immobilized cells retained considerably high production of **2** in the second passage, but its production decreased rapidly on the third passage.

After culture for 60 days, we transferred the beads to growth medium to examine the viability of the entrapped cells. The cells divided actively and leaked from the beads

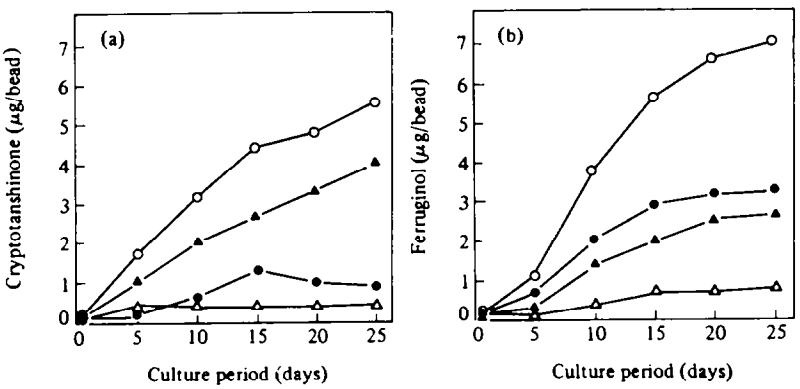


Fig. 1. Increases in cryptotanshinone (a) and ferruginol (b) contents in immobilized cultured cells of *S. miltiorrhiza*. Immobilized cells were cultured in production medium (medium without Fe-EDTA but with 1 ppm of kinetin). The content in medium is the amount released per bead into the medium. ○, Total (in the cells, gel and medium); ●, cells; △, gel; ▲, medium.

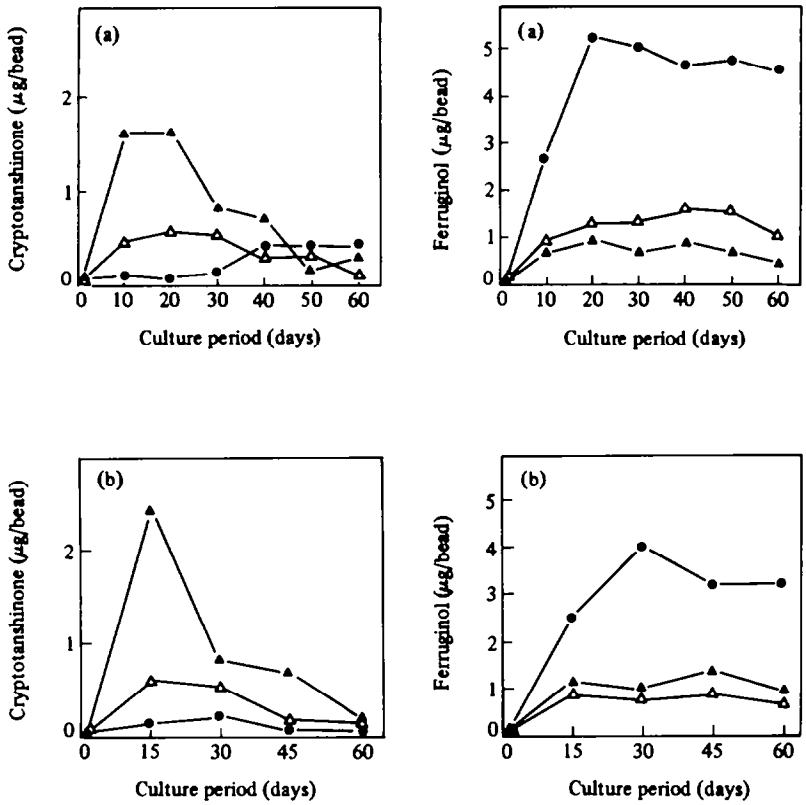


Fig. 2. Changes in cryptotanshinone and ferruginol content in immobilized cultured cells of *S. miltiorrhiza*. The immobilized cells (5 beads/ml) were inoculated into production medium (medium without Fe-EDTA but with 1 ppm of kinetin). The medium was renewed at 10-day (a) or 15-day (b) intervals. The content in medium is the amount released per bead into the medium. ●, Cells; △, gel; ▲, medium.

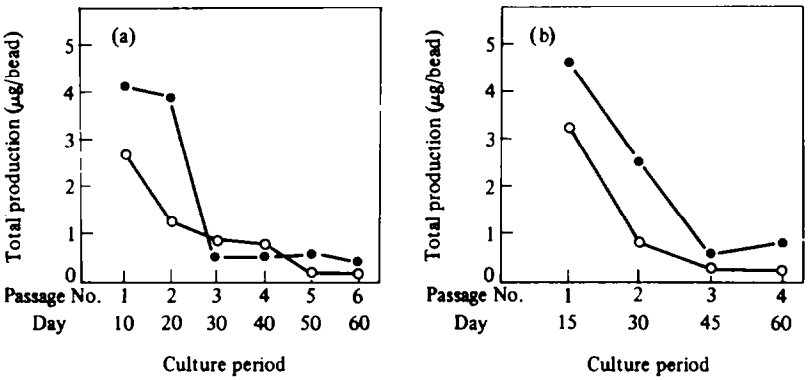


Fig. 3. Changes in 'total production' by immobilized cultured cells of *S. miltiorrhiza*. Immobilized cells (5 beads/ml) were cultured in production medium (medium without Fe-EDTA but with 1 ppm of kinetin), and the medium was renewed at 10-day (a) or 15-day (b) intervals. 'Total production' is the total amount of cryptotanshinone (○) or ferruginol (●) newly produced during each passage.

into the medium, indicating the high viability of the immobilized cells. Thus the decreases in 'total production' of **1** and **2** by immobilized cells during successive passages may be due to negative feedback effects of accumulated lipophilic metabolites (e.g. **2**) rather than to death of immobilized cells. Hence, if a method can be developed by which these lipophilic metabolites can be released from the cells by membrane permeabilization [3] or by con-

tinuous extraction of the metabolites from the medium [1], the immobilized cells should produce **1** and **2** in larger quantities on successive passages.

EXPERIMENTAL

Plant material and method of culture. Cultures of a cell line (line A5) selected as described in the previous paper [7] were used. All stock suspension cultures were grown on a rotary shaker (100 rev/min) at 25° in the dark in Murashige–Skoog (MS) medium [8] containing 2,4-D (0.1 ppm) and kinetin (0.1 ppm).

Preparation of immobilized cells. Immobilized cells were prepared by the alginate method, as follows. Cells were suspended in 3% Na alginate and then the suspension was added dropwise to 0.1 M $CaCl_2$ soln. The Ca alginate beads were collected with nylon cloth (20 mesh), washed with sterile H_2O and cultured in production medium [7] on a rotary shaker (100 rev/min) at 25° in the dark.

Extraction and determinations of cryptotanshinone (**1**) *and ferruginol* (**2**). For determination of the cell contents of **1** and **2**, the beads were solubilized in 1 M KPi buffer (pH 7) and the cells collected on a tetrafluoroethylene polymer filter (10 μm pore size; Polyfrone paper PF-2, Toyo Kagaku Sangyo Co., Osaka), dried at 80° for 2 hr and extracted with $CHCl_3$ in a Soxhlet for 3 hr. The filtrate was also extracted with EtOAc for determination of the contents of **1** and **2** in the gel. The contents of **1** and **2** were determined by GC as described elsewhere [9].

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