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Enhanced Production of Shikonin by Using Polyurethane-entrapped *Lithospermum erythrorhizon* Cells

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Polyurethane Foam에 포괄시킨 *Lithospermum erythrorhizon* 세포에 의한 Shikonin 생산

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Production of shikonin derivatives by *Lithospermum erythrorhizon* cells by using polyurethane foam was investigated. Shikonin derivatives were effectively adsorbed mostly by phase distribution to polyurethane matrices and their production increased significantly compared to the suspension culture. The enhanced production of shikonin was probably due to more facilitated cell to cell contact and lowered intracellular shikonin concentration, both of which are known to be favorable for plant secondary metabolite production. In order to improve the process productivity, cell culture was conducted under various culture conditions: Of them, Schenk and Hildebrandt medium containing indole-3-acetic acid (1.75 mg/l) and kinetin (0.1 mg/l) was considered most appropriate for shikonin production. Production of shikonin increased about 4.5 times in the Schenk and Hildebrandt medium containing indole-3-acetic acid (1.75 mg/l) and kinetin (0.1 mg/l) when compared to the same medium containing *p*-chlorophenoxyacetic acid (2.0 mg/l) and kinetin (0.1 mg/l). When polyurethane was used as the support material, a single-stage system was more preferred to the conventional two-stage culture system in terms of shikonin productivity.

Introduction

Since 1970's, there have been lots of attempts to use plant cells as a source to produce various biologically active substances. Plant cell culture technology has been rapidly developed, and a commerical production of plant secondary metabolites, shikonin, was first established in Japan (1).

Immobilization techniques have also been successfully applied to plant cell culture technology (2, 3). Entrapment of growing plant cells within inert polysaccharides (4) and their adsorption to porous polymer materials (5) are the typical examples. Plant cells were protected by immobilization from external environmental factor such as shear stress, and cellular activity itself could be further enhanced by more facilitated cell to cell contact. Ease of scale up and improvement in the productivity of the bioreactor system are the other merits to be considered.

In this study, production of shikonin derivatives by *Lithospermum erythrorhizon* cell with polyurethane foam was investigated. We herein report the results that shikonin was effectively adsorbed onto polyurethane and its production in-

Key words: *Lithospermum erythrorhizon*, shikonin, polyurethane foam-entrapped cell culture, cell to cell contact, one stage culture, two stage culture
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creased significantly compared to the normal suspension culture of the same cell line.

Materials and Methods

Cell line and culture media

Cell line used in this study was *Lithospermum erythrorhizon* KCTC PCL 52001 which was obtained from Korean Collections for Type Cultures, at Genetic Engineering Center, KIST . Basal media were Schenk and Hildebrandt medium (6) and M-9 (7). Schenk and Hildebrandt medium containing 2 mg/*l* of *p*-chlorophenoxyacetic acid and 0.1 mg/*l* of kinetin was designated as the SHND medium; whereas, Schenk and Hildebrandt medium containing 1.75 mg/*l* of indole-3-acetic acid and 0.1 mg/*l* of kinetin was designated as the SHIK medium. The pH of the media was adjusted to 5.8 before sterilization. The cell line was maintained by regular subculture in SHND liquid or agar medium every eight days.

Cultivation methods

Cells were cultivated in 250 ml Erlenmeyer flask containing 40 m*l* medium in a gyratory shaking incubator at 25°C and 100 rpm under darkness. After 9 days of the cultivation, 10% (v/v) of the precultured cells were added to the specified culture medium in a single-stage culture: In a two-stage culture, 25% (v/v) of the precultured cells, separated by using stainless steel mesh (pore size: 30 μm) from the first stage, were added to the second stage containing a specified culture medium. Polyurethane foam, which was cut into cube forms of size 0.5×0.5×0.5 cm, was added to the medium such that its weight constituted 1% (w/v) of the culture broth.

Analytical methods

After separating the cultured cells by using stainless steel mesh, fresh cell weight (FCW) was measured and then dry cell weight (DCW) was determined by drying the sample in an oven at 95°C to a constant weight. Sugar concentration was determined by DNS method (8). Shikonin was extracted with chloroform and its content was determined by the method of Mizukami *et al* (9).

Polyurethane foam (Sam Kwang Polyurethane Co., Seoul, Korea) was washed with ethanol and distilled water and then dried. A sheet of dried polyurethane foam was cut into cube forms with the lateral length 0.5 cm.

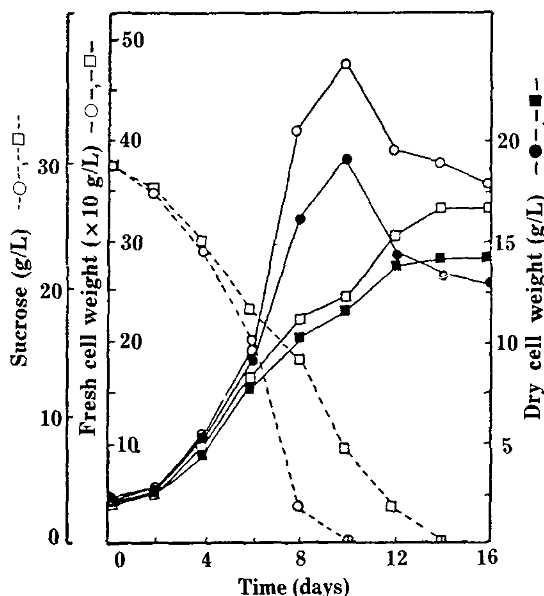

Fig. 1. Time courses of sucrose consumption and cell growth in SHND and SHIK medium.
(-○-, -●-: SHND medium; -□-, -■- : SHIK medium)

Results and Discussion

Suspension culture

When *L. erythrorhizon* cells were cultivated in a flask, sucrose consumption and accompanied cell growth were shown in Fig. 1. In SHND medium, cell growth reached up to 18.85g DCW/*l* (453g FCW/*l*) after 10 days and sucrose was almost completely consumed at this point. In SHIK medium, cell growth was observed to be slightly delayed and reached up to 14g DCW/*l* (330g FCW/*l*) after 14 days. Diminishing trend of cell mass thereafter was considered due to the autolysis of cells. Similiar observations were reported in the other plant cell culture system (10, 11).

Growth of cell mass is closely related to the consumption of sucrose in the medium. Growth yield based on the sucrose consumption was found to be 0.54g DCW/g sucrose and 0.47g DCW/g sucrose in SHND and SHIK medium, respectively. Maximum specific growth rates calculated from the semilogarithmic plots of cell mass versus time were 0.291/day and 0.286/day in SHND and SHIK

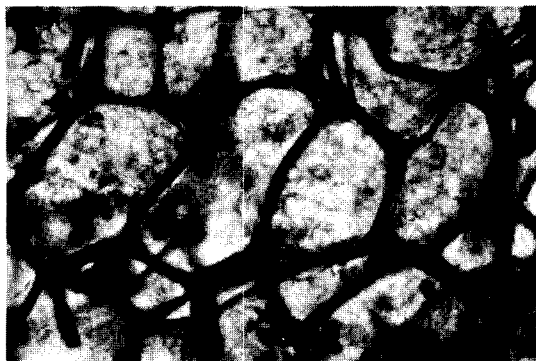

Fig. 2. Cell growth in polyurethane foam pore in SHND medium after 12days (200X).

medium, respectively. Therefore, for the growth of *L. erythrorhizon* cells, SHND medium containing *p*-chlorophenoxyacetic acid was found more effective than the SHIK medium which contains indole-3-acetic acid.

Cultivation with polyurethane foam

Production of shikonin in a single-stage culture: The cells precultured in SHND medium were cultivated in the same medium in the presence of polyurethane foam. It was observed with time that the cells were entrapped in the pores of polyurethane foam (Fig. 2) and proliferated with an active induction of shikonin (Fig. 3A). In this case, cell growth was observed to be delayed to some extent compared to the suspension culture (Fig. 1); cell mass reached up to 17g DCW/*l* after 12 days. In the suspension culture, however, shikonin was not induced at all, while when polyurethane foam was used in the medium, peculiar color of shikonin began to appear after 8days and 25 mg/*l* of shikonin was accumulated within 20 days.

Increased production of plant secondary metabolites has been reported with immoblilized growing plant cells (12). In the present study, we have observed that the production of shikonin increased significantly by culture with polyurethane foam comparing to the suspension culture in the same medium. The increase was considered probably due to the enhanced cell to cell contact by confining the cells to polyurethane pores and compartmentation of intracellular shikonin to polyurethane site. Both effects are considered favorable for the production of plant secondary metabolites. Adsorption of shikonin to polyurethane matrices also lowers the chance of decomposition, and should result in an improved recovery yield.

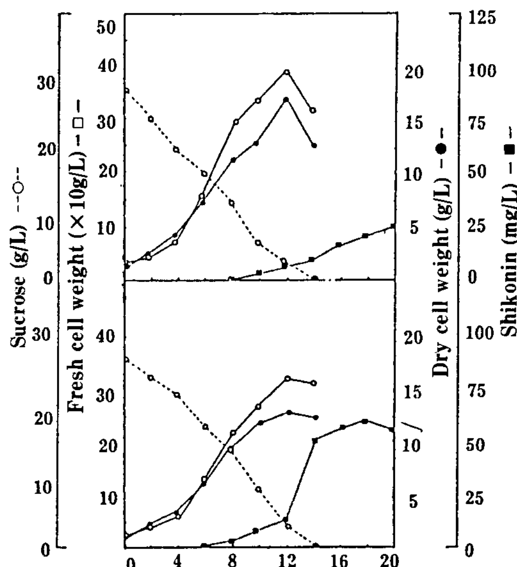

Fig. 3. Cell growth and shikonin production in SHND medium (A), and SHIK medium (B) with polyurethane foam.
(preculture in SHND medium)

Further increased production of shikonin was observed when the cells were cultured in SHIK medium with polyurethane foam (Fig. 3B). Cell mass reached up to 12 g DCW/*l* after 12 days and the peculiar color of shikonin could be observed as early as 6 days and 57 mg/*l* of shikonin was finally produced after 18 days. Although a reduced cell growth was observed in this case, shikonin production increased more than two times. This is probably due to the fact that indole-3-acetic acid is more favorable for shikonin production than p-chlorophenoxyacetic acid. Mamoru *et al* (13) reported that the synthetic auxin, 2,4-D (2,4-dichlorophenoxyacetic acid) is inhibitory to shikonin production. Therefore *p*-chlorophenoxyacetic acid, which is a derivative of 2,4-D, is also considered to inhibit the shikonin production.

Production of shikonin was even more improved when the cells were precultured and then cultivated with polyurethane foam in SHIK medium. The time courses of shikonin production and cell growth were shown in Fig. 4. Cell mass reached up to 12g DCW/*l* after 12 days. The peculiar color of shikonin began to be observed at the very first days and 112 mg/*l*

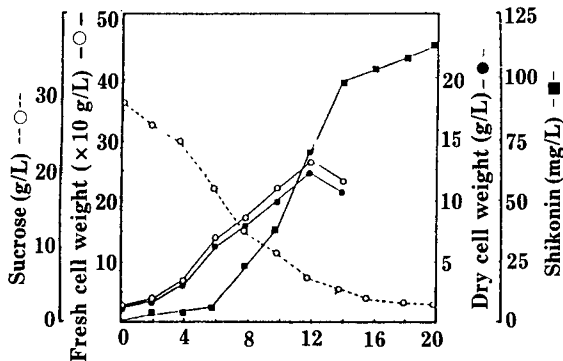

Fig. 4. Cell growth and shikonin production in SHIK medium with polyurethane foam.
(preculture in SHIK medium)

Table 1. Comparison of parameters related to biomass growth, substrate consumption, and shikonin production.

Parameter \ Culture condition	Suspension culture		Culture with Polyurethane foam		
	SHND →SHND	SHND →SHIK	SHND →SHND	SHND →SHIK	SHIK →SHIK
Final cell mass (g DCW/l)	18.85	14.	17.45	12.63	12.48
Final shikonin conc. (mg/l)	0.0	0.0	25.	55.	112.
μ (1/day)	0.106	0.068	0.087	0.083	0.085
q_s (g/g cell/day)	0.182	0.167	0.128	0.186	0.168
q_p (mg/g cell/day)	0.0	0.0	0.072	0.218	0.449

of shikonin was produced after 20 days. When compared with the cultures which are grown in SHND-SHND medium system, shikonin production increased more than 4.5 times. The results obtained from the series of the experiment were summerized in Table 1.

Production of shikonin in a two-stage culture: It has been reported that shikonin production is commercially carried out in a two-stage batch culture (1); cells were propagated in a large quantity in the first stage and then shikonin was produced with an appropriate medium in the second stage. The conventional two stage culture technique has been again applied in the present study with polyurethane foam. After precultured in SHND or SHIK medium, cells were harvested and transferred to M-9 medium con-

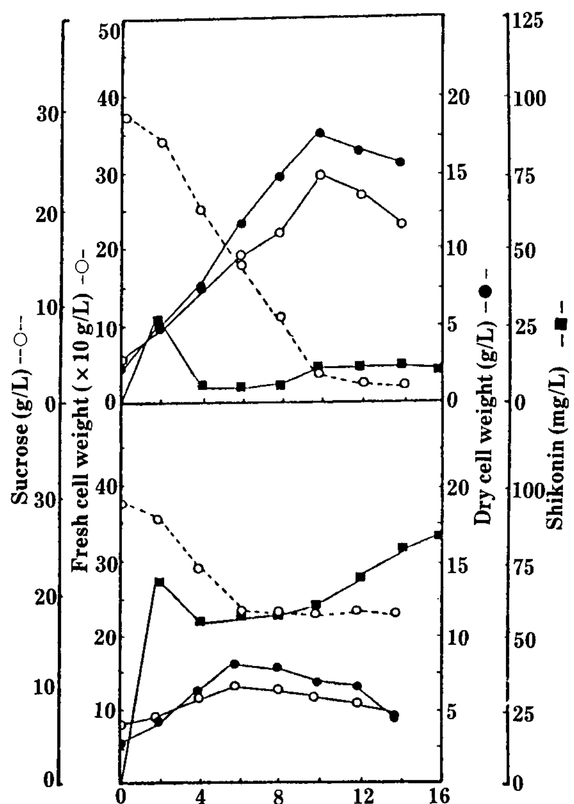

Fig. 5. Time courses of cell growth and shikonin production in the second stage M-9 medium with polyurethane foam.
(First stage cell growth in SHND medium (A) and SHIK medium (B))

taining polyurethane foam, and the results were shown in Fig. 5(A and B). The peculiar color of shikonin was observed from polyurethane matrices in a few hours. After two days shikonin production decreased and then again increased slowly with time. It is not clear at the present point why the concentration of shikonin decreases in the early fermentation phase, it is probably due to the decomposition of shikonin accompanied by autolysis of the cells.

From these results, it can be noted that the single stage culture (SHIK-SHIK medium system: 112 mg shikonin/l/20 days, Fig. 4) was more effective for shikonin production than the two stage culture (SHIK medium to M-9 medium system: 77.5 mg shikonin/l/23 days, Fig. 5B). This is considered due to the fact that effective induction of shikonin is supported by slow cell growth and the enhanced productivity in SHIK medium system.

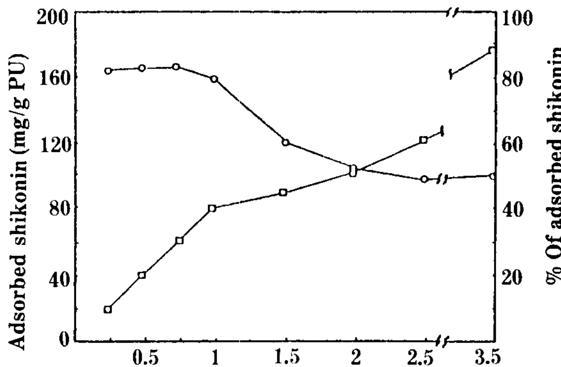

Fig. 6. Adsorption of shikonin on polyurethane foam. (After shikonin was dispersed in distilled water, it was adsorbed to polyurethane foam by shaking in 250 m*l* flasks containing 0.4 g polyurethane/40 m*l* shikonin soln. On a gyratory shaking incubator at 25°C, 100 rpm for 48 hours.)

Adsorption of shikonin to polyurethane

Adsorption of shikonin to polyurethane matrices was tested. The patterns of shikonin adsorption to polyurethane matrices were shown in Fig. 6. The adsorption of shikonin to polyurethane was proportional to the shikonin concentration of the solution up to the concentration of 1.0 g/m*l*, but with an increase of shikonin concentration the adsorbed amount decreased and the percent adsorption reached an equilibrium about 50% at the concentrations higher than 2 mg/m*l*. In view of this result, it was thought that phase distribution is the major adsorption mechanism of shikonin to polyurethane matrices.

Conclusion

In this study, we have observed that the cells of *Lithospermum erythrorhizon* produced shikonin in a higher yield by culture with polyurethane foam than the conventional suspension culture system. Shikonin was effectively adsorbed to polyurethane matrices and its *in situ* extraction was believed economically quite feasible. Decomposition of shikonin was also considered to be reduced significantly by its compartmentation to the polyurethane site. However, most of the improved production of shikonin was considered due to more facilitated cell to cell contact and lowered intracelluar shikonin concentration, both of which are known to be favorable for plant secondary metabolite production.

In a trial to improve the process productivity, we have found that a single-stage culture (SHIK-SHIK medium system) was more effective for shikonin production than the well-known two-stage culture (in this study, SHIK-M-9 system). It was probably due to the fact that effective induction of shikonin was supported by slow cell growth and further enhanced cellular productivity in the SHIK medium system.

요 약

L. erythrorhizon 세포를 polyurethane foam과 함께 증식시킬 경우 shikonin 유도체가 polyurethane에 효과적으로 흡착됨과 동시에 polyurethane을 사용하지 않은 경우와 비교하여 shikonin 생산량이 현저히 증가하였다. 이같은 증가는 세포를 polyurethane pore에 고정하여 증식시킴으로써 원활한 세포간 접촉을 유지하고 세포 내에 shikonin 농도를 저하시켜 shikonin 생성에 좋은 조건을 제공함에 기인한 것으로 생각되었다. 공정의 생산성을 높이기 위하여 여러가지 배양시스템이 검토되었는데, indole-3-acetic acid(1.75 mg/*l*)와 kinetin(0.1 mg/*l*)을 함유하는 Schenk-Hildebrandt 배지(SHIK 배지) 시스템이 가장 효과적이었다. *p*-Chlorophenoxyacetic acid (2.0 mg/*l*)와 kinetin(0.1 mg/*l*)를 함유하는 Schenk-Hildebrandt 배지(SHND 배지) 시스템에 비교하여 SHIK 배지 시스템에서 Shikonin 생성량은 약 4.5배 증가하였다. Polyurethane을 세포를 고정화하는 지지체로 사용할 경우에는 현재 행하여지고 있는 2단계 배양보다 1단계 배양이 더욱 효과적이며 경제적으로도 매우 유리할 것으로 판단되었다.

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