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Bioreactor Cultures of *Lithospermum erythrorhizon* for Shikonin Production with *In Situ* Extraction

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동시 추출을 겸한 생물반응기에서 *Lithospermum erythrorhizon* 배양에 의한 shikonin 생산

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Plant cell cultures of *Lithospermum erythrorhizon* were performed in stirred tank and packed-bed reactors with *in situ* extraction by *n*-hexadecane. The specific shikonin production and volumetric shikonin productivity of stirred tank reactor reached 1.5 mg shikonin/g cell and 400 μg shikonin/(L.day), respectively. In packed-bed reactor with calcium alginate-immobilized cells specific shikonin production and volumetric productivity reached 2.0 mg shikonin/g cell and 2857 μg shikonin/(L.day), which were 1.3 and 7.1 times higher than those of stirred tank reactor, respectively. The higher shikonin production and productivity of packed-bed reactor seemed to be due to high cell loading capacity of calcium alginate immobilized cells in packed-bed reactor and improved cell-cell contact.

Higher plants represents a valuable resources for a great variety of specialty biochemicals including pharmaceuticals, flavors, fragrances, pigments, and other fine chemicals. Currently, most of these chemicals are produced by cultivation of whole plant, followed by extraction and purification of the desired chemicals. Recent progress in plant cell and tissue culture techniques have provided an altenative to whole plant cultivation for prodution of plant-derived chemicals. But successful exploitation of these culture techniques requires large scale cultivation in novel bioreactors providing optimal conditions for cell growth and production of the desired products.

Large scale cultivation of plant cells is hampered by the fragile nature of the cells, low growth rates, complex systems of cellular metabolic pathways. Nevertheles, the large scale cultivation (20 k*l*) of *N. tabacum* (1) and the successful industrial production of shikonin have been achived.

The successful exploitation of plant cell culture technology requires an understanding of the cellular information (physical, biochemical, and genetic factors affecting metabolite production) an reactor design variables (concerning bioreactor performances including mixing, oxygen transfer, and rheological characteristics of cultured cells). Advantages and disadvantages of various types of reactors such as stirred tank, bubble column, air lift, rotating drum, and a few specialized reactors for immobilized cells have been discussed in many articles (1-14).

The major advantages of plant cell immobilization are that it provides high cell concentration, better cell-cell contact, and more favorable conditions for cell

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differentiation (7). It also eliminates cell wash-out in a continuous system and protects shear stress (7). Plant cells require only a small amount of oxygen for their metabolism when compared to aerobic microorganism. Thus oxygen limitation is less severe in immobilized plant cells.

The product of interest should be released from the immobilized cells into a medium so it can be removed from the culture vessel while the cells remain. However, many kinds of valuable plant secondary metabolites are rarely released from the cells in suspended or immobilized cell cultures, and they have very low solubility in water.

In order to remove the desired products from the cells variations in pH, the use of permeabilizing agents (15-17), and *in situ* extraction (18-20) or adsorption (21-23) have been tried. Among the above techniques *in situ* products removal (extraction or adsorption) has been found the most suitable methods. Furthermore, *in situ* removal has been used to enhance alkaloid excretion by immobilized cells (24) and hairy roots (21) and shikonin by suspension and immobilized cell cultures (20) to stimulate secondary metabolite production.

In this study reactor operations of stirred tank reactor for suspension culture and packed-bed reactor for immobilized cell with *in situ* extraction were performed to investigate the effect of cell immobilization and reactor characteristics on shikonin production.

Materials and Methods

Cell line and culture media

Lithospermum erythrorhizon KCTC PCL 52001 was obtained from the Genetic Engineering Center, Korea Institute of Science and Technology. and maintained at our laboratory in the form of suspension culture. The cells were grown in SH medium (25) with 30g/*l* sucrose and 10^{-5} M *p*-chlorophennoxyacetic acid (*p*CPA) and 10^{-6} M kinetin. The pH of the medium was adjusted to 5.8 before autoclaving.

Cell cultivation was conducted in the dark on 50 m*l* of SH medium in 250 m*l* Erlenmeyer flasks capped with aluminum foil at 25°C and shaken at 100 rpm on a rotary shaker.

For shikonin production M-9 medium with 30g/*l* sucrose and 10^{-6} M indole-3-acetic acid (IAA) was used (26). The pH was adjusted to 5.8 before autoclaving.

Immobilization of plant cells

Cultured suspension cells of *L. erythrorhizon* were immobilized by using calcium alginate. Sixty m*l* of a suspension culture of plant cells (12g dry cell/*l*) grown to the stationary phase (10 days) were harvested and washed with distilled water.

For immobilization the cells were added to 60 m*l* of M-9 medium containing 0.9g sodium alginate and mixed gently. The cell-alginate mixture was fed dropwise by a syringe into a flask containing 300 m*l* of 0.1 M $CaCl_2$. The droplets instantaneously reacted with $CaCl_2$ to form spherical beads of 4.5 mm average diameter. The beads were kept in the solution for 1 h to ensure the complete precipitation. Finally, the beads were rinsed with distilled water and used for the experiments.

Reactor design for shikonin production

Stirred tank reactor: Stirred tank reactor was designed to produce shikonin from suspension cell cultures. The reactor was made of pyrex glass with a height of 116 cm and a inner diameter of 11 cm, and had a total volume of 1520 m*l* and working volume of 500 m*l* (27). When operating, suspension culture was performed with *in situ* extraction (50 m*l* *n*-hexadecane). To provide the oxygen necessary for the maintenance of cell viability, sterilized and humidified air was directly pumped to the culture medium (100 m*l*/min). For sufficient mixing and aeration floating magnetic stirrer bar was used to agitate the culture broth and to avoid cell grinding and high shear stress. By circulating temperature controlled water into the water jacket the temperature of the reactor was maintained at 25°C in dark (Fig. 1). Producd shikonin was dissolved in the *n*-hexadecane layer and the solvent was measured for shikonin production.

Packed-bed reactor: A packed-bed reactor was designed to continuously produce shikonin from immobilized plant cell cultures. The reactor, with a height of 16.5 cm and a diameter of 3.5 cm, had a total volume of 160 m*l* and working volume of 70 m*l*. Stainless steel mesh size of 250 mesh was used for medium sparger (27). The column was then packed with immobilized cell beads with a void fraction of 0.37.

To provide the oxygen necessary for the maintenance of cell viability, the aerated medium at the reservior was circulated through the packed cell beads. The aerated medium flow rate was about 20 m*l*/min. Continuous extraction of produced shikonin was per-

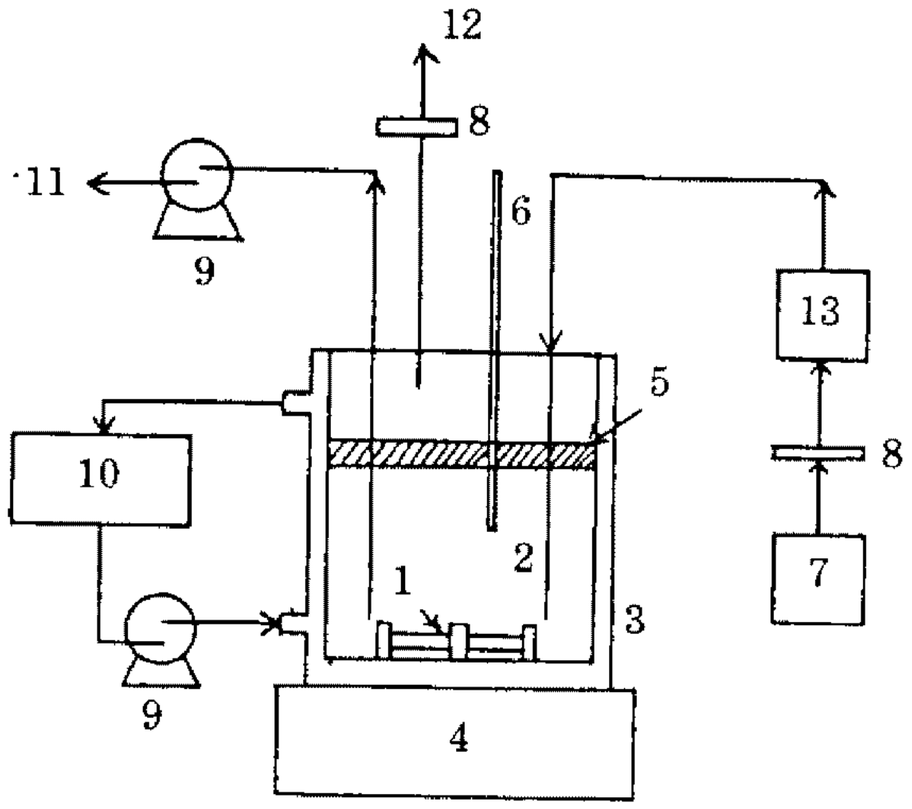

Fig. 1. Schematic diagram of experimental setup for suspension culture with *in situ* extraction in stirred tank reactor.

(1. floating stirring bar, 2. medium, 3. water-jacketed fermenter, 4. magnetic stirrer, 5. *n*-hexadecane, 6. thermometer, 7. air generator, 8. air filter, 9. peristaltic pump, 10, water bath, 11. sampling, 12. air out, 13. humidifier).

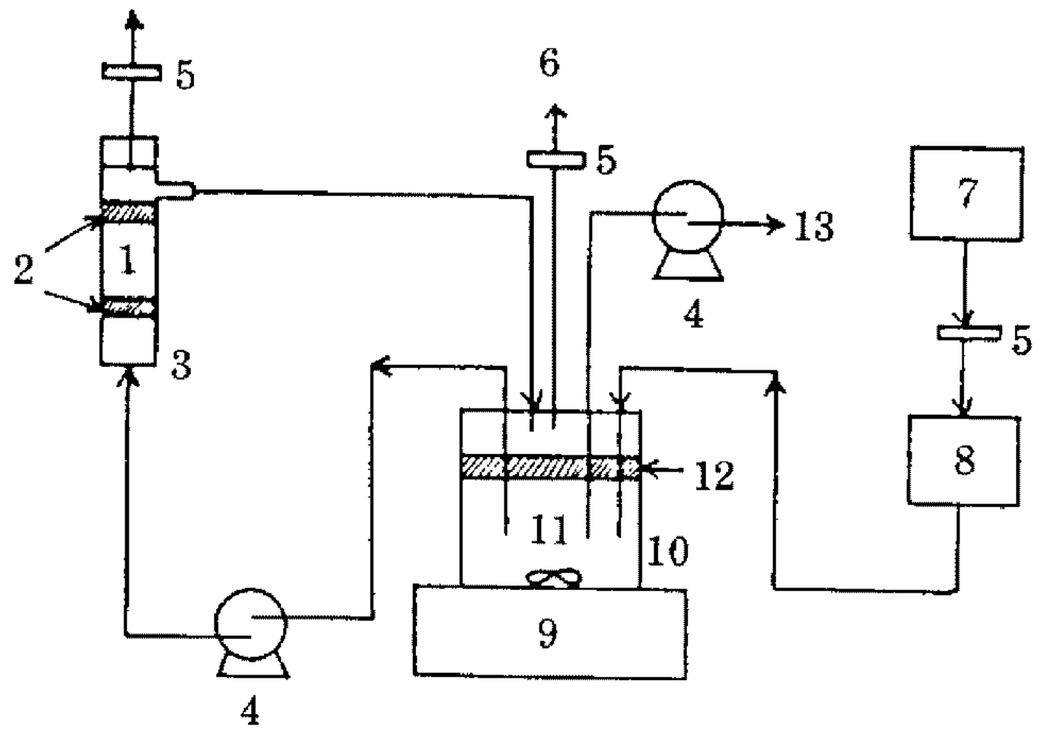

Fig. 2. Schematic diagram of experimental setup for immobilized cells with *in situ* extraction in packed-bed reactor.

(1. calcium alginate bead, 2. glass bead, 3. packed-bed reactor, 4. peristaltic pump, 5. air filter, 6. air out, 7. air generator, 8. humidifier, 9. magnetic stirrer, 10. extractor and aerator, 11. medium, 12. solvent, 13. sampling).

formed at medium reservoir by 50 m*l* of *n*-hexadecane. All the experimental apparatus were placed in a temperature controlled incubator at 25°C in dark (Fig. 2). Shikonin was dissolved in the *n*-hexadecne layer and the solvent was used to measure shikonin.

Analytical methods

Shikonins in fresh cell and culture medium were extracted by $CHCl_3$ for 3 h with vigorous mixing. To measure the amounts of shikonin dissolved in $CHCl_3$ and *n*-hexadecane in which shikonin was extracted *in situ,* 5 m*l* of 2.5% KOH solution were added to a test tube containing 1 m*l* of the solvent and the tube was shaken vigorously for 10 min. For the case of *n*-hexadecane 2 m*l* of $CHCl_3$ were added to the test tube to settle the *n*-hexadecane layer. The solution was centrifuged at 4000 rpm for 10 min, and the absorbance of the blue supernatant solution produced was measured by spectrophotometry at a wavelength of 622 nm (28). All spectrophotometric measurements were made with a Bausch & Lomb spectrophotometer (Spectronic 21).

The concentration of suspended plant cells were determined by removing aliquots from well mixed suspensions and placing the samples into pre-weighted culture tubes. The samples were washed and centrifuged three times and placed in a 70°C oven to constant weight. To determine the concentration of immobilized cells, calcium alginate beads were dissolved in a 0.2 M citrate buffer (pH 5.0) (2 M NaOH, 22 m*l*; 2 M citric acid, 10 m*l*; distilled water, 68 m*l*) and shaken for 30 min at 30°C. The freed cells were rinsed with distilled water, centrifuged three times, dried, and weighed (17).

Results and Discussion

Shikonin productions were carried out by using stirred tank and packed-bed reactor. In stirred tank reactor suspension culture was performed with *in situ* extraction. Fifty milliliters of *L. erythrorhizon* cells (10g/*l*) grown 10 days in SH medium were transferred to the reactor containing M-9 medium (500 m*l*) and *n*-hexadecane (50 m*l*).

Fig. 3. shows the time course of shikonin production in stirred tank reactor. Shikonin production was linearly increased during the first 9 days and after then its specific shikonin production was maintained at 1.5 mg/g cell. Its shikonin production profile is somewhat similar to that of shake flask, but its shikonin production stopped earlier. The cells were seemed to be damaged by shear caused by stirring magnetic bar. Others also observed that excess shear disturbed cell growth and cell metabolism (6,29). When considering volumetric shikonin productivity, 400 g shikonin/(L·day) was obtained based on the working volume.

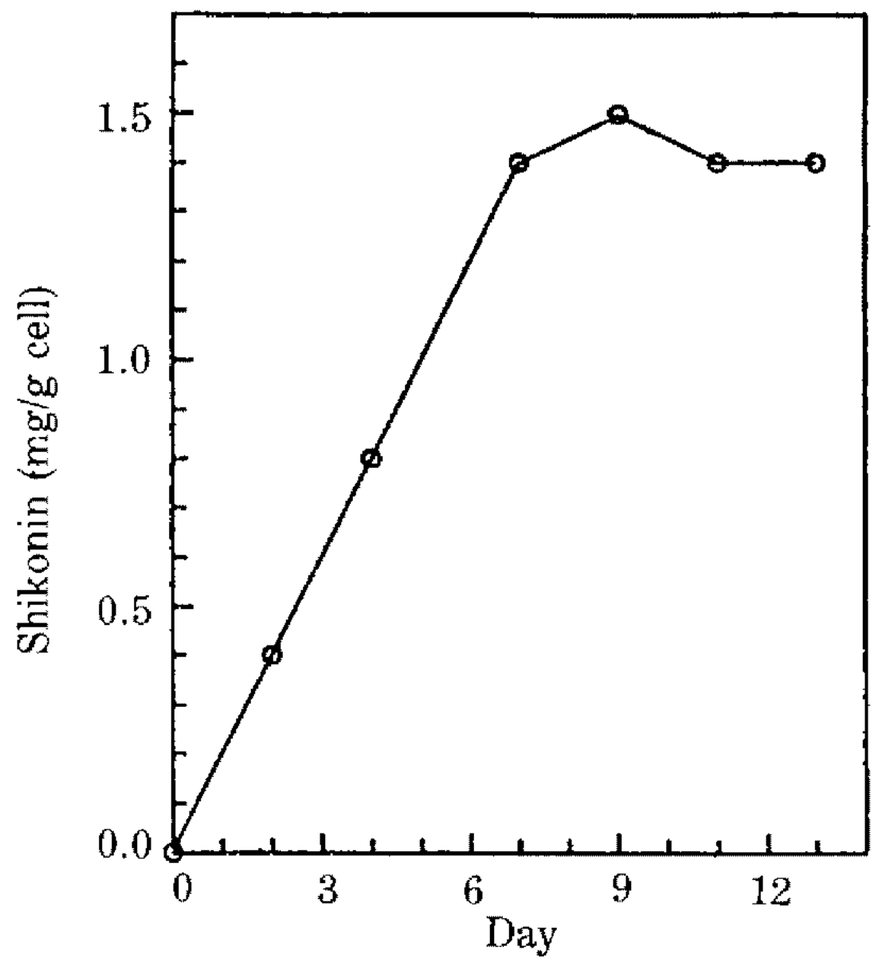

Fig. 3. Time courses of shikonin production of stirred tank reactor with *in situ* extraction.

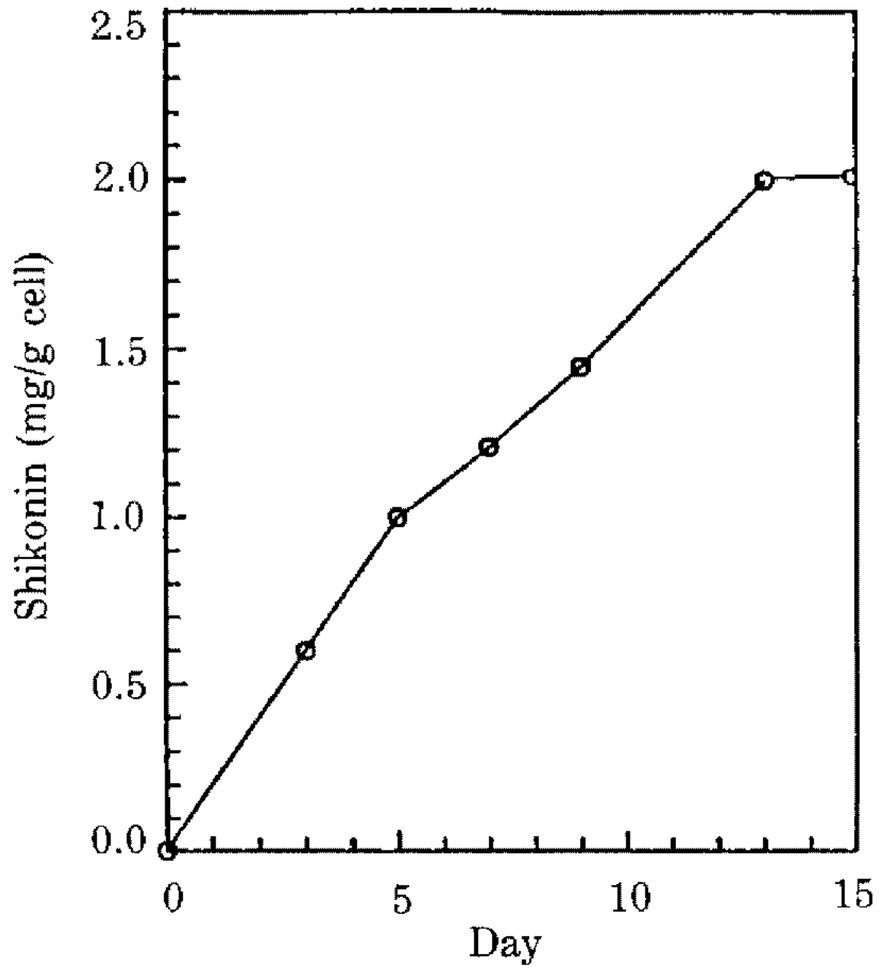

Fig. 4. Time courses of shikonin production of calcium alginate immobilized cells in packed-bed reactor with *in situ* extraction.

In packed-bed reactor 50 m*l* of calcium alginate immobilized cells (average bead diameter of 4.5 mm) were transferred to the reactor. Glass beads of equivalent diameter were mixed with alginate beads to prevent medium plugging and alginate bead rising. Five hundred milliliters of aerated M-9 medium was continuously recycled, and shikonin was extracted by *n*-hexadecane (50 m*l*) at the medium reservoir.

Fig. 4 shows the time course of shikonin production in packed-bed reactor with *in situ* extraction. Shikonin production was continuously increased during 13 days of the experiments. Specific shikonin production was 2.0 mg/g cell at 13 day which was about 30% higher than that of the stirred tank reactor. The increased specific shikonin production seems to be caused by cell immobilization effect (19). The volumetric shikonin productivity 2875 μg shikonin/(L·day) could be obtained, which was 7.1 times higher than that of stirred tank reactor. However, some operational difficulties are to be overcame in the packed-bed reactor. Channeling and insufficient mixing in the reactor may cause medium deficiency in some region including oxygen transfer limitation.

Considering the above results immobilized cells in air lift or bubble column reactor with *in situ* extraction is favorably considered for shikonin production.

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요 약

식물세포인 *Lithospermum erythrorhizon*을 교반 반응기와 calcium alghinate에 고정화된 상태로 충전층 반응기에서 *n*-hexadecane으로 동시 추출하면서 shikonin을 생산하여 각각의 생산성을 비교하였다. 교반 반응기에서 shikonin의 비생산과 부피생산성은 각각 1.5 mg shikonin/g cell과 400 μg shikonin/(L·day)였고 충전층 반응기에서는 각각 2.0 mg shikonin/g cell과 2857 μg shikonin/(L·day)였으며 이는 각각 교반 반응기에 비하여 1.3, 7.1배 높은 것이다. 충전층 반응기에서 shikonin의 생산성이 높은 것은 calcium alginate 입자에 세포가 고농도로 축적되어 단위 반응기 부피당 세포의 부하 능력이 높고 또한 세포가 서로 접촉하기가 쉽고 고정화 입자내의 환경이 세포가 분화하기에 좋은 조건을 형성하기 때문인 것으로 사료된다.

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