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PRODUCTION OF BERBERINE IN IMMOBILIZED PLANT CELL CULTURE OF *Thalictrum rugosum*

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Abstract—*Thalictrum rugosum* cells were immobilized in calcium alginate, where they continued to live with their biological activity. The immobilized living cells performed the production of berberine in both shake flasks and an airlift bioreactor. Berberine formation was growth associative and most of the berberine produced was stored intracellularly. Rapid hydrolysis of sucrose and preference of glucose over fructose during the growth stage was observed. Phosphate-deficient media increased berberine production and prevented the dissolution of alginate beads. The behavior of immobilized cells grown in an airlift reactor was compared with that of the corresponding shake flask culture with respect to growth and berberine production. The rate of cell growth and berberine production in an airlift reactor operation was higher than those in packed-column reactor operation due to a better oxygen transfer.

INTRODUCTION

Plant cell and tissue-culture technique has been developed as an attractive alternative for the production of valuable compound instead of agricultural (farming) techniques. The major valuable chemicals from plant cell culture are the secondary metabolites, which are biosynthetically derived from the primary metabolites. Though plant tissue culture techniques have been developed since 1940, the significant advances have been done in the last decade for the large scale commercial production of valuable compounds from plants in a fermentation type environment.

Immobilization of plant cells onto a suitable carrier has received increasing attention as an alternative to suspension cell culture to provide for favorable cell differentiation, to give the advantage of ease of processing in a bioreactor operation and to improve the production of secondary metabolites [1-3]. Immobilized plant cells give an advantage of case of control over the size of aggregates and cell concentration compared with suspension cell culture. Adverse shearing effects imposed by fluid motions are also eliminated or greatly reduced when cells are protected by immobilization. The protection against high shear stress resulted in increased cell densities and made the cells stable in an otherwise shear-sensitive plant cell line [4]. Another advantage is the possibility of reusing the biocatalyst. Immobilization of plant cell helps decouple the growth and product formation phases, which allows the biocatalyst (immobilized plant cells) to be recycled. The continuous flow-through process can be achieved by immobilization of plant cell without complete washout and may be used at dilution rates in excess of the maximum specific growth rate of the culture. This is very important consideration of plant cells that, in general, grow very slowly. From a process engineering perspective, the most important advantage of the use of immobilized plant cells is the case of processing in a large scale production.

Since the immobilization of plant cells was introduced as a beneficial aid for the production of secondary metabolites, many kinds of immobilization techniques have been investigated. The predominant immobilization methods are the entrapment of cells within gels or membranes, adsorption and covalent linkage of cells on the surface of polymers and encapsulation [1,3]. These methods enable the immobilization of plant cells to be carried out under mild conditions so as to minimize the damage. Gel entrapment has been a widely used type of immobilization method in plant cells because entrapment process is easy and there is no chemical modification of the cell wall with formation of the gel structure around the cell [1,2]. The water soluble substrates and nutrients can pass freely

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through the gel to provide the cells with essential nutrients.

Plant cells are sensitive to shear which can result in lysis of cells during fermentation. To overcome this barrier, development of bioreactors which focus on minimizing mechanical shear stress has been investigated for large scale cultivation of plant cells. Several types of bioreactors have been used in plant cell culture. Stirred-jar bioreactors, frequently used for microbial fermentations, can cause damage to plant cells because of high shear [4]. Airlift reactors have the advantages of low shear, low energy requirement, good mixing, easy scale up, and avoidance of complex seals and glands which increases the reliability in sterile condition [3,4]. Because of these advantages, airlift reactors have been used for various plant-cell-suspension cultures such as strawberry cells [5], *Helianthus annuus* [6] and *Catharanthus roseus* [7].

In this study, the model system was berberine production from cultured *Thalictrum rugosum* cells. Berberine, a yellow quaternary isoquinoline alkaloid, has been used as an intestinal antiseptic agent, and as a fluorescence marker in several area of medicinal research [8]. The cells were immobilized in calcium alginate beads and the production of berberine was investigated in shake flasks and in an airlift reactor.

MATERIALS AND METHODS

1. Plant cell culture and culture media

Thalictrum rugosum cultures were provided by Dr. Peter Brodelius (Institute of Plant Biology, University of Zurich, Switzerland). Cell suspension cultures have been maintained on Murashige and Skoog (MS) medium prepared from MS salt mixture (GIBCO Laboratories, Grand Island, NY) with the addition of 2 μM 2,4-D, vitamin stock solution and 30 g/L of sucrose as carbon source. The pH of medium was adjusted to 6.0 before autoclaving. After autoclaving, the pH of medium became 5.8. Cell suspension culture were cultivated on a rotary shaker at 180 rpm and 25 °C in normal room light. The pH of medium was adjusted to 6.0 before autoclaving.

2. Immobilization of plant cells

Thalictrum rugosum cells were immobilized by using calcium alginate. The following procedures were done under sterile conditions. Five-day-old cells were sieved through a nylon net of 1 mm mesh size. The sieved cells were washed with calcium-free medium on a ceramic filter. Cells (20 g fresh weight) were mixed with 3% alginate solution (80 g) in culture medium. Alginate beads of 3.5-4 mm in diameter were made by dropping the alginate/cell suspension into medium fortified with 50 mM $CaCl_2$ solution. The beads were kept in the solution for 30 min and then rinsed with culture medium before experiments.

3. Cell concentration measurement

When the samples were taken, the alginate beads were separated from the liquid medium and suspended in 50 ml 0.1 M sodium citrate solution (pH 6.0). The beads were fully dissolved after shaking the suspension for 30 min on a rotary shaker. The cells were collected by filtration and dried for dry weight measurement.

4. Alkaloid analysis

Intracellular berberine was extracted with HPLC-grade methanol. A 2.0 gram of beads by fresh weight in 20 ml of methanol was sonicated at 125 W for 1 hr. A filtered sample (10 μl) of extract or medium was injected into an HPLC system. Quantitative berberine analysis was carried out by HPLC system, Spectroflow 400 (Kratos Corp., Ramsey, NJ), under the following conditions; Column: SUPERCOSIL LC-18-DB (Supelco Inc., Bellefonte, PA), 15 cm × 4.6 mm. Flow rate: 2 ml/min. Mobil phase: 1 mM tetrabutyl ammonium phosphate in water, adjusted to pH 2 with phosphoric acid (60%) and acetonitrile (40%). Detection: 271 nm with a UV detector (Kratos Corp., Ramsey NJ).

5. Sugar analysis

A HPLC system was used for the simultaneous analysis of sucrose and its hydrolyzed products, glucose and fructose under the following conditions; Column: SUPELCOSIL LC-NH_2, 25 cm × 4.6 mm. Flow rate: 2 ml/min. Mobile phase: 75% acetonitrile and 25% water. Detection: refractive index (RI) detector (Perkin-Elmer Corp., Wilton, CT).

6. Airlift reactor

An airlift fermentor (Model 500 Series, LH Fermentation, Inc., England) was used to produce secondary metabolites from immobilized plant cell culture (Fig. 1). The vessel was made of glass with a permanent water jacket and the working volume was 2.4 liter. A waterbath (Haake-Buchler Instrument, Inc., Saddle Brook, NJ) which has both a cooling unit and heater was used for temperature control by circulating through the water jacket of the fermentor. The mixing and aeration were done by sterile gas from an air pump through a flow meter and an air filter (Balston, Ltd., Maidstone, England). Dissolved oxygen (DO) was controlled by a DO controller. Foam was controlled by the addition of antifoam, DCA (Cerex Corporation, Gar-

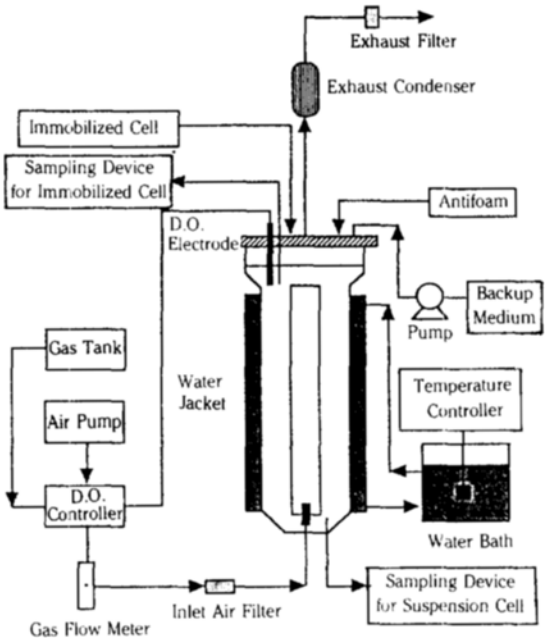

Fig. 1. The configuration of an airlift reactor system.

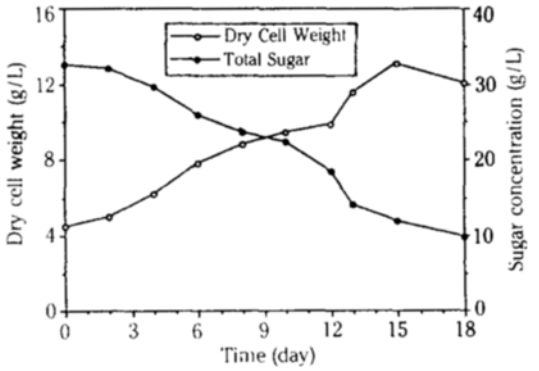

Fig. 2. Time course change of dry cell weight and sugar consumption of immobilized cells in shake flasks.

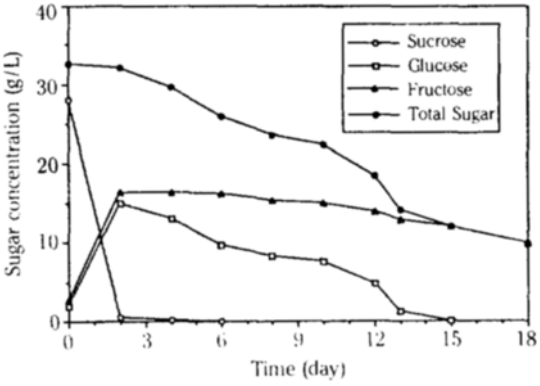

Fig. 3. Hydrolysis of sucrose and the sugar consumption of immobilized cells in shake flasks.

thersburg, MD). A cell scraper was installed to prevent the accumulation of cells at the interface and to give better mixing. The innoculation was performed through a large opening on a headplate by pouring in the hood. Samples were taken by a hooded sampler which was attached to a sampling tube that extents to the top of liquid medium in the vessel.

RESULTS AND DISCUSSION

1. Batch culture in shake flasks

Calcium alginate-entrapped *Thalictrum rugosum* cells were cultured with the standard culture medium (See Materials and Methods) in a shake flask with a working volume of 50 mL to investigate the effect of aggregation on berberine production. Figure 2 shows the change in dry cell weight and sugar consumption in *T. rugosum* cells. The cells entrapped in alginate were somewhat restricted in growth. Dry cell weight was maximum at the 15th day of culture, which was twice as long than in suspension culture [9]. From the pattern of extracellular sugar depletion and conversion, sucrose, the only carbon source, was found to be hydrolyzed quickly to glucose and fructose, demonstrating the existence of an extracellular-acting invertase, as shown in Figure 3. Just 2 hours after inoculation, the conversion of sucrose to its monomeric sugars was significant. The preference of glucose over fructose during the growth stage was observed. Fructose was formed in the medium through the hydrolysis of sucrose, and scarcely decreased during the cell growth. This result suggests that the entrapped cells seem to utilize no fructose derived from sucrose as a carbon source.

Figure 4 shows the production and distribution of berberine. Cell concentration increased steadily for the initial growth phase, but the berberine concentration did not follow the cell growth. After the 4th day of culture, berberine production increased as the cell concentration increased. But berberine formation became steady at the last part of the cell growth. Berberine production in immobilized cells was slightly growth associated but that in suspended cell was highly growth associated [9]. Most of the berberine was released after inoculation and then the cells took up the extracellular berberine. After that, a small amount of berberine existed in the medium. This result suggested that immo-

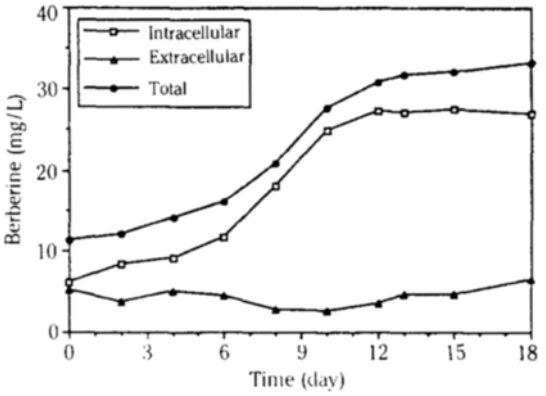

Fig. 4. Time course change of berberine distribution of immobilized cells in shake flasks.

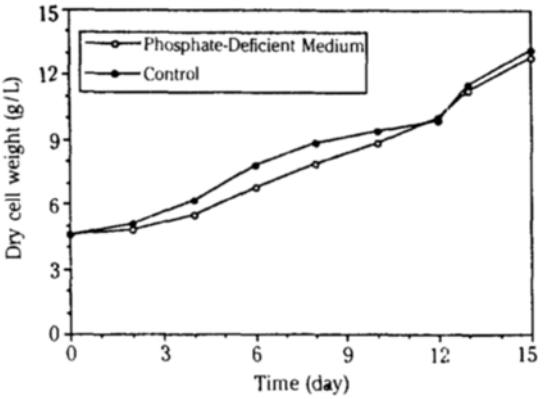

Fig. 5. Cell growth of immobilized cells in phosphate-deficient medium.

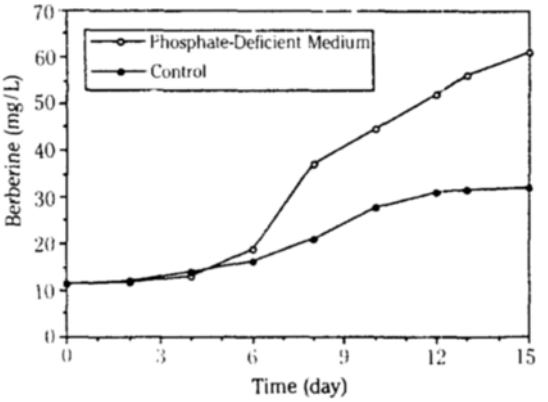

Fig. 6. Berberine production of immobilized cells in phosphate-deficient medium.

bilized *Thalictrum rugosum* cells have a capacity to store the berberine in the vacuole space and to maintain an intracellular accumulation system.

2. Enhancement of berberine production by phosphate limitation

The concentration of phosphate in the medium is very important both for cell growth and product formation in plant cell culture. In addition to the carbon and nitrogen source, phosphate is one of the key substances controlling cell metabolism. When the phosphate was removed from the medium in suspended *T. rugosum* cell culture, cell growth was very low and berberine production increased, but cell growth and berberine production were constant at levels higher than 1.75 mM phosphate concentration [10]. This result suggested that there exists phosphate inhibition effect on berberine production. Berlin et al. [11] reported that suspension cell culture of *T. rugosum* released protoberberine alkaloids into medium when cells were transfered to fresh medium lacking phosphate. Phosphate and chelating ions in the medium dissolved calcium alginate beads, resulting in the release of the released free cells from beads as the experiment proceeded [9].

In order to investigate the effect of phosphate, phosphate-limited condition was tested for calcium alginate entrapped *T. rugosum* cell. Phosphate deficient medium was used for cell culture but a growth medium with 1.75 mM phosphate concentration was used to make calcium alginate beads. Total phosphate concentration for the culture was 0.5 mM including beads and medium. Berberine production and cell growth are shown in Figure 5 and 6, respectively. Dry cell weight in phosphate deficient medium was nearly the same as that in growth medium, but berberine production in phosphate deficient medium increased significantly more than two times after 8th day of culture. Extracellular berberine levels were nearly the same as that in growth medium. In order to clarify the effect of repeated use of a phosphate deficient medium, mediums for both cultures were replaced at the 12th day of culture. Dry cell weight in phosphate deficient medium increased until the 27th day of culture after medium replacement, but that in growth medium increased only until the 23rd day of culture and then decreased as shown in Figure 7. Figure 8 shows berberine production in phosphate deficient medium increased continuously, but that in growth medium increased and then decreased. This might be due to the effect of released free cells as shown in Figure 9. The amount of released free cell from beads increased in both cultures but the amount of released free cell in phosphate deficient medium was significantly less than that in growth medium as shown in Figure 10.

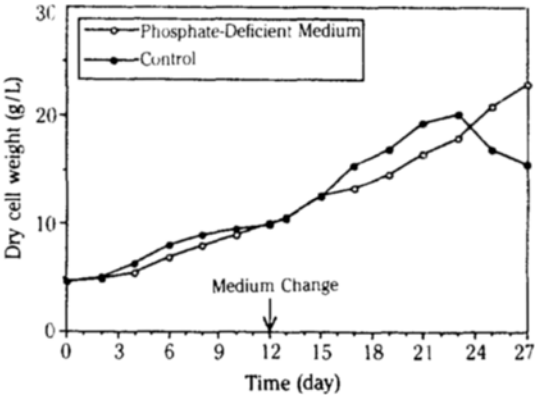

Fig. 7. Cell growth of immobilized cells in phosphate-deficient medium with medium replacement.

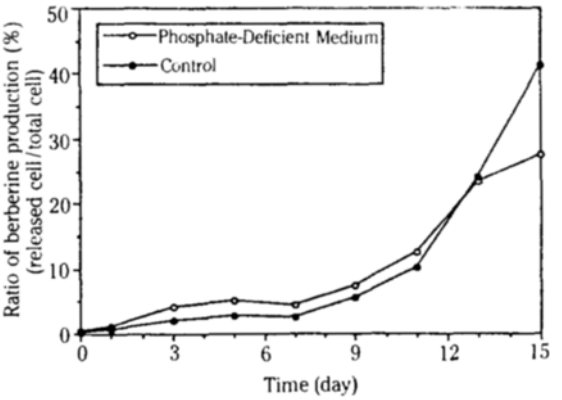

Fig. 9. Effect of released cell from bead on berberine production in the second culture.

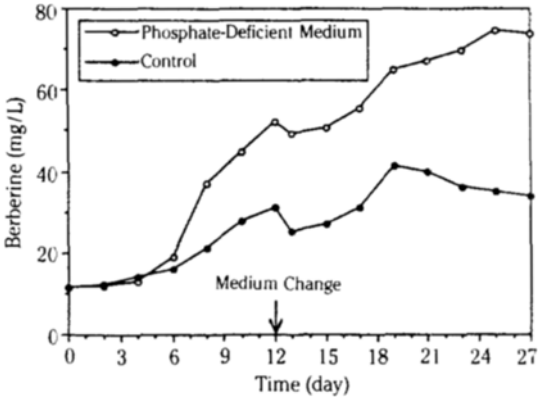

Fig. 8. Berberine production of immobilized cells in phosphate-deficient medium with medium replacement.

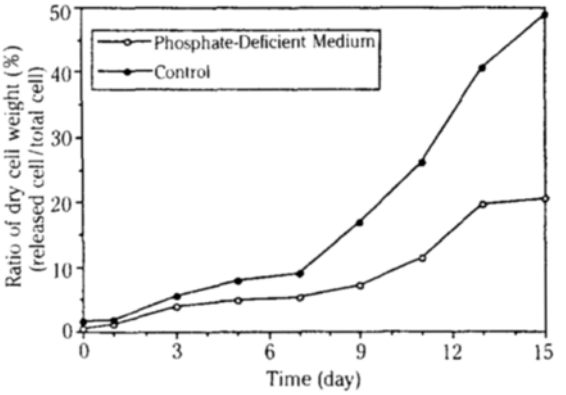

Fig. 10. Effect of phosphate-deficient medium on the release of cell from bead in the second culture.

These results suggest that phosphate-limited conditions increase berberine production and prevent the beads from dissolving. The reason for the enhancement of berberine production might be that limiting the concentration of phosphate can cause the use of an intracellular phosphate pool, which in turn stimulates the berberine production if a phosphate inhibition effect on berberine production exists.

3. Airlift reactor operation

To study the large scale performance of cell growth and berberine production with immobilized cells, an airlift reactor was operated with calcium alginate entrapped *T. rugosum* cells. Experimental conditions were as follows: working volume; 2.4 L, air flow rate; 100 cc/min and initial loading amount of beads; 350 g (20% cell loading based on fresh cell weight).

Figure 11 shows the variation of cell growth and sugar consumption. Immobilized cells grew well and continued to live with their biological activity in an airlift reactor. But cell growth was slower and the final cell density was lower than those for cultures in shake flasks. The sugar consumption pattern was almost the same as that in shake flasks but the consumption rate was low because of the low cell growth rate. Figure 12 shows that the production and distribution of berberine during the experiment. The distribution of berberine shows that small amounts of berberine exist in medium. The immobilized culture of *T. rugosum* has a capability to store the berberine produced inside the cells in an airlift reactor system. Growth associative product formation was observed like in shake flasks, but rate of berberine production was lower than that in shake flasks due to low cell growth rate. Similar low rate of cell growth and production in an airlift reactor was reported by Scragg et al. [7] in serpentine forma-

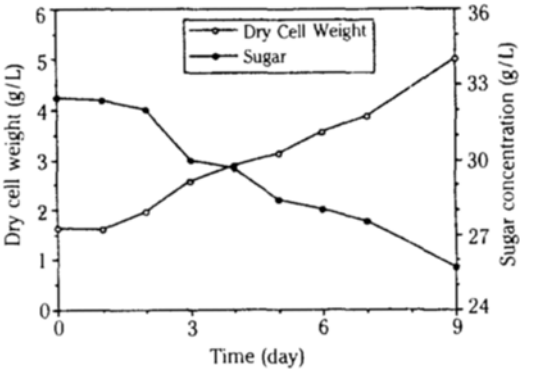

Fig. 11. Cell growth and sugar consumption of immobilized cells in an airlift fermentor.

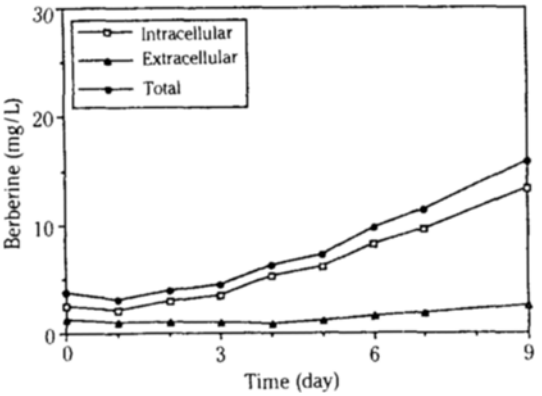

Fig. 12. Berberine production of immobilized cells in an airlift fermentor.

tion by suspension cultured *Catharanthus roseus* cells. They suggested that carbon dioxide and ethylene, which could be easily stripped out by air sparging, has been shown to affect plant cell physiology. The low rate of cell growth and production of secondary metabolites by plant-cell-suspension culture in an airlift reactor might be due to stripping of some important factors, gases and volatile compounds, for cell growth and product formation by continuous feeding of air to achieve the circulation of cells [10,12]. This interpretation might be applied to immobilized plant cell culture in an airlift reactor. To increase the production of secondary metabolites by immobilized plant cell culture in a large scale the stripping effect of air in bioreactors should be investigated more.

In order to check the aeration effect, an airlift reactor was operated with 50 cc/min air flow rate which was half of the air flow rate used in the previous experiment. Figure 13 shows the variation of cell growth and berberine production. As cells grew inside the

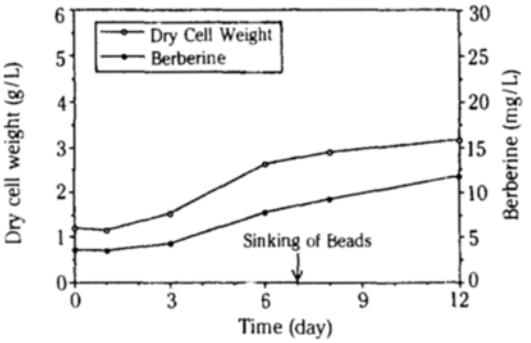

Fig. 13. Effect of aeration on cell growth and berberine production of immobilized cells in an airlift reactor.

beads, the density of the beads increased until the beads could not be circulated with 50 cc/min air flow rate. The beads settled at the 7th day of operation. After the beads sank, packed-column reactor operation started and the cell growth rate decreased. This might be due to less oxygen transfer of packed-column reactor operation since entrapped cells inside the beads required a high amount of oxygen compared to the suspended cells [13]. Berberine production rate also decreased because of low growth rate. These results suggest that airlift reactor operation might be better than packed-column reactor operation due to better oxygen transfer in immobilized plant cell culture.

Since berberine produced in immobilized *Thalictrum rugosum* cell was stored intracellularly as found in the above experiments, permeabilization process to secrete the intracellularly stored berberine for the repeated use of cells, and *in situ* product separation process to separate the berberine secreted and to enhance berberine formation are needed. An integrated process with permeabilization using permeabilizing agent and *in situ* product separation using immobilized adsorbents in an airlift reactor is developed by Choi [9].

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