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IN SITU BERBERINE SEPARATION WITH IMMOBILIZED ADSORBENT IN CELL SUSPENSION CULTURES OF *Thalictrum rugosum*

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Abstract—The use of suspended and alginate-entrapped XAD-7, polycarboxyl ester resin, for the *in situ* separation of berberine, isoquinoline alkaloid, produced from plant cell culture of *Thalictrum rugosum* was investigated. XAD-7 could adsorb the berberine and the amount of berberine adsorbed on XAD-7 depended on pH. The neutral form of berberine was adsorbed onto XAD-7 and the adsorption isotherm for berberine showed a Langmuir-type appearance. *In situ* berberine removal enhanced the production of secondary metabolites in cell suspension culture of *Thalictrum rugosum*. Addition of XAD-7 at the exponential phase of cell growth was the most effective for enhancement of berberine production. In chitosan-treated cell culture to permeabilize intracellular berberine, berberine secretion was significantly accelerated by addition of alginate-entrapped XAD-7 at the stationary phase of cell growth and thus more than 70% of the produced berberine could be adsorbed to alginate-entrapped XAD-7.

INTRODUCTION

To enhance process economics through improvements in the primary recovery stages, it is necessary to develop a separation technique which can concentrate the bioproduct, often with some degree of selectivity for the product of interest, and be economical from both capital and operation cost standpoints in large scale cell culture. In addition to the above advantages, *in situ* product separation can enhance the production of secondary metabolites by removing feedback regulation mechanisms nd nonspecific inhibitors in plant cell culture [1, 2]. For *in situ* product separation of plant cell culture, liquid-liquid and solid-liquid systems have been investigated [3, 4]. Two-phase culture, where the products were extracted into the nonaqueous phase, has been investigated to remove the feedback regulatory mechanism in plant cells. The continuous release of products and the extraction to the nonaqueous phase would make possible the continuous production because the storage capacity of the cells would not become limiting. It is also possible that the nonaqueous phase can remove the nonspecific inhibitors of secondary metabolism and retain volatile compounds where product feedback effects are not observed to be limiting. The integration of permeabilization and *in situ* product separation by two-phase culture could be feasible for the continuous production of products in immobilized plant cell bioreactors with the repeated use of biocatalyst.

Liquid-liquid culture systems for plant cells consisting of an aqueous nutrient phase and of a lipophilic phase, have been used with success for the continuous extraction and accumulation of lipophilic constituents out of the culture medium for shikonin production and for the bioconversion of geraniol [5, 6]. Water insoluble triglyceride was used to retain volatile hydrocarbons in *Thuja occidentals* cell [7]. A liquid-liquid extraction column has been used to recover capsaicin from an immobilized plant cell bioreactor containing *Capsicum frutescens*, in which capsaicin production were increased by its removal from the aqueous phase and food grade sunflower oil was used as a suitable non-toxic extractant [3]. Suspension cultures of *Nicotiana tabacum* have been successfully grown in aqueous, two-phase systems comprised of polyethylene glycol (PEG) and dextran in a modified LS medium [8].

Liquid-solid culture systems for plant cells consisting of an aqueous nutrient phase and of solid polar adsorbents, have been preferred because many products of plant cells are expected to be of polar character and bound weakly to the lipophilic phase of liquid-liquid systems. The polymeric resin XAD-4 has been

used for the recovery of indole alkaloids and could concentrate the alkaloids by two order of magnitude over solvent extraction in *Catharanthus roseus* cells [9]. Amberlite XAD-4 is an aromatic adsorbent, composed of styrene divinyl benzene copolymers [10]. Polycarboxylic ester resin XAD-7 was found to be more selective in absorbing indole alkaloids than XAD-4 despite a lower capacity [11]. When XAD-7 was added to the culture medium, the formation of total indole alkaloids was stimulated and increased the ratio of desired product to other metabolites [12].

In this study, the use of XAD-7 was examined for *in situ* separation of berberine produced from cultured *Thalictrum rugosum* cells as a model system Polycarboxylic ester resin, XAD-7, was examined to analyze and quantify berberine adsorption, especially for pH and concentration dependences. To prevent surface fouling and to separate adsorbents from cells for the repeated use of cell and adsorbents, alginate entrapped adsorbent was investigated.

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. Suspended cells were cultivated on a rotary shaker at 180 rpm and 25℃ under normal room light.

2. Batch Experiment Procedure

For the batch experiment in shake flasks, cells in the late exponential growth phase, which are usually 5-6 days old, were used. To avoid heterogeneity of the inoculum, all the cells from different flasks were collected in a pre-autoclaved large flask and mixed well by shaking. The cells were filtered through Whatman No. 1 filter paper on a Buchner funnel under slight vacuum and washed with fresh medium which was prepared according to the purpose of experiment. 5g of cells by fresh weight was inoculated into a 125 m*l* Erlenmeyer flask containing 50 m*l* of medium. The cultures were incubated at 25℃ on a shaker at 180 rpm. Two or three replicas of flasks were sacrificed for analysis of samples. After filtration, the cells were collected for cell mass measurement and intracellular product determination. The filtrates were usually stored in the refrigerator for extracellular product and sugar assays.

3. Cell Mass Measurement

For dry cell weight (DCW), suspension cells were filtered with dried and pre-weighted Whatman No. 1 filter paper under slight vacuum. Filtered cells were washed with distilled water and dried in a oven at 60℃ to constant weight.

4. Alkaloid Analysis

Intracellular berberine was extracted with HPLC-grade methanol. 0.5g of cells by fresh weight in 20 m*l* of methanol was sonicated at 125 W for 1 hr. A filtered sample (10 μ*l*) 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 m*l*/min. Mobile 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

An 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 m*l*/min. Mobile phase: 75% acetonitrile and 25% water. Detection: refractive index (RI) detector (Perkin-Elmer Corp., Wilton, CT).

6. Preparation of Adsorbent

The neutral polymeric resin, XAD-7, was obtained from Supelco Inc. (Bellefonte, PA). Prior to use, the resins, XAD-7, were soaked in methanol for 24 hour and then washed with 2 liter distilled water. The washed resins were air dried on the filter paper by pulling a vacuum in the filter flask. The resins were sieved through the nylon nets (mesh size: 250 μm and 500 μm) to get suitable size distribution. The resins were autoclaved in distilled water for 15 min at 121℃. One gram of the autoclaved resin was added to the culture flask.

7. Immobilized Adsorbent

The neutral polymeric resin, XAD-7, was sieved through the nylon nets (mesh size: 250 μm and 500 μm). 2g alginic acid IV was dissolved in water (98 m*l*) by heating under stirring. 20g of XAD-7 was mixed with alginate solution (80g) at room temperature. Alginate beads of 4.35 mm in diameter were made by dropping alginate/resin suspension into 1% $CaCl_2$ solution under continuous stirring. The beads were allow-

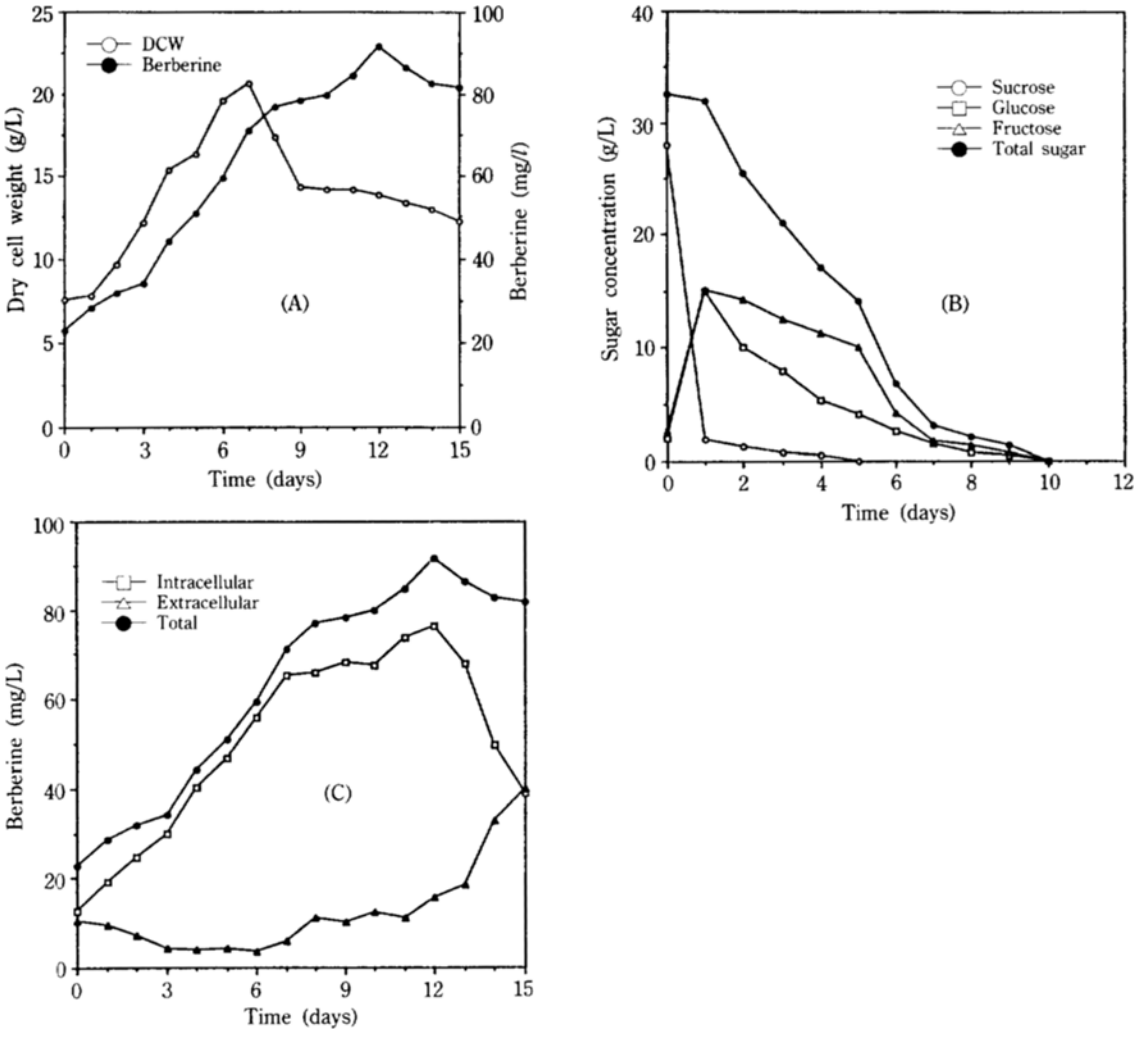

Fig. 1. (A) Time course behavior of cell growth and berberine production in batch culture. (B) Hydrolysis of sucrose and the consumption of sugars in batch culture. (C) Time course change of berberine distribution in batch culture.

ed to form for 30 min and then collected by filtration. Beads were washed with distilled water and then were autoclaved in 0.5% $CaCl_2$ solution to maintain the integrity for 15 min at 121℃. The sterilized beads were stored in sterilized water.

RESULTS AND DISCUSSION

1. Batch Culture

For accurate analysis of the kinetic behavior of cell growth and product formation in *Thalictrum rugosum* cell suspension culture, a batch experiment was carried out in shake flasks and samples were taken every day. Fig. 1(A) shows the time course changes of cell growth and berberine production. The lag phase for cell growth existed to about the first day of cultivation. Dry cell weight reached a maximum at the 7th day of inoculation and then decreased continuously.

From the pattern of extracellular sugar consumption, sucrose, the only carbon source, was hydrolyzed quickly to glucose and fructose, demonstrating the existence of an extracellular-acting invertase as shown in Fig. 1(B). It was found that only the hexose sugars were taken up into plant cells [13]. 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 is evident.

The typical secondary product accumulation in plant

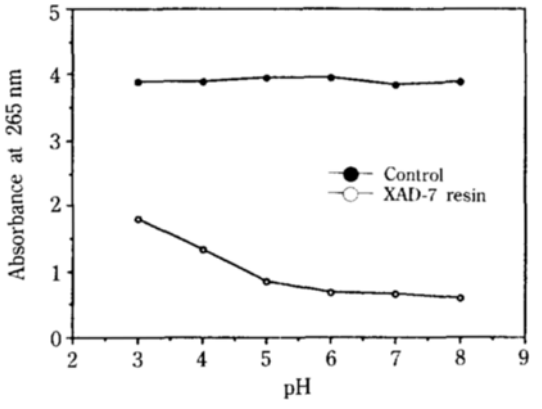

Fig. 2. Berberine adsorption on XAD-7 at various pH.

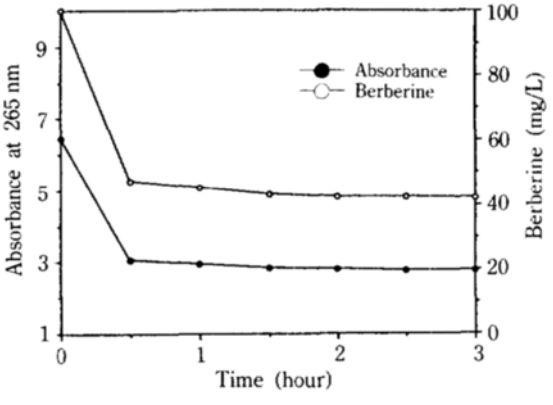

Fig. 3. Time course behavior of berberine adsorption on XAD-7.

cell cultures is nongrowth-associated [14] but the berberine production is mixed growth-associated as shown in Fig. 1(C). The distribution of berberine shows that a small amount of berberine existed in the medium until the 12th day of culture and then its level increased at the late stage of culture, probably due to cell lysis. Before the cell lysis occurred, extracellular berberine was less than 15% of the total berberine. Berberine was released from the cells and also decreased after the cell lysis, perhaps, due to enzymes excreted during cell lysis that might degrade berberine. This suggests that suspension cultures of *T. rugosum* have a capability to store the berberine in the vacuole space [15] and to maintain an intracellular product accumulation system.

2. Adsorption of Berberine on XAD-7

To investigate *in situ* berberine separation in *T. rugosum* cell culture, polycarboxylic ester resin, XAD-7, was examined to analyze and quantify berberine adsorption, especially for pH and concentration dependences. Fig. 2 showed the result from an experiment designed to examine the potential for berberine adsorption on XAD-7 (control: no XAD-7, initial berberine conc.: 60 mg/L, 0.5g XAD-7/50 mL). In the control without XAD-7, the increase of pH from 3 to 8 resulted in constant value in the UV absorbance. When XAD-7 was used, the UV absorbance was reduced due to the adsorption of berberine. Adsorption decreased as pH increased up to 6 and then absorbance became constant. This decrease might be due to the increase of neutral form of berberine since pK_a of berberine is 2.47.

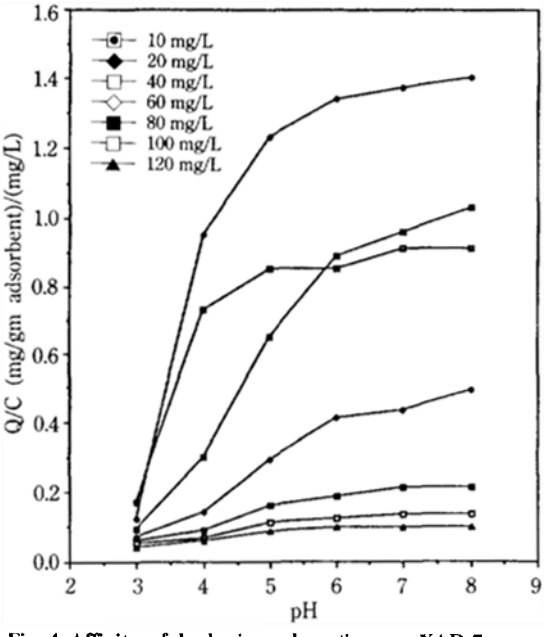

Fig. 4. Affinity of berberine adsorption on XAD-7 as a function of pH.

Fig. 3 showed the result for an experiment designed to examine the time to reach equilibrium for berberine adsorption on XAD-7 (pH: 6, 2g XAD-7/200 mL, initial berberine conc.: 100 mg/L). The UV absorbance of solution was reduced rapidly due to the adsorption of berberine until 30 min. After 1 hour, absorbance became constant, e.g., equilibrium was reached. From the next experiments, the solution with XAD-7 were allowed to equilibriate for at least 1 hour. After equilibration, aqueous samples were withdrawn and analyzed for dissolved berberine.

Dependence of berberine adsorption on pH and concentration was examined as shown in Fig. 6. Equilibrium ratio (Q/C), a measure of affinity of berberine, could be calculated by

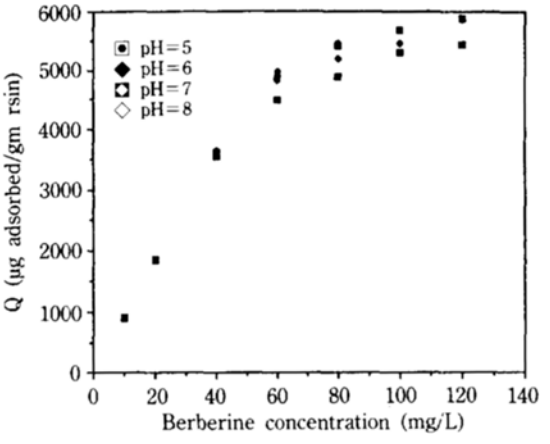

Fig. 5. Adsorption isotherm for berberine based on the concentration of the neutral form of berberine.

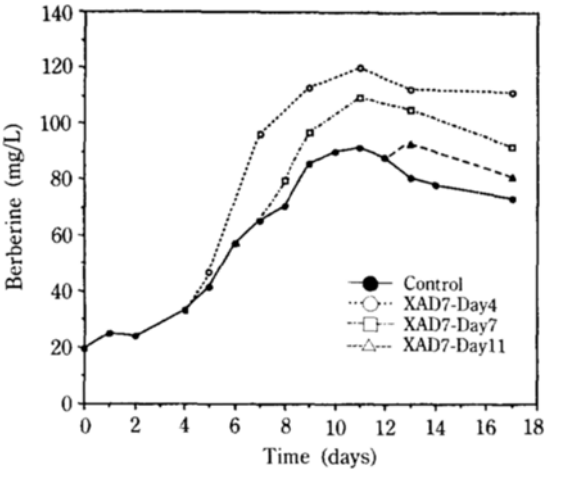

Fig. 6. Effect of suspended XAD-7 on berberine production.

$$\frac{Q}{C}=\frac{(C_o-C)}{C}\ \frac{V_B}{A_s} \tag{1}$$

, where C and C_o were the measured and initial berberine concentration, respectively; V_B was the liquid volume; A_s was the amount of adsorbent in shake flask; and Q was adsorbent loading, amount of berberine per mass of adsorbent (mg adsorbed/g adsorbent).

Fig. 4 showed that the affinity increased with the increase of pH up to 6 and then became nearly constant. This result suggested that the uncharged form of berberine was adsorbed on XAD-7 because aqueous concentration was the same as concentration of uncharged form over pH 6 due to 2.47 pK_a value of berberine.

If only neutral form of berberine could be adsorbed, the equilibrium on the adsorbent could be expersed as [11, 16]:

$$C^+ \leftrightarrow C^o + H^+ \tag{2}$$

$$C^o + a_v \leftrightarrow C_a^o \tag{3}$$

where C^+ and C^o were the protonated and neutral forms of berberine, respectively. C_a^o and a_v were the adsorbed berberine and sorption site, respectively. The total concentration of the dissolved berberine, C, is

$$C = C^o + C^+ \tag{4}$$

Based on the Hendersen-Hasselbach relation [16], the protonated berberine concentration could be expressed as a function of pH of solution and pK_a of the berberine:

$$C^+ = C^o\, 10^{(pK_a - pH)} \tag{5}$$

To find the neutral berberine concentration, Eq. (4) could be rearranged with Eq. (5):

$$C^o = \frac{C}{(1+10^{(pK_a - pH)})} \tag{6}$$

Fig. 5 showed the adsorbent loading, Q, as a function of C^o, based on data from various pH. The result suggested that the neutral form of berberine was adsorbed onto XAD-7. This result was consistent with the result that adsorption onto XAD-7 was due to hydrophobic interactions [10]. In Fig. 5, adsorption isotherm for berberine showed a Langmuir-type appearance. This result suggested that berberine would be adsorbed at the monolayer coverage.

3. *In situ* Separation with Suspended XAD-7

To examine the possibility of *in situ* product separation, 0.5g of adsorbents was added per 50 m*l* culture medium at different growth stages of *T. rugosum* cell. Addition of adsorbents increased berberine production as shown in Fig. 6. The increase of berberine production might be due to the removal of the some nonspecific inhibitors, not of feedback regulation mechanisms because exogenous berberine concentration did not affect the berberine production significantly [17]. Berberine production was the highest when adsorbents were added at the 4th day of culture, the middle of the exponential phase of cell growth. This result suggests that the growth phase was the best time for the addition of adsorbents to stimulate berberine production.

The variation of pH after addition of adsorbents was shown in Fig. 7. When the pH went over 6 after the 13th day of culture, berberine concentration decreas-

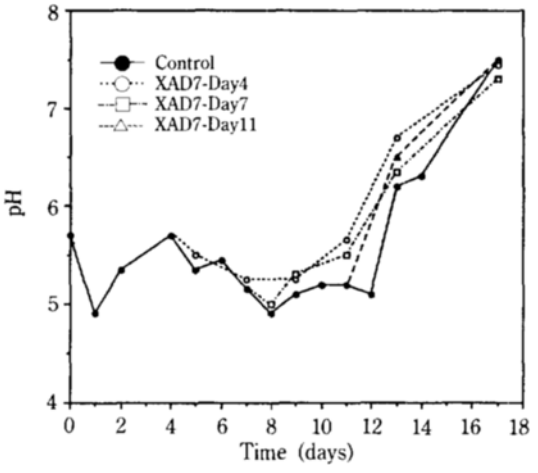

Fig. 7. Effect of suspended XAD-7 on pH.

ed. The reason for that phenomenon might be that adsorption of berberine on adsorbents is a function of pH or that degradation of berberine occurred as the cells became lysed. The effect of pH on the adsorption isotherm for berberine was shown in Fig. 5 and the neutral form of berberine could be adsorbed onto XAD-7. Since an increase of pH from 5 to 8 did not affect berberine adsorption significantly, degradation of berberine might be the main cause of the decrease of berberine concentration after the 13th day of culture.

Cell concentration was not determined due to the interference of adsorbents in this experiment. Surface fouling of adsorbents was observed during this experiment. To prevent surface fouling and to separate adsorbents from cells for the repeated use of cell and adsorbents, alginate entrapped adsorbent was investigated in the following experiments.

4. *In situ* Separation with Immobilized Adsorbent

Calcium alginate-entrapped XAD-7 was tested for berberine separation in this experiment because immobilized XAD-7 could be used easily in bioreactor operation while maintaining the advantages of the prevention of surface fouling and easy separation of adsorbents from cells for the repeated use of cell and adsorbents [17, 18]. The permeabilization process had to be integrated with *in situ* product separation process for intracellularly stored product in plant cell culture. For the repeated use of cells, a permeabilizing agent should have to be used to secrete the intracellularly stored product. Chitosan caused *Thalictrum rugosum* cells to secrete the intracellularly stored berberine by perturbing the cell membrane without damage to cells [17]. Chitosan was used as a permeabilizing agent and immobilized XAD-7 was used as a product adsorbent in this experiment.

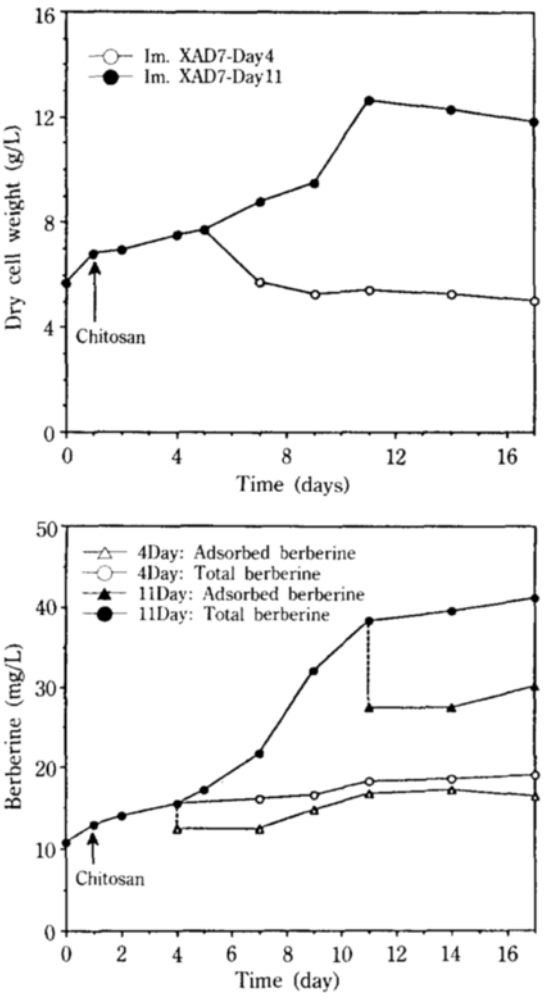

Fig. 8. (A) Influence of immobilized XAD-7 on dry cell weight in chitosan-treated cell. (B) Influence of immobilized XAD-7 on berberine production in chitosan-treated cell.

1 mg of chitosan per g fresh cell weight was added at the 1st day of culture to *T. rugosum* cells, based on the results for optimum strategy of chitosan addition. About 10g of immobilized XAD-7 (20% XAD-7 loading) was added at the 4th day of culture (exponential phase of cell growth) and the 11th day of culture (stationary phase of cell growth). Variation of cell growth in chitosan-treated cell with immobilized XAD-7 was shown in Fig. 8(A). After addition of chitosan, the cell growth rate became lower and the lag phase continued until the 4th day of culture. After immobili-

zed XAD-7 was added at the 4th day of culture, dry cell weight decreased and cell growth stopped. Though cells with XAD-7 grew rapidly without addition of chitosan and cells without XAD-7 grew with addition of chitosan in the previous experiments, cells with XAD-7 could not grow with addition of chitosan. The reason might be that proteins and electrolytes needed for cell growth as well as berberine, secreted by chitosan, were adsorbed onto adsorbent. The leakage of proteins and electrolytes by chitosan was reported [19] and a number of secreted materials could be adsorbed onto the adsorbent due to hydrophobic interactions when used in polar solvents (e.g., water) of XAD-7 [10]. To prevent growth inhibition of chitosan-treated cell, immobilized XAD-7 was added at the 11th day of culture, when cell growth reached to stationary phase.

Berberine production after addition of chitosan increased rapidly after the lag period as shown in Fig. 8(B). After immobilized XAD-7 was added at the 4th day of culture, berberine concentration increased slightly and then became nearly constant due to growth inhibition. Most of the produced berberine was adsorbed to immobilized XAD-7 with secretion of intracellular berberine but total amount of produced berberine was low. After immobilized XAD-7 was added at the 11th day of culture, when total amount of produced berberine reached to nearly maximum, total berberine concentration increased slightly and up to 70% of the berberine was adsorbed onto immobilized XAD-7. The secreted and adsorbed berberine concentration by chitosan and immobilized XAD-7 was significantly higher than extracellular berberine concentration in control culture. As more berberine was adsorbed onto immobilized XAD-7, more intracellular berberine was released. These might be due to membrane perturbation by chitosan treatment and shifting of chemical equilibrium of berberine in the medium and inside the cells. As a result, more than 70% of the berberine could be separated onto immobilized XAD-7 with the addition of the permeabilizing agent.

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NOMENCLATURE

A_s : amount of the adsorbent [g]
C : berberine concentration [mg/mL]
C_a^o : adsorbed berberine concentration [mg/mL]
C_o : initial berberine concentration [mg/mL]
C^+ : concentration of protonated forms of berberine [mg/mL]
C^o : concentration of unprotonated forms of berberine [mg/mL]
Q : resin loading capacity [mg adsorbed/g adsorbent]
V_B : liquid volume of berberine solution [mL]

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