한국생물공학회지 제4권 제3호
Korean J. Biotechnol. Bioeng.
Vol. 4. No. 3. 271-275 (1989)

Batch Time Course Behaviors of Growth and Berberine Production in Plant Cell Suspension Cultures of *Thalictrum rugosum.*

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Thalictrum rugosum 식물세포배양에 있어서 시간에 따른 세포성장 및 Berberine 생산의 변화

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ABSTRACT

Batch growth of plant cell suspension cultures of *Thalictrum rugosum* was studied to clarify the kinetic behaviors. It was found that the product formation was growth associated. The specific growth rate was 0.20-0.25 day^{-1} at the growth phase and the FW / DCW ratio was an interesting parameter which represented the status of the cells or the status of sugar concentration. The cell yield was 0.36 g cells / g sugar. The maximum berberine level was 139 mg / L of which 120 mg / L was intracellular. In terms of the specific content of berberine, the product was 1.10% of dry cell weight. At the growth phase, the relationship between the specific growth rate and sugar concentration was described well by Monod kinetics.

INTRODUCTION

Higher plants synthesize a wide range of important products including primary and secondary metabolites such as pharmaceuticals, pigments, flavors, fragrances, and pesticides. Plant derived pharmaceuticals and intermediates represent an annual United States market of 9 billion dollars at the consumer level, while aroma components represent a worldwide market of 1.5 billion dollars (1). The production of secondary metabolites via cell suspension culture has some advantages over the conventional isolation from intact plant. These advantages include: stability of supply, freedom from disease and vagaries of climate, closer relationship between supply and demand, freedom from political interference, growth of large amounts of plant tissue in minimal amount of space, ability to control the growth condition, ease of harvesting the cells, ease of extracting the desired product, and possibility of synthesis of novel products (2, 3). However, commercialization has been limited because of technological obstacles. In order to develop a successful process for the production of a secondary metabolite, a series of studies are required on factors affecting productivity such as selection of overproducing strains, optimization of process variables, development of process strategies, and bioreactor operations.

Berberine is a quaternary isoquinoline alkaloid found in several plant species and its biosynthetic pathway is well known. It has been used as antibacterial, antimalarial and stomach drug in the Orient. In addition to its clinical use, berberine is used as a fluorescent marker in several areas of medical research. Many attempts have been made to produce berberine in callus or cell suspension cultures (4, 5), mostly in Japan, and recently it is under research for the commercial production (6).

In this study, we have investigated the time course behaviors of cell growth and product formation in *Thalic-*

trum rugosum cell suspension cultures to comprehend their kinetic characteristics in detail. These data will give some ideas on the secondary metabolite production in plant cell suspension cultures. They also can be used for further study and will be helpful for the development of industrial processes.

MATERIALS AND METHODS

Plant Cell Cultures and Media

The cell suspension culture of *Thalictrum rugosum* was kindly provided by Dr. Brodelius (Institute für Biotechnologie, ETH, Zürich, Switzerland) and has been maintained on Murashige and Skoog (MS) medium prepared from MS salt mixture (GIBCO laboratories, Grand Island, NY, USA) with the addition of 2 μM of 2-dichlorophenoxyacetic acid (2, 4-D), vitamin stock solution and 30 g / L of sucrose as carbon source. The pH was adjusted to 6.0 with 1N KOH. The suspension cultures were grown in 125 ml Erlenmeyer flasks with 50ml of medium on a Gyrotory shaker (Model G10, New Brunswick Scientific Co., Inc., Edison, NJ, USA) at 200 rpm. The temperature of the culture room was 25 ℃ and exposed to 18 hr of cool white fluorescent light per day. Subcultures have been done weekly by 1 : 3 dilution.

Batch Experiment Procedures

For the batch experiment in shake flasks, cells in the late exponential growth phase, which are usually 5-6 days old, have been used. To avoid heterogeneity of the inoculum, all the cells from different flasks were collected in a preautoclaved 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. As fresh weight, 5 g of cells were inoculated into a 125 ml Erlenmeyer flask containing 50 ml of medium. Two or three replicas of flasks were sacrificed samples. After filtration, the cells were collected for cell mass measurement and intracellular product determination. The filtrates were stored in the refrigerator for extracellular product and sugar assay.

Growth Measurement

The cell suspension was transferred to a 50 ml graduated cylinder and allowed to settle for 30 min. The packed cell volume (PCV) was measured by recording the volume of the pellet as a function of the volume of the culture. For the fresh weight (FW) determination, cell suspensions were filtered and washed with distilled water. The water was removed by draining fully under vacuum until no drops of water appeared and the weight was measured on a pre-weighed aluminum weighing tray. After measuring FW, the cells were dried in an oven at 60 ℃ to constant weight to determine the dry cell weight (DCW).

Sugar Analysis

An HPLC system, SP 8000 (Spectra Physics Co., Inc., Piscataway, NJ, USA) was used for the simultaneous analysis of sucrose and its hydrolyzed products, glucose and fructose. A SUPELCOSIL LC-NH_2 column (25 cm × 4.6 mm, Supelco Inc., Bellefonte, PA, USA) was used with a refractive index (RI) detector (Perkin-Elmer Corp., Wilton, CT, USA) and a Hewlett-Packard integrator(Model 3390A, Hewlett-Packard Co., Avondale, PA, USA). The mobile phase was 75% acetonitrile and 25% water and the flow rate was 2 ml / min with constant flow mode at ambient temperature. The mobile phase was degassed by placing in a sonic bath (Branson Co., Shelton, CT, USA) before use. All the samples were filtered through a 0.45 μm membrane filter before injection.

Alkaloid Analysis

The samples for intracellular berberine analysis were collected by taking 0.5 g of cells by fresh weight. To each sample, 20 ml of HPLC grade methanol was added and the suspension was sonicated at 125 W for 10 min. The filtrates obtained during the cell mass measurement were collected for the analysis of extracellular berberine in the medium. A 0.45 μm membrane filtered sample (10 μl) was injected into the HPLC system with an UV detector (Kratos Corp., Ramsey, NJ, USA). A SUPELCOSIL LC-18-DB column was used with a Supelco LC-18 precolumn. The mobile phase was 1 mM tetrabutylammonium phosphate in water adjusted to pH 2 with phosphoric acid (60%) and acetonitrile (40%). The flow rate was 2 ml / min and the measuring UV wavelength was 265 nm.

RESULTS AND DISCUSSION

In order to clarify the kinetic behaviors of cell growth

and product formation for *Thalictrum rugosum* cell suspension cultures, a batch time course experiment was done in shake flasks. Samples were taken every day for accurate analysis of kinetic behaviors. Fig. 1 shows the time course changes of cell growth, product formation, sugar consumption, and pH. Dry cell weight went to a maximum at the 7th day after inoculation which was 15.5 g / L and decreased continuously afterward. The specific growth rate was between 0.20 day $^{-1}$ and 0.25 day $^{-1}$ at growth phase. This indicates that cell doubling time is 2.8 days when cells grow the fastest. There was a good relationship between the three different methods of cell mass measurement (PCV, FW,and DCW) before the stationary phase was reached as shown in Fig. 2. However, it is impossible to correlated these three methods after the cells stop growing.

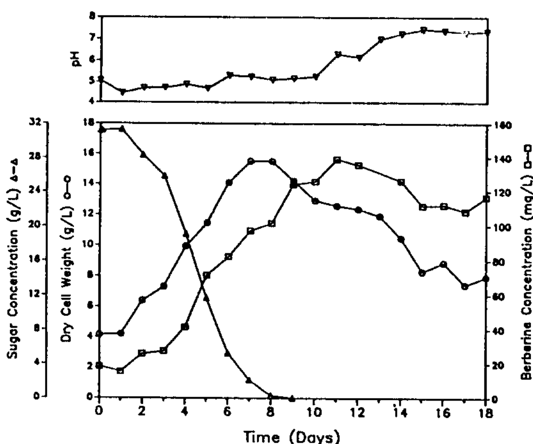

Fig. 1. Time course profiles of cell growth, berberine production, sugar consumption, and pH in batch culture of *T. rugosum* cell suspension.

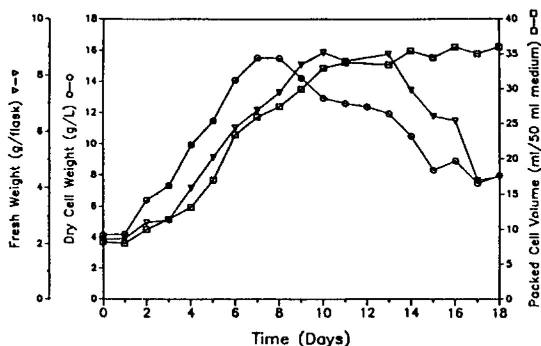

Fig. 2. Time course changes of the different methods of cell mass measurement.

If the ratio of fresh weight to dry cell weight versus culture time is plotted, the relationship is easier to see. As shown in Fig. 3, the FW / DCW ratio increased rapidly at the stationary phase of growth. The FW / DCW ratio is an interesting parameter which shows the status of the cells or the status of sugar concentration. Drapeau *et al.* (7) also found that the FW / DCW ratio increased as sucrose concentration dropped in both *Catharanthus roseus* and *Dioscorea deltoidea* cultures. This phenomenon may be either a substrate-controlled process, wherein external sugar concetration governs internal starch content, or an osmotic effect, wherein external osmotic potential governs the degree of cell expansion.

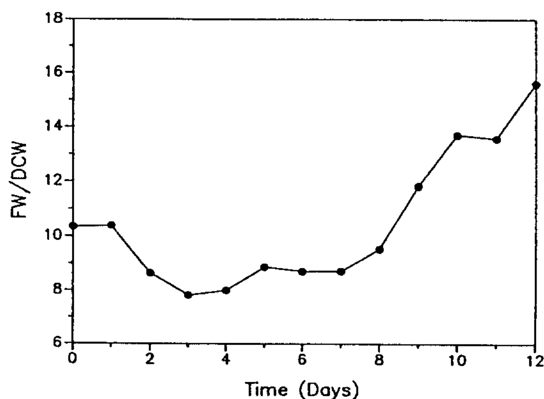

Fig. 3. Time course change of the ratio of fresh weight to dry cell weight during batch culture in shake flasks.

The increase of water content in the cell leads to an increased value of the fresh weight or packed cell volume. When the cells were grown in an airlift fermentor, this phenomenon occurred in almost the same way. However, when the cells were grown in a mechanically agitated fermentor, the FW / DCW ratio did not change although the sugar concentration dropped. The cells may not enlarge because of the shear stress (8). The characteristics of the FW / DCW ratio in the bioreactors were discussed detail elsewhere (9).

The pH of the medium did not change much during the growth phase and was between 4.5 and 5.2. After cells ceased to grow, it kept on increasing up to 7.4. This suggests that pH control during the growth phase is not quite necessary because it is stable. However, we do not know the optimum pH for cell growth and product formation. If the optimum pH does not lie between 4.5

and 5.2, pH should be controlled.

Sucrose, the only carbon source, was hydrolyzed very quickly to glucose and fructose, demonstrating the existence of a membrane-bound or an extracellular invertase. This conversion and subsequent consumption of each sugar are shown is Fig. 4. Just 2 hours after inoculation, the conversion of sucrose to its monomeric sugars was significant. The preference for glucose during the growth stage was not marked. The cell yield was calculated as 0.36 g cells / g sugar.

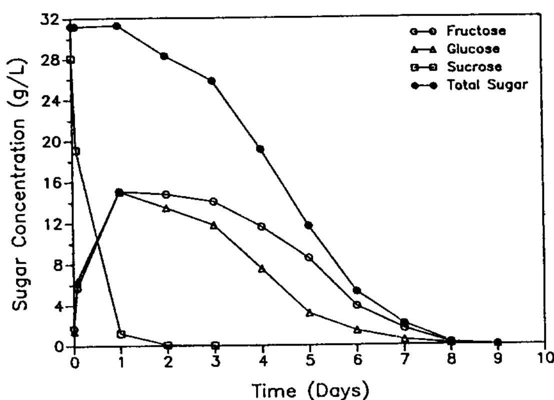

Fig. 4. Hydrolysis of sucrose and the consumption of sugars in batch culture.

The product formation was growth-associated unlike the general characteristics of the other secondary metabolites which are produced after cell growth. There were small amounts of extracellular berberine in the medium until the 10th day and its level jumped quickly at the late stage of the experiment. The distribution of berberine is shown in Fig. 5. The occurrence of alkaloids in the medium suggests that the cells have the ability to excrete the products into the medium, instead of accumulating them mainly within the vacuoles. At the cell death phase, cells became lysed and berberine was released into the medium. Before the cell lysis occurred, extracellular berberine was less than 10% of the total berberine produced. In contrast, Nakagawa *et al.* (10) reported that 89% to 92% of the berberine produced by cell suspension cultures of *Thalictrum minus* was continuously released into the medium. Therefore, the distribution characterisitics of the same products seem to strongly depend on plant species and cell strains. The distribution of products is a very important factor for downstream processing in commercialization. The total berberine level was the highest at the 11th day reaching 139 mg / L of which 120 mg / L was intracellular. In terms of the specific content of berberine, the product was 1.10% of dry cell weight.

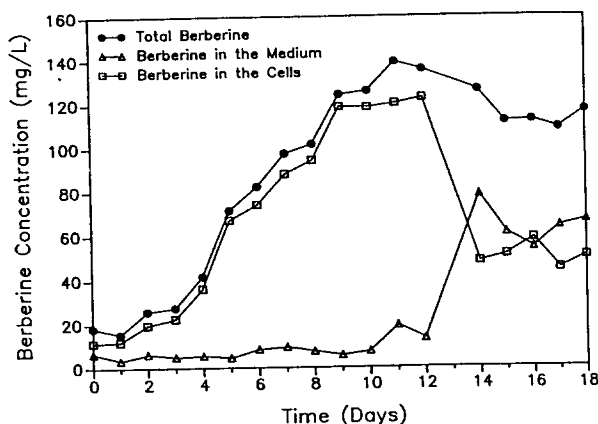

Fig. 5. Time course change in distribution of berberine during batch culture.

The growth rate and production rate were calculated by differentiating nonlinear-regressed values. This is plotted in Fig. 6. Even though there was a slight difference in times which gave the maximum rates, it is clear that berberine production is growth-associated. This was the same in other berberine-producing systems. Berberine production in *Thalictrum minus* culture proceeded almost parallelled with cell growth (11). According to Suzuki *et al.* (12), their time course experiment with *thalictrum flavum* cultures showed that berberine production started within a few days after inoculation and increased almost

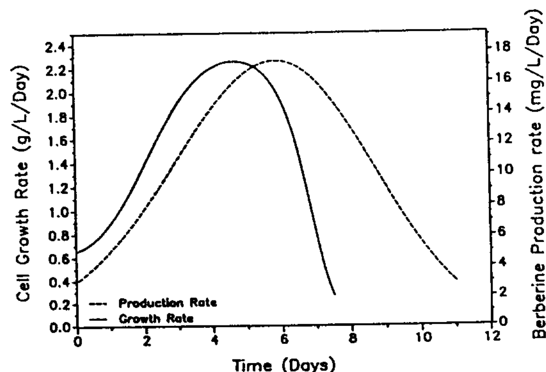

Fig. 6. Time course behaviors of growth and production rates.

in parallel with cell growth. Therefore, we can conclude that berberine is produced growth-associatedly regardless of the plant species.

At the growth phase, the relationship between the specific growth rate and sugar concentration was described well by Monod kinetics. A Lineweaver-Burk plot of the specific growth rate of *T. rugosum* in the form

$$\frac{1}{\mu} = \left(\frac{K_s}{\mu_{max}}\right)\left(\frac{1}{S}\right) + \frac{1}{\mu_{max}}$$

shows a K_s value of 6.76 g / L. This value is much higher than those for microorganisms, but is acceptable for plant cells. Plant cells in their natural environment are exposed to phloem fluid that can contain sucrose at concentration up to 250 g / L. When sugar concentration in the phloem fluid drops due to lack of photosynthetic activity, meristematic growth must slow down so that sugar can be conserved for maintenance needs (7). The Lineweaver-Burk plot for growth rate and total sugar concentration is shown in Fig. 7.

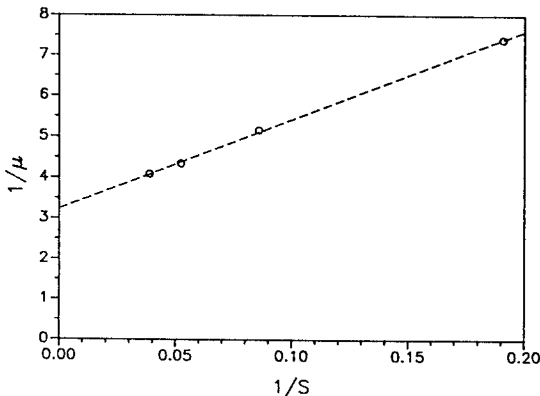

Fig. 7. Lineweaver-Burk plot for specific growth rate and total sugar concentration.

요 약

본 논문에서는 *Thalictrum rugosum* 식물세포의 회분식 배양에 있어서 동역학적 특성들을 연구하였다. 본 실험에서 alkaloid의 생성은 일반적인 2차대사 산물의 생성 특성과 달리 세포성장에 비례함이 확인되었다. 세포 성장기의 비증식속도는 0.20~0.25 day^{-1}이었으며 FW / DCW 비가 배양상태를 나타내는 중요한 인자임을 알수 있었다. Cell yield는 0.36 g cells / g sugar이었으며 berberine은 139 mg / L 까지 생성되었고 그중 120 mg / L는 세포내에 존재하였다. 이를 specific content로 계산하면 dry cell weight의 1.10%가 된다. 세포 성장기에서 비증식속도와 당농도간의 관계는 Monod kinetics로 잘 기술될 수 있었다.

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(Received November 21, 1989)