한국생물공학회지 제7권 제3호
Korean J. Biotechnol. Bioeng.
Vol. 7. No. 3. 222-228(1992)

자당 수용액을 이용한 *Thalictrum rugosum* 식물세포배양에서의 berberine 생산성 증진

김동일
인하대학교 공과대학 생물공학과

Enhanced Berberine Production in Sucrose Solution by Plant Cell Suspension Cultures of *Thalictrum rugosum*

Dong Il Kim
Department of Biological Engineering, College of Engineering, Inha University

ABSTRACT

The effects of sucrose solution on cell growth and berberine production were studied in*Thalictrum rugosum* cell cultures. Application of sucrose solution without any other components enhanced berberine accumulation significantly in spite of slower cell growth. At high sucrose concentration, cells became very compact and four-fold increase in specific berberine content was achieved. Optimum concentration of sucrose in plain water to maximize the productivity was found to be 8%. Time course experiment in 8% sucrose solution showed that more than 5 days were required to utilize the advantage of this system efficiently. Addition of vitamins, growth regulators, and inorganic salts into the solution was not effective in increasing berberine productivity.

INTRODUCTION

There are continuing interests in plant cell culture as a controllable source of a variety of important chemicals. In order to produce these compounds successfully, a series of studies are required on factors affecting productivity. First of all, the composition of the culture medium is extremely important for secondary metabolites. Particularly, sucrose is one of the most important components for cell growth as well as for the formation of secondary metabolites. In general, raising the initial sucrose levels leads to an increase in the secondary metabolite yields of cultures (1). For example, above 2% of sucrose concentration enhanced the production of indole alkaloids by not only stimulating growth but also increasing the rate of product synthesis(2). Increased sugar concentration has also been reported to be beneficial for shikonin production (3) and diosgenin production (4). Increase of sucrose concentration beyond a certain limit suppressed cell growth and alkaloid synthesis. It could be due to osmotic shock or repression of biosynthetic pathways.

On the other hand, the stimulatory effect of sucrose solution without any other nutrients on *Catharanthus* alkaloids production has been well-known for many years and it was suggested as a production medium even though growth was suppressed due to the absence of major nutrients.

Perhaps the stimulatory effect of high initial levels of sucrose in water is to raise the osmotic potential. In addition, removal of phosphate and nitrate from the medium may also be responsible for the enhancement in secondary metabolism. Generally, depletion of these nutrients is known to be associated with growth limitation and concomitant stimulation of secondary metabolism(1). The induction effect of sucrose solutions on alkaloid accumulation is also greatly dependent on the concentration of sucrose and the presence of other components. Knobloch and Berlin (5) found that solutions of 8%(w/v) sucrose were optimal, whereas at zero concentration (dilution into water) no significant alkaloid formation could be observed in *Catharanthus roseus* cultures.

In this study, although berberine production is growth-associated which is totoally different from the complete decoupling of growth and product formation in *C. roseus* culture, the same sucrose solution was applied to *Thalictrum rugosum* culture in the hope that specific berberine yield could be increased in compensation for the growth inhibiting effects.

MATERIALS AND METHODS

Plant Cell Culture and Culture Medium

The cell suspension culture of *Thalictrum rugosum* was kindly provided by Dr. Peter Brodelius (University of Lund, Lund, Sweden) 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 2,4-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 50 ml of medium on a gyratory shaker(Model G10, New Brunswick Scientific Co., Edison, NJ, USA) at 200 rpm. The temperature of the culture room was 25℃ and cultures were exposed to 18hr of cool white flurorescent light per day. Subcultures have been done weekly by 1 : 3 dilution.

Batch experiment procedure

For batch experiments in shake flasks, cells in the late exponential growth phase have been used. To avoid heterogenetiy of the inoculum, all the cells from different flasks were collected in a pre-autoclaved 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, 5g of cells were inoculated into a 125ml Erlenmeyer flask containing 50 ml of medium. Twp or three replicas of flasks were sacrificed for samples. After filtration, the cells were collected for cell mass measurements and intracellular product determination. The filtrates were assayed for extracellular product and sugars.

Growth Measurement, Sugar and Alkaloid Analysis

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 and alkaloid were analyzed as described previously(6).

RESULTS AND DISCUSSION

Sucrose Effects without Any Other Components in the Medium

Cells of *Thalictrum rugosum* were inoculated into sucrose solution (1%, 3%, 5%, 8%, and 10% in w/v) without any other nutrients, growth regulators, or vitamins. For comparison, control cultures with normal conditions were prepared. The growth results after 7 days of culture are shown in Fig. 1 and the change in the ratio of fresh weight to dry weight (FW/DCW) is in Fig. 2. In a control culture, 19.0 g/l of dry cell weight was obtained and the value of FW/DCW ratio was 9.49. As expected, plain water did not support cell growth at all, but even after 7 days of culture cell lysis could not be found. As shown in Fig. 1, sucrose solution could support cell

growth to some extent without any other nutrients. In concentrations of 3% to 8% of sucrose, cell mass almost doubled. This increase in cell mass might have come from the supply of residual nutrients which were carried over from the inoculum. Knobloch and Berlin (5) reported that their sucrose solution culture contained only the minerals, growth regulators, and vitamins, which were transferred from the parent culture with the inoculum volume and that the low concentration of these compounds probably limited growth. Sucrose solution containing 5% sucrose supported the best cell growth at 13.2 g/l as dry cell weight. Higher concentration seemed to inhibit cell growth. In *Catharanthus* culture, high sucrose concentration also inhibited culture growth under identical conditions of nutrient deficiency. The ratio of fresh weight to dry weight was strongly dependent upon sucrose concentration. The FW/DCW decreased significantly as sucrose concentration increased. This suggests high sugar concentration makes cells more compact. The FW/DCW value at 10% sucrose was much lower than half of the value at 1% sucrose (6.47 at 10% and 14.63 at 1%). In plain water, this value was even higher, i.e., 16.62. In general, FW/DCW indicates the degree of water content inside cells. At the end of normal batch culture, this value went up rapidly because of the lack of sugar. When the sugar is depleted, cells begin to accumulate water to utilize intracellular carbon sources or there may be an osmotic effect. Drapeau *et al.* (7) and Battat *et al.* (8) have also noted that the ratios of fresh weight to dry cell weight were influenced by sugar concentration although they used normal medium. The severe change in FW/DCW value is the cause of the difference in sucrose concentrations which resulted in maximum DCW and FW. By FW, 3% sucrose gave the maximum cell mass, not 5%.

In addition to a strong influence on growth, this experiment showed that the use of high concentration of sucrose as production medium was feasible. Despite a lower cell growth than in the control culture, sucrose solution at high concentration maintained a considerable increase in the production of berberine both in terms of

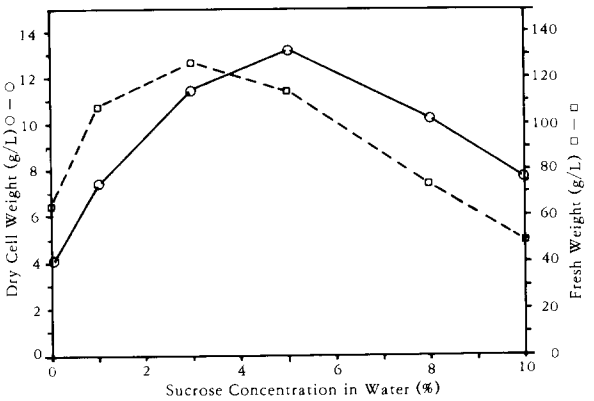

Fig. 1. Importance of sucrose concentration in water without any other nutrients on cell growth(7 days after inoculation).

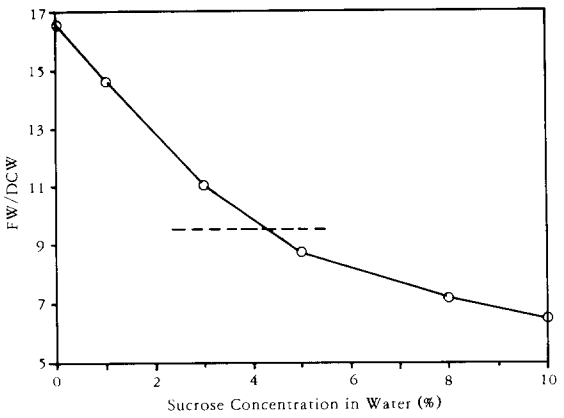

Fig. 2. Changes of FW/DCW at various concentration of sucrose in warer without any other nutrient (broken line shows the value of control which is 9.49)

volumetric and specific content. These are illustrated in Fig. 3 and Fig.4. A control culture produced 78.1 mg/l of berberine which was 0.41% of DCW. At 10 % sucrose, berberine content as % of DCW was 1. 64, partly due to low cell mass. This means that the same amount of cells produce four times higher content of berberine. This four-fold increase in specific berberine content is almost the same as the value obtained by Funk *et al.*(9) using a yeast elicitor. In order to increase the berberine content both on a volumetric basis and on a cell mass basis,

high concentration of sucrose was required. Among the concentrations examined in this experiment, 8% was the optimum as it supported the best volumetric production of berberine while maintaining growth to some extent.

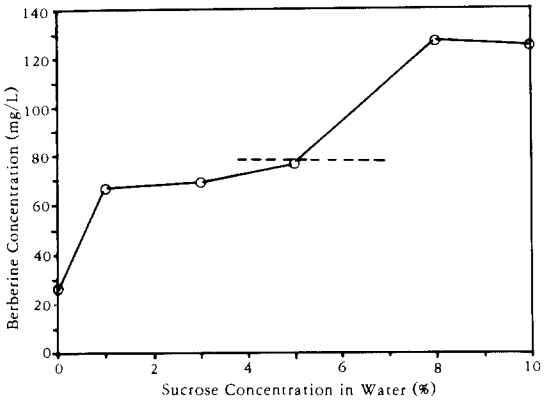

Fig. 3. Effect of sucrose concentration without nutrients on berberine production on a volumetric basis (berberine concentration of control culture was 78.1 mg/1).

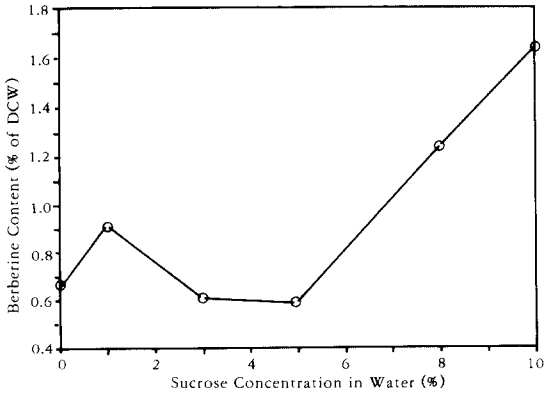

Fig. 4. Berberine production in the basis of cell mass by changing sucrose concentration in water without any other nutrients(berberine content of control culture was 0.41% of DCW).

The evident decoupling of growth and berberine production in a sucrose solution at the end of the cultivation, not in batch time course experiment, is very interesting because berberine is produced growth-associatedly in normal culture. This strongly suggests that simple increase of cell mass does not enhance product level and that the growth-associated characteristic of berberine production is somewhat different form that of primary metabolite production. Thus, the growth-associated production of secondary metabolites should be classified uniquely.

In order to use 8% sucrose solution as production medium, however, some problems have to be solved. A two-stage culture may be necessary in order to employ production medium, but careful design of the process strategy should be done because both the growth-associated characteristic of berberine production in batch culture and the decoupling of growth and product formation at the end of the cultivation in sucrose solution have to be considered together. This was already discussed previously in more detail with the results from a two-stage culture (10,11).

Time Course Behaviors with 8% Sucrose Production Medium

For the strategy utilizing an 8% sucrose production medium, precise responses of the culture should be known to optimize berberine production. The exact time when the culture produces the maximal amount of product has to be found to enhance the productivity of the process. A time course experiment in 8% sucrose solution was carried out to manifest the exact behavior of the culture. The inoculation density in the beginning was about 13 g/1 as dry cell weight so as to simulate a two stage culture. The trends for cell growth, berberine production, and sugar consumption are illustrated in Fig. 5. Until the 5th day of the culture, cell mass did not increase much. After that time, a sudden increase was observed in two days and 23 g/1 of final cell mass was obtained. Knobloch and Berlin (5) reported that there was a similar lag-phase of two days after cell transfer to 8% sucrose solution in *Catharanthus roseus* culture.

It is of interest to follow berberine production under conditions completely different from the general growth related pattern of batch culture in

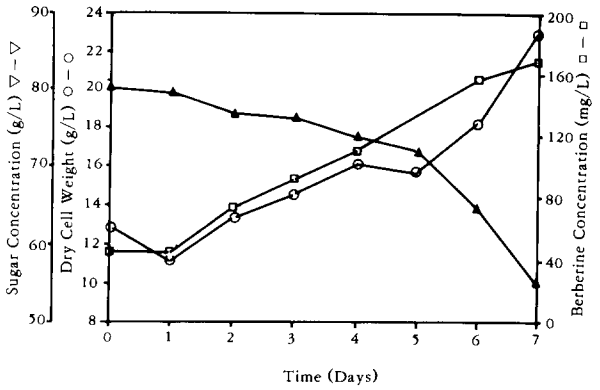

Fig. 5. Time course changes of cell mass, berberine production, and sugar consumption in 8% sucrose solution.

normal medium. Even though cell mass did not increase much until the 5th day, berberine production was continuously increasing regardless of growth. On the contrary, the rapid increase of cell mass did not promote berberine production between the 5th and 7th day. Therefore, we can say that the slow growth rate or suppression of growth due to the omission of nutrients other than sugar promotes product formation significantly and that a minimum of 5 days are required after media change to maximize berberine production if we use 8% sucrose solution as a productioin medium. The consumption of suger was also faster after the 5th day. The cell yield was 0.41 g cells/g sugar which was slightly higher than that in normal medium. The pH was maintained between 5.0 to 6.1 even though there were no inorganic salts.

Changes in FW/DCW ratio were as expected based on the previous discussion. This is shown in Fig. 6. The production of berberine is displayed again in Fig. 7 as specific content. The decrease of berberine content after the 6th day was due to the rapid cell mass increase without concurrent increasase of product. Fig. 8 shows the distribution of berberine and explains that strict intracellular accumulation is maintained. As depicted in Fig. 9, the hydrolysis of sucrose and consumption of monomeric sugars was similar as that seen in normal medium(12).

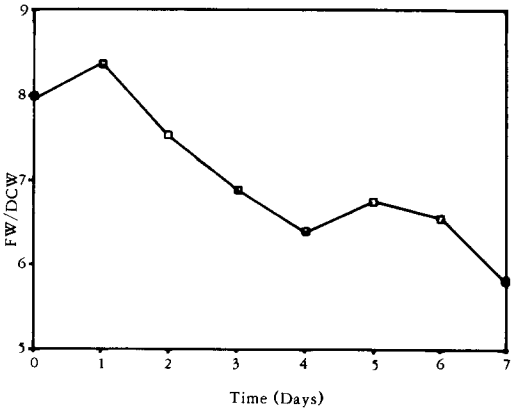

Fig. 6. Changes of FW/DCW ratio in 8% sucrose solution.

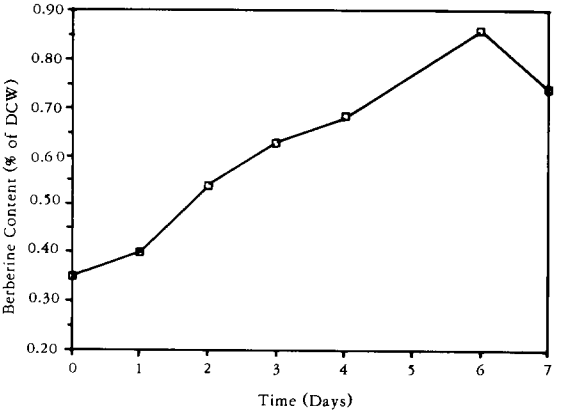

Fig. 7. Profile of berberine production as specific content.

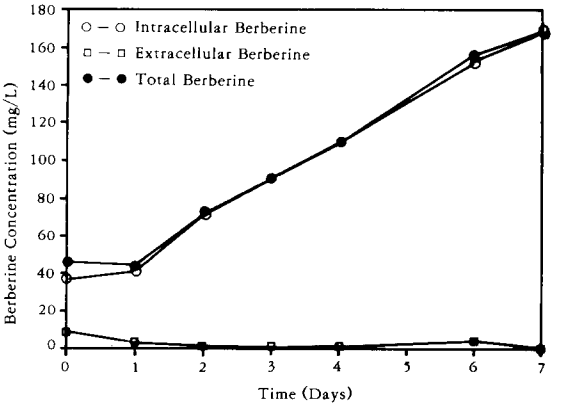

Fig. 8. Distribution profiles of berberine in 8% sucrose solution.

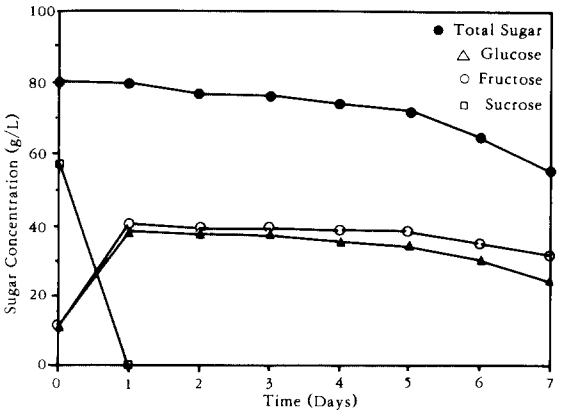

Fig. 9. Hydrolysis of sucrose and consumption of each sugar in 8% sucrose solution.

In brief, increase of product level with severe loss of cell growth is apparent in 8% sucrose solution and more than 5 days are required after media change to utilize the advantage of this system efficiently.

Effects of Other Components in 8% Sucrose Productioin Medium

The effects of vitamins, growth regulators, and inorganic salts were examined to find out if any one of them could enhance the productivity. All the samples were collected after 7 days of culture. Normal vitamins used in MS medium, 2,4-D, IAA, and MS salts were added separately or in combination into 8% sucrose solution. As can be seen in Fig. 10, Fig. 11, and Fig. 12, addition of vitamins and growth regulators such as 2,4-D and IAA had no effect either on growth or berberine production.

With regard to cell growth, addition of inorganic salts was favorable in producing as high as 25.1g/l of cell mass. However, it was of as good with respect to specific product formation because the volumetric level of berberine was almost the same as that in the production medium without salts, despite a much higher cell mass. This result leads to an important question. Which one is more important in comparing product formation, specific productivity or volumetric productivity? Careful evaluation of productivity is necessary to decide an optimum parameter or a strategy. In general, volumetric productivity is more meaningful and considered first, but specific productivity shougld be taken into account together for the economic production. Enhancing both productivities is, of course, desirable. However, this is not always the case. If it is not true

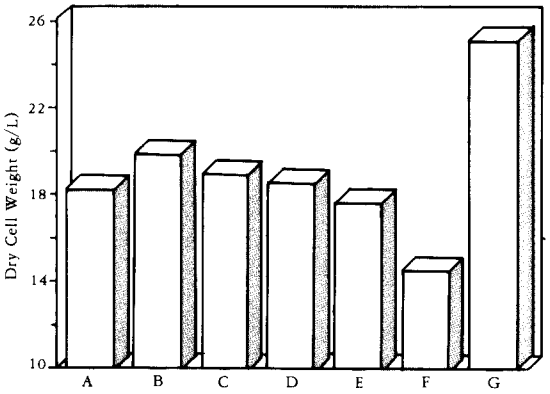

Fig. 10. Results of cell growth for 7 days after addition of other components in 8% sucrose solution ; A-8% sucrose only in water ; B-8% sucrose and vitamins in water ; C-8% sucrose and 1μM IAA in water ; D-8% sucrose, vitamins, and 1μM IAA in water ; E-8% sucrose and 2μM 2,4-D in water ; F-8% sucrose, vitamins, and 2 μM 2,4-D in water ; G-8% sucrose, vitamins, 2 μM 2,4-D and Murashige and Skoog salts in water(normal M&S medium with 8% sucrose).

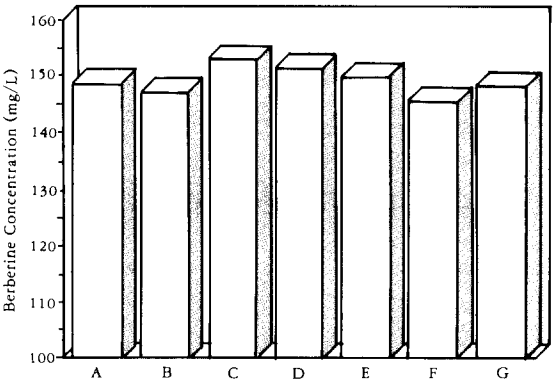

Fig. 11. Influences of other components on volumetric production of berberine. Descriptions of each item are the same as those in Fig. 10.

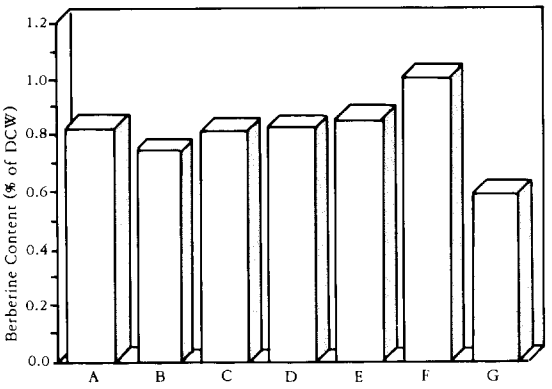

Fig. 12. Influences of other components on specific production of berberine. Descriptions of each item are the same as those in Fig. 10.

for a certain case, a careful consideration has to be made combining other factors such as economics, ease of process, etc. In this context, fed-batch culture with sugar feeding can be another good choice by reason of the following points. High sugar concentrations may support a high cell density as in case G in this experiment with high level of volumetric berberine production. In addition, the process is much easier and more economical than two stage culture from an engineering point of view.

ACKNOWLEDGMENT

This work was supported by a research grant from Inha University. The support is deeply appreciated.

요 약

Thalictrum rugosum 세포배양에서 세포증식 및 berberine 생산에 미치는 자당 수용액의 영향을 연구하였다. 순수한 자당 수용액내에서는 무시할 만한 세포증식에도 불구하고 상당한 berberine 생성의 증가가 있었다. 자당의 농도가 높은 경우 세포의 수분함유량이 매우 낮아졌으며 세포당 berberine 생성량은 4배나 증가되었다. 최적 자당 농도는 8%로 확인되었다. 8%자당 수용액상에서의 회분배양 결과, 이 방법의 장점을 최대한 효과적으로 이용하기 위해서는 접종후 최소 5일 이상은 지나야 함을 알 수 있었다. 비타민, 생장조절제, 무기염류 등의 첨가는 생산성 향상에 도움이 되지 않았다.

REFERENCES

1. S. H. Mantell and H. Smith (1983), *Plant Biotechnology*, (S. H. Mantell and H. Smith, eds), 75, Cambridge University Press, Cambridge.
2. J. M. Merillon, M. Rideau, and J. C. Chenieux (1984), *Planta Med.*, **50**, 497.
3. Y. Fujita, Y. Hara, T. Ogino, and C. Suga (1981), *Plant Cell Rep.*, **1**, 59.
4. B. Tal, J. Gressel, and I. Goldberg (1982), *Planta Med.*, **44**, 111.
5. K.Knobloch and J. Berlin (1980), *Z. Naturforsch*, **35c**, 551.
6. D.-I. Kim, H. Pedersen, and C.-K. Chin (1991), *Biotechnol Bioeng.*, **38**, 331.
7. D. Drapeau, H. W. Blanch, and C. R. Wilke (1987), *Planta Med.*, **53**, 373.
8. E. Battat, J. S. Rokem, and I. Goldberg (1989), *Plant Cell Rep.*, **7**, 652.
9. C. Funk, K. Gügler, and P. Brodelius(1987), *Phytochemistry*, **26**, 401.
10. D.-I. Kim, H. Pedersen, and C.-K. Chin (1990), *J. Biotechnol.*, **16**, 297.
11. D.-I. Kim, H. Pedersen, and C.-K. Chin (1991), *J. Biotechnol.*, **21**, 201.
12. D.-I. Kim(1989), *Korean J. Biotechnol. Bioeng*, **4**, 271.