Biotechnol. Bioprocess Eng. **2000**, 5: 350-354

Growth Promotion of *Taxus brevifolia* Cell Suspension Culture Using Conditioned Medium

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Abstract The growth promotion of a *Taxus brevifolia* cell suspension culture was investigated using conditioning factors. The conditioning factors produced and secreted from cultured cells usually stimulate cell division and the production of secondary metabolites. Therefore, the effective incubation time for the optimal secretion of conditioning factors was firstly determined for the promotion of cell growth. Conditioned media obtained by cultivating for 2 and 5 days showed the promotion of initial cell growth during the early cell growth period. However, the positive effect of the conditioning factors on the initial cell growth did not continue because of the depletion of the medium nutrients. Accordingly, the addition of a carbon source to the conditioned medium prolonged the positive effect on the cell growth. The addition of sucrose to the conditioned medium resulted in the maximum cell density being reached 4 days earlier compared to the control group and an increased substrate yield.

Keywords: conditioning factor, conditioned medium, *Taxus brevifolia*

INTRODUCTION

As the sources of various biochemical substances, higher plants have been used to produce pharmaceuticals, flavors, fragrances, and insecticides. Traditionally, these substances have been obtained using direct extraction from the intact plant on a large scale; however, certain of these substances are actually produced through chemical syntheses. Plant cells grow faster than the plants itself, are not limited to geographic or weather conditions, and can promote the production of secondary metabolites by metabolic regulation under optimal conditions through artificially controlling the environment [1]. However, the comercialization of plant cell cultures is not yet possible due to the difficulty of large-scale cultivation, low yields, the instability of cell lines, relatively slow growth characteristics compared to microbial cells, and difficulties in purification and separation [2].

Although secondary metabolites do not affect the important basic metabolism of cells, they help in the physiological activation of cells and carry out the function of a defense mechanism against various pathogens. The biosynthetic pathway of these secondary metabolites of plants is very complicated, implying a complex relationship among various enzymes related to the production of the metabolites [3]. Plant cell biotechnology is currently being considered as a method of producing secondary metabolites and related research on increased productivity at a reduced cost needs to be continued [4]. In addition, to increase the productivity of secondary metabolites, researches are progressing in bioreactor design, optimization of culture conditions, elicitation, signal transduction, and redirecting the biosynthetic pathway in order to scale up and commercialize the plant cell culture process [5].

At the time of inoculation, a plant cell culture needs to maintain a minimum inoculation concentration. If the inoculation level is under the minimum concentration, the initial cell division becomes difficult since the conditioning factors that are either secreted from the cells or present in the culture do not accommodate the conditions required for growth. Normally, it is the cell that produce the conditioning factors that change the surrounding environment to accommodate cell growth. However, if a conditioned medium containing these conditioning factors is provided, inoculation under the minimum concentration would be possible and cell growth could be promoted [6,7]. Yet, these conditioning factors have not been clearly identified, and only a few potential substances have been reported. When the initial cell concentration is relatively low and the phosphate concentration in the medium is high, cell growth has been observed to be reduced, indicating that an excess amount of phosphate acts as a negative conditioning factor for cell growth [8]. In contrast, Sun *et al.* [9] reported that extracellular calmodulin promotes plant cell growth through a physiological interaction with an unknown extracellular component or various factors in the cell wall. Conditioning factors not only promote cell growth but also increase productivity of the secondary metabolites. When anthocyanin is produced by a strawberry suspension culture, the productivity has been reported to increase with a conditioned medium

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and the ratio of the anthocyanin components changes [6,10]. The growth-stimulating effects of these conditioning factors and increased productivity of secondary metabolites has been reported to be possible in various types of plant cells [7]. Another study related with this subject reported that anthocyanin production from a rose suspension culture was enhanced when using a conditioned medium obtained from a strawberry suspension culture [11].

Accordingly, the present study attempted to improve productivity by promoting the growth of a *Taxus brevifolia* cell culture, which produces the plant-originated anti-tumor agent paclitaxel. Therefore, a conditioned medium was introduced to obtain growth conditions without a lag phase during the initial growth period and prolong the growth-stimulating effect for the promotion of cell growth.

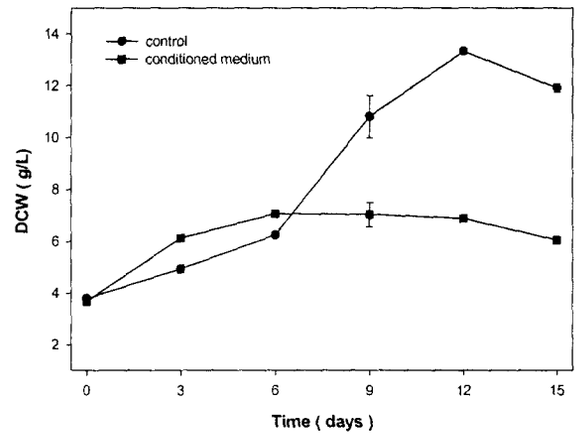

Fig. 1. Effect of conditioned medium on initial cell growth in suspension culture of *T. brevifolia*.

MATERIALS AND METHODS

Suspension Cell Culture and Media

The *Taxus brevifolia* (Pacific yew) suspension cell culture used in the present study was induced from an explant of a Pacific yew tree provided by Dr. Henrik Pedersen (Rutgers University, USA) and the cells were grown in Schenk and Hiderbrandt (SH) medium [12] with the addition of 20 g/L of sucrose as the carbon source. Five mg/L of α-naphtaleneacetic acid (NAA) and 0.2 mg/L of 6-benzylaminopurine (BAP) were added as growth regulators along with a vitamin concentrated solution supplement. The pH was set at 5.8 with the addition of 1 N NaOH before autoclaving and a subculture was done every 12 days. The cell culture was performed in a rotary shaker under dark conditions at 120 rpm while maintaining a temperature of 25°C.

Preparation of Conditioned Medium

The conditioned medium was prepared from the *T. brevifolia* suspension cell culture. Eighty mL of the culture broth including cells was inoculated into 170 mL of the new culture medium and subculture was taken every 12 days. Based on the time lapse after each subculture, different conditioned media were made by separating the cells and the culture in a vacuum using a Whatman No. 1 filter in a Buchner funnel under sterile conditions.

Analytical Methods

After the removal of the culture medium in a vacuum using a Whatman No. 1 filter paper, the cells were washed with the same amount of distilled water twice to eliminate any remaining sugar on the cell surface and then dehydrated under vacuum. The filtered cells were transferred to an aluminum weighing-tray and the fresh cell weight (FCW) was measured using a chemical balance. After measuring the FCW, the cells were dehydrated in a drying oven at 60°C until a constant weight was achieved then the dry cell weight (DCW) was measured.

To determine the amount of sugar used relative to the cell growth, the concentrations of sucrose, glucose, and fructose were analyzed using an HPLC system consisting of a pump (Model 910, Young-In Scientific Co., Korea), refractive index (RI) detector (Waters Co., USA), and a carbohydrate column (4.6 × 250 mm) as a stationary phase. As for a mobile phase, a mixture of acetonitrile and water at the ratio of 80:20 was used under isocratic conditions with a flow rate of 1.0 mL/min.

RESULTS AND DISCUSSION

Effect of Conditioned Medium in *Taxus brevifolia* Suspension Culture

To observe the initial cell growth of *T. brevifolia* due to a conditioned medium, experiments were conducted using the conditioned medium obtained 5 days after subculture. As shown in Fig. 1, when the conditioned medium obtained on the 5th day after subculture was used, the results showed no lag phase, which is normally experienced during the initial cell growth period, plus the initial cell growth increased compared to that of the control group. The maximum cell concentration was seen on the 6th day after applying the conditioned medium, after which there was no more cell growth and the stationary phase finished. This result may be due to intracellular sugar depletion, as shown in Fig. 2. When the specific growth rate was calculated on the 3rd day, the rate was 0.08 and 0.11 day^{-1} for the control group and the group using the conditioned medium, respectively, thereby indicating that the specific growth rate increased by 38% when the conditioned medium was used.

A conditioned medium usually promotes cell growth. Immobilized *Catharathus roseus* cells cultured in the

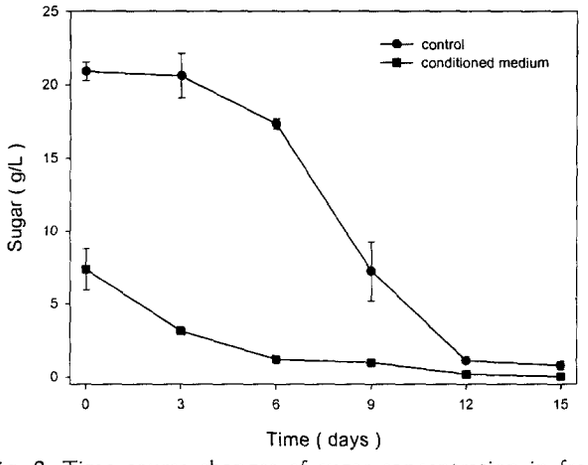

Fig. 2. Time course changes of sugar concentration in fresh medium and conditioned medium.

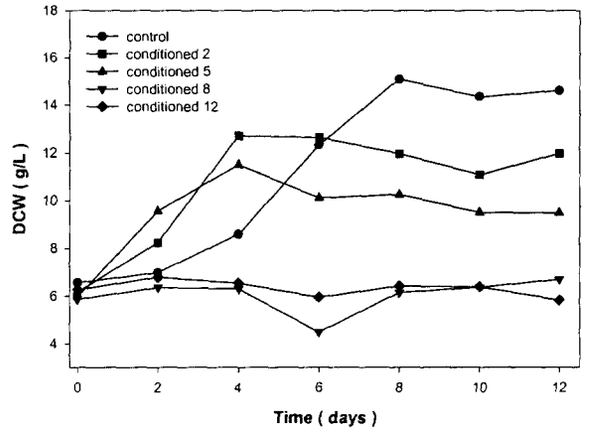

Fig. 3. Appropriate conditioning time for initial cell growth promotion in *T. brevifolia* suspension culture. The conditioned numbers (2, 5, 8, 12) indicate the conditioning period (days).

presence of a conditioned medium enhanced both the fresh-weight accumulation and the growth rate [13]. However, in the previously published reports [6,10], no clear effects of conditioned media on strawberry suspension cell growth were obtained during the early period of cultivation. According to Mori and Sakurai [7], the plant type is a determining factor as to whether or not a plant is capable of stimulating cell growth.

Determination of Optimal Conditioning Time

Fig. 3 shows the results of the experiment conducted to determine the most effective conditioning time for the initial cell growth of *T. brevifolia*. As the conditioning periods, 2, 5, 8 and 12 days were applied for the conditioned medium, representing the lag phase, initial exponential growth phase, late exponential growth phase, and stationary phase, respectively. In the case of the conditioned medium obtained on the 2nd and 5th

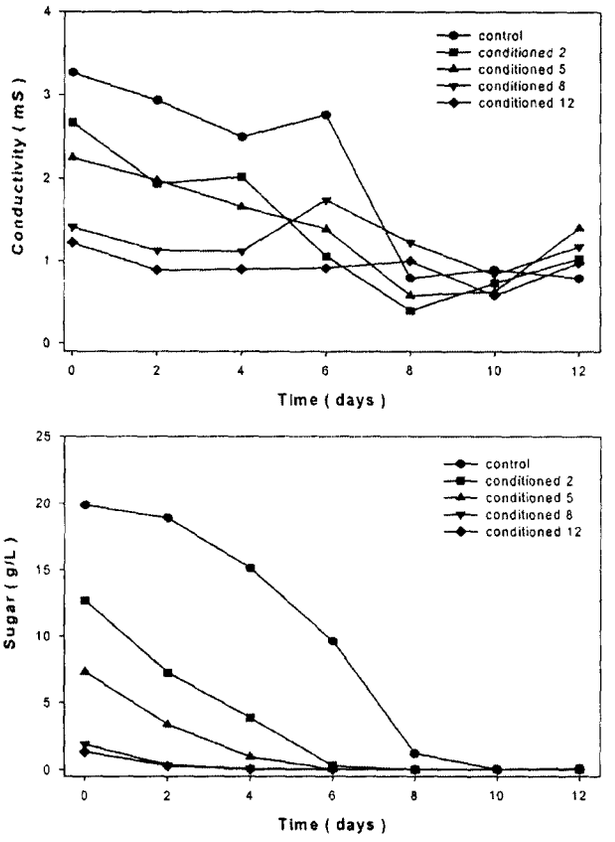

Fig. 4. Time course change of sugar concentration and conductivity in various conditioned media. The conditioned numbers (2, 5, 8, 12) indicate the conditioning period (days).

day, the initial cell growth was promoted, whereas with the other days, the growth was reduced compared to that of the control group. During the initial two days when the lag phase was shown in the control group, the growth effect was good and the best effect was seen in the conditioned medium obtained on the 2nd day. This result was due to the fact that the cell growth did not continue because of a relative lack of nutrients within the conditioned medium obtained on 5th day. As shown in Fig. 4, when the pattern of conductivity change was examined, since the amount of salt did not appear to be short, the depletion of sugar would seem to have caused the key effect. However, the maximum cell concentrations in both of the two conditioned media were low compared to that of the control and the positive effect on the initial cell growth did not last. Therefore, to maintain the effect of stimulating the initial cell growth, the supply of additional nutrients would appear to be necessary. As a result, cell growth without a lag phase and a higher maximum cell concentration could be achieved. In addition, the effect of the intracellular salt concentration on growth also needs to be considered. All subsequent experiments used the conditioned medium obtained on the 5th day.

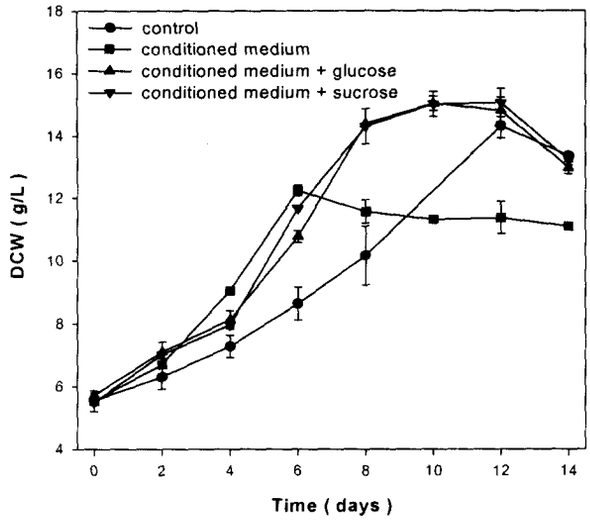

Fig. 5. Time course change of dry cell weight on effect of sugar addition into conditioned medium during lag phase.

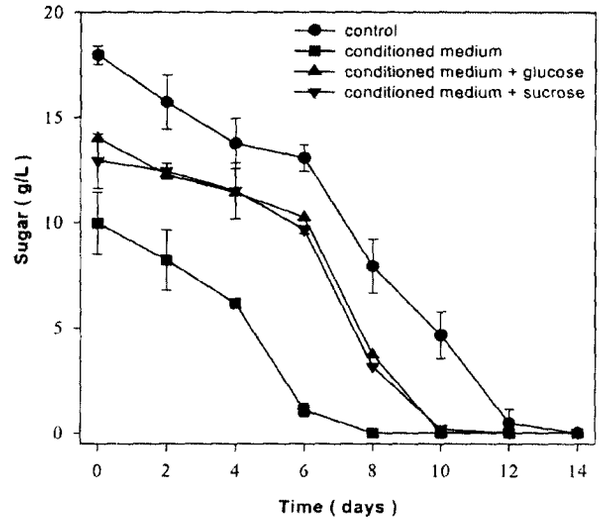

Fig. 6. Time course change of sugar concentration on effect of sugar addition into conditioned medium during lag phase.

Addition of Sugar for Extension of Conditioning Effect

It was confirmed that the initial growth-stimulating effect due to the conditioning factors did not last because of the lack of nutrients. Thus, an experiment to prolong the production promoting effect was conducted by adding the conditioned medium before the minimum inoculum density was achieved. Compared to the initial sugar concentration of 20 g/L in the control culture, glucose and sucrose were added separately in the conditioned medium so that the predicted sugar concentration would be 15 g/L. The effects of the sugar addition in the conditioned medium on the cell growth are shown in Fig. 5. Although an increase in the initial cell growth was observed compared to that of the control when the conditioned medium was used as a growth medium, the cells rapidly entered into the stationary phase on the 6th day when no additional sugar was used. In contrast, when glucose or sucrose was additionally provided, a steady cell growth was observed and the same maximum cell concentration as that obtained in the control culture was achieved 4 days earlier. Fig. 6 shows that the initial sugar concentration after adding sugar into the conditioned medium was 15 g/L and Fig. 7 shows that the osmotic difference relative to the sugar concentration difference was maintained until the latter stage of the culture, indicating that the cell growth occurred more actively and effectively despite a relative low concentration of sugar during the initial stage. When only the conditioned medium was used, the sugar ran out on the 6th day of cultivation, reconfirming that the cell growth ceased due to the depletion of sugar. To confirm that the effective cell growth occurred due to the conditioning factors, the cell yield against the substrate was also analyzed. The result, as shown in Fig. 8, shows that the yield increased by 0.34 compared to that of the control when additional sugar was provided to the conditioned medium.

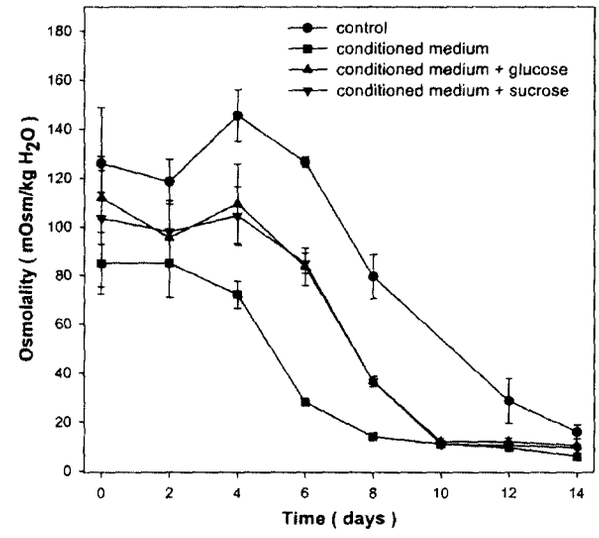

Fig. 7. Time course change of osmolarity on effect of sugar addition into conditioned medium during lag phase.

Effect of Sugar Addition Time

Based on the results observed for prolonging the growth-stimulating effect due to the conditioning factors through adding sugar during the initial culture stage, the possibility as to whether a similar effect would be seen with the addition of sugar after the stationary phase was also investigated. Therefore, the effect on the growth with the addition of glucose and sucrose was examined when the conditioned medium was used 4 days after the beginning of the stationary phase. For the control culture, glucose and sucrose were added into the same volume of SH basal medium. As shown in Fig. 9, no similar growth-stimulating effect was observed when the sugar was added during the sta-

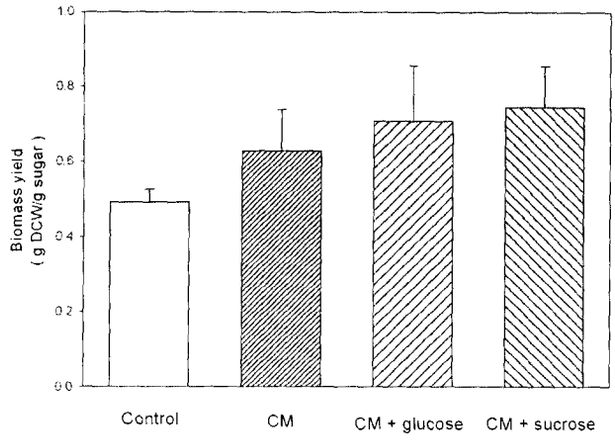

Fig. 8. Comparison of biomass yield on effect of sugar addition into conditioned medium during lag phase.

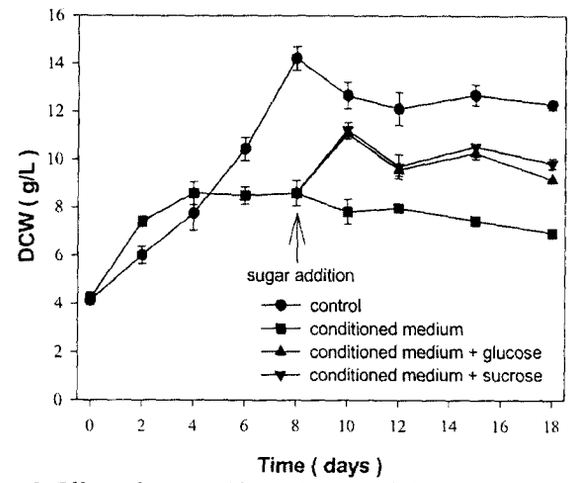

Fig. 9. Effect of sugar addition into conditioned medium during stationary phase.

tionary phase as when the sugar was added during the initial stage, and only the maintenance of the stationary phase was observed.

CONCLUSION

Initial cell growth stimulation with the application of a conditioned medium in suspension cultures of *Taxus brevifolia* was investigated. The initial cell growth-stimulating effect observed the using conditioned medium was not a supplemental effect of low osmotic pressure but rather the result of the conditioning factors. For suspension cultures of *T. brevifoila*, the optimal conditioning time for the preparation of the conditioning factors applicable for cell growth promotion was 5 days. An important factor for maintaining the initial cell growth-stimulating effect due to the conditioning factors was an additional supply of sugar. This additional sugar supplementation promoted cell growth, decreased the time taken to reach the maximum cell concentration, and increased the substrate yield. The time when the sugar was added to the conditioned medium was important and was found to be more effective when it was during the initial culture stage.

Acknowledgements This work was supported by special research funding for biotechnology program from Inha University (2000).

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[Received August 9, 2000; accepted October 3, 2000]