Kinetic Studies of Paclitaxel Production by Taxus canadensis Cultures in Batch and Semicontinuous with Total Cell Recycle

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Suspension cultures of Taxus canadensis were elicited with methyl jasmonate (MJ) under defined headspace ethylene concentrations. Kinetic studies of growth, nutrient consumption, pH variation, and paclitaxel accumulation were conducted in batch cultures and semicontinuous culture with total cell recycle. A dramatic increase of paclitaxel was obtained when the cultures were elicited with 100 μM MJ, but cell growth was thereby arrested. Supplementation of acetyl-CoA and MJ to the culture proved to be another way to improve paclitaxel yields. Using semicontinuous culture with total cell recycle, paclitaxel accumulation was increased by a factor of 4.0 relative to that in the batch culture during 35 days of cultivation.

Introduction

Higher plants are suppliers of indispensable raw materials and drugs in food and pharmaceutical industries. Among these is Taxol (generic name paclitaxel), which has been approved by the FDA for use in treatment of lung, ovarian, and breast cancers; clinical trials are underway for treatment of other cancers. While many academic and industrial research groups around the world are pursuing a plant cell culture route of production, Phyton (Ithaca, NY) is leading the development of a plant cell culture process for production of paclitaxel. With their German subsidiary, Phyton Gesellschaft für Biotechnik GmbH, a large-scale (75 m³) process is being developed (Dörnenburg and Knorr, 1995) under license to Bristol-Meyer Squibb, which has committed $25 million for FDA approval of a commercial process.

The accumulation of paclitaxel and other taxanes is thought to be a biological response to specific external stimuli. Our past work, which has identified approaches that enhance paclitaxel productivity in cell culture, established a complex interdependence of ethylene and methyl jasmonate (MJ) in affecting paclitaxel biosynthesis (Mirjalili and Linden, 1996; Phisalaphong and Linden, 1999). The optimal paclitaxel production in suspension cell cultures of Taxus canadensis was obtained using 7 ppm ethylene, 15% O₂, and 0.5% CO₂ headspace gas composition, with 100–200 μM MJ elicitation after 8 days of growth.

Another consideration for production-scale fermentation is the process strategy. Preliminary kinetic studies reported here indicate that paclitaxel production is nongrowth associated. When growth is minimal, conditions favor the production and accumulation of paclitaxel. Therefore, prolonging the stationary phase of the cultures was attempted to increase volumetric productivity. However, the long-term maintenance of the batch culture is not possible since nutrients are depleted along with the accumulation of toxic products. Continuous processes can be used to maintain cell growth at steady state. However, the productivity of a CSTR would be limited due to the loss of cells with the outlet stream. Continuous culture with cell recycle may be used as an alternative way to maintain higher productivity over a long period.

In this work, spinner flasks provided an ideal, totally inert cell growth environment with a concave dimple in the bottom to improve circulation and turbulence. Agitation was achieved by means of a magnetically driven, suspended, flat-bladed turbine rotating at 80 rpm, the lowest speed that still maintained a uniform distribution of cells throughout the liquid. Batch operation to observe the kinetic activities in the spinner flask was performed, along with a semicontinuous culture with total cell recycle culture to check the possibility of improving paclitaxel production. The results were compared with those from cultures in shake flasks with agitation at 130 rpm in the dark at 23 °C with headspace gas composition control, as described below.

Materials and Methods

Plant Materials and Maintenance. The cell line used, Taxus canadensis Marsh (C93AD) (Ketchum and Gibson, 1996), was kindly provided by the laboratories of M. L. Shuler (School of Chemical Engineering, Cornell University, Ithaca, NY) and D. A. Gibson (USDA/ARS Plant Nutrition Laboratory, Ithaca, NY). The maintenance of the culture by transfer every 14 days showed the average specific growth rate, μ = 0.1 day⁻¹. Approximately 80% of the total taxanes in a culture were accumulated in the extracellular medium. The growth medium used was the same as that described in Ketchum et al. (1999). The maintenance of cultures was carried out as follows: 10 mL of 14-day-old suspensions (approximately 2.5 mL of cell volume) were transferred by pipetting into 40 mL of fresh medium in 125-mL Erlenmeyer flasks. The culture flasks were capped with 28-mm-i.d. Belco silicone dosures (Vineland, NJ) and kept in a New Brunswick Scientific model G-25 (Edison, NJ) shaking incubator in the dark at 130 rpm and 23 °C. Chemicals were from Sigma (St. Louis, MO).

Experimental Studies. In this study, batch and semicontinuous T. canadensis cultures with total cell
and 160 mL of fresh medium with 100 μM MJ was added. Spinner bottles were placed in a laminar incubator at between 13 and 16 g L⁻¹. Total sugar in the semicontinuous system was maintained negligible in the production phase after elicitation with 100 μM MJ. Additional MJ was not added to the system studied here, growth rate was negligible in the production phase after elicitation with 100 μM MJ (see preliminary kinetic studies discussed below). Hence, fresh medium was fed based on the sugar utilization information in the semicontinuous culture. As sucrose was depleted and the remaining sum of glucose and fructose was reduced to about 15 g L⁻¹, the medium was changed. Spinner bottles were placed in a laminar flow hood, the cells were allowed to settle, 160 mL of cell free medium in the spinner flask was taken by aspirating, and 160 mL of fresh medium with 100 μM MJ was added. MJ was added with fresh medium, because the condition without MJ would return the culture to the nonelicited state that favored growth and diminished secondary metabolite formation. Additional MJ was not added to batch cultures because concentrations greater than 200 μM were toxic to this culture. Using this strategy, the total sugar in the semicontinuous system was maintained at between 13 and 16 g L⁻¹.

Results and Discussion

Preliminary Batch Kinetic Studies. In the following kinetic studies, MJ and acetyl-CoA were added according to the following experimental design: (A) control; (B) 100 μM MJ; (C) 20 μM acetyl-CoA + 100 μM MJ. These data are given in Figure 1. After 23 days of growth, paditaxel levels increased by 40−75% in the systems treated with acetyl-CoA (Figure 1C). A preliminary batch experiment exhibited levels of paditaxel similar to control when the culture was supplemented by the precursor acetyl-CoA without elicitation by MJ (data not shown). Effects of the acetyl-CoA supplementation with MJ elicitation were comparable qualitatively to those of the MJ treatments in terms of cell growth and sugar uptake. Also note from Figure 1 that cultures without MJ addition were linear (not logarithmic) in terms of growth and sugar consumption. The growth yield (Y_\\text{xb}) decreased from approximately 0.5 in treatments without MJ (Figure 1A) to 0.3 in treatments with MJ (Figure 1B). On the other hand, product yields, Y_\\text{px} and Y_\\text{ph}, obviously improved with MJ elicitation (0.47 and 0.20, respectively) but were significantly better with precursor supplementation (0.82 and 0.32, respectively). These data were from duplicated treatments (error bars in Figure 1 represent standard deviations). Yield coefficients were calculated from the average of day 23 values.

Sugar Utilization and Cell Growth in Spinner Flask Cultures. The patterns of sugar utilization in the spinner flask (500-mL working volume) were similar to those in the shake flasks (50-mL working volume) (data not shown). Sucrose in the culture medium was completely hydrolyzed to fructose and glucose within 12 days. Glucose was utilized at a faster rate than fructose, as shown in Figure 2 for the batch process and Figure 3 for the semicontinuous process. This pattern of sugar utilization has been observed using other Taxus sp. cell lines (Hirasuna et al., 1996; Wickremesinhe and Arteca, 1994; Srinivasan et al., 1995; Mirjalili and Linden, 1995). The uptake rate of fructose could be affected by the oxygen supply rate; cells grown at the lower oxygen supply were reportedly unable to utilize fructose (Taticzek et al., 1994).

The average sugar uptake rate of the batch culture in the spinner flask was about 0.50 g L⁻¹ day⁻¹ during the first 12 days and then declined to about 0.15 g L⁻¹ day⁻¹. The total sugar uptake of the batch culture elicited with 100 μM methyl jasmonate in the spinner flask was about 9.6 g L⁻¹, which was almost the same as the value observed in the shake flasks (9.2 g L⁻¹). The patterns of sugar profiles in the semicontinuous culture (Figure 3) were the same as those in the batch culture (Figure 2).
until the transfer of fresh medium was initiated on day 16. Therefore, the total sugar profile in the semicontinuous culture with cell recycle was maintained between 13 and 16 g L\(^{-1}\); that of the batch culture gradually declined from 16 to 10 g L\(^{-1}\). Cumulative sugar uptake in the semicontinuous culture continuously increased after transfer of fresh medium into the system (Figure 4), while the cumulative sugar uptake in the batch culture increased only slightly after day 12. Over the entire 35 days of the experiment, the total sugar consumption in the semicontinuous culture was about 28 g L\(^{-1}\), 3 times that observed in the batch culture.

From the analysis of cell concentration at the end of the experiments (day 35), the cell concentration was 2.68 g dry wt/L in the batch culture and 3.11 g dry wt/L in the semicontinuous culture. In our preliminary kinetic studies and in a report by Fett-Neto et al. (1994), the total sugar uptake was closely associated with the increase in culture biomass. The total sugar consumption, which was the sole estimate of cell growth, in the semicontinuous culture was 3-fold that of the batch culture. The cell concentration was only 1.2-fold relative to the batch culture, because cells do not grow after elicitation. The difference is represented by the quantity of secondary metabolites that accumulated in the respective cultures. The cell concentrations in both the batch and semicontinuous cultures were rather low, because cell growth is dependent on MJ concentration. The spinner flask cultures were ended at 35 days for convenience. As can be seen from Figure 6 (below), the production of taxol was continuing; it would have been interesting to explore longer culture periods.

From the comparison of the batch cultures in the shake flasks and in the spinner flask, the kinetics of sugar utilization in both systems were nearly the same, but cell growth differed. Cell growth in the spinner flask was only 0.63 that observed in the shake flask. During the kinetic studies in the spinner flasks, even though the agitation was at the low speed, a large amount of cell debris was observed. Some cell debris also flowed out with medium when medium was transferred in the semicontinuous culture. These losses presumably had a partial effect on the apparent decrease in cell concentration and cell growth rate (data not shown). Cell debris, however, was not observed in cultures in shake flasks. The effects of shear stress on cell lysis and growth rate have been reported in many plant cell lines. Zhong et al. (1994) reported a decrease of Perilla frutescens viability with both shearing time and magnitude of shear rate. Majer et al. (1993) reported a strong inhibition of growth of Catharanthus roseus at agitation speeds of 4.2 rev s\(^{-1}\), although metabolic activity was still observed. From the study of T. cuspidata in a rotary wall vessel system by Sun and Linden (1999), rotation at shear stress more than 5.2 s\(^{-1}\) damaged the plant cells and eventually lead to cell death. Seki et al. (1995) and Pestchanker et al.
(1996) observed decreases of growth rate of Taxus sp. when the culture was scaled up from shake flasks into reactors.

**pH Profiles in Spinner Flask Cultures.** In batch culture, the medium pH increased rapidly from the initial pH 4.0 to 5.4 in 6 days and remained relatively constant between pH 5.4 and 5.8 (Figure 5). In semicontinuous culture after feeding fresh medium, the pH of the culture medium dropped to about 4.7, and then rapidly increased to 5.5 within 2–3 days. Overall, the pH of the culture medium was maintained at 5.6 ± 0.2 in both batch and semicontinuous cultures. This pattern of pH profiles was also observed in the preliminary kinetic studies (data not shown). Wickremesinhe and Arteca (1994), in studying suspension cultures of Taxus media, found no substantial differences between the pH values of the medium after 21 days of growth when initial media pH values were adjusted to between pH 3.0 and 7.0; the final pH at day 21 was always 5.4–5.6. Fett-Neto et al. (1994) suggested that the transient increase of the internal pH of the cells was due to the uptake of NH$_4^+$. Ion flux may stimulate the initial release of paclitaxel into the medium, as also previously proposed for the release of alkaloids by Cinchona spp. suspension (Parr et al., 1986).

**Paclitaxel Analysis.** Growth rates of the cultures in the spinner flasks were less than those in the shake flasks. In the preliminary kinetic studies, elicitation using 100 μM MJ strongly inhibited cell growth (Figure 1). Therefore, elicitation with 100 μM MJ and control of the headspace gas composition in the kinetic studies in spinner flasks were initiated 16 days after culture transfer. Profiles of paclitaxel accumulation in batch culture and semicontinuous culture were similar before the transfer of fresh medium. The maximum paclitaxel accumulation obtained in the batch culture at day 33 was 0.75 mg L$^{-1}$. In semicontinuous culture with total cell recycle, 160 mL of fresh medium with 100 μM MJ was transferred into the flask at days 16, 20, and 28 according to the sugar information (Figure 3). Although the paclitaxel concentrations were slightly diluted from the transfers of medium, significant increases were observed. The total amount of paclitaxel secreted into the medium in the semicontinuous culture with cell recycle during 35 days of cultivation reached 3.0 mg L$^{-1}$, which was 4.0-fold compared to the maximum observed in the batch culture (Figure 6). After day 20, the accumulation of paclitaxel was linear, at average rates of 0.20 mg L$^{-1}$ day$^{-1}$. Seki et al. (1995) showed that a perfusion culture
of T. cuspidata using suspension or immobilized cells in 300-mL shake flasks (100-mL working volume) without elicitation, the paclitaxel productivity increased by a factor of 10 compared to that in batch culture. However, lower concentrations were obtained (0.3 mg L⁻¹ in perfusion culture versus 1.8 mg L⁻¹ in batch culture).

Overall comparisons of the batch cultures in the shake flasks and the spinner flask and the semicontinuous culture with cell recycle in the spinner flasks are summarized in Table 1. In batch cultures, the profiles and the amounts of sugar uptake of the cultures in the spinner flask and in the shake flasks were nearly the same, but the total paclitaxel production and cell growth rates differed. No significant difference in sugar uptake was observed between cultivation in the shake flasks and spinner flask. Growth yield (Y_{px}) and production yield (Y_{px}) of the cultures in the spinner flask were only 60% of those observed in the shake flasks. The lower cell concentrations, which resulted in lower paclitaxel production in the spinner flask, may be related to differences in hydrodynamic shear. From the studies by Pestchanker et al. (1996) in suspension cultures of T. cuspidata, the productions of paclitaxel in a Wilson-type bioreactor (WR) and shake flasks were the same (22 mg L⁻¹). Cell growth in the WR was only 0.43 of that observed in shake flasks (136 vs 310 mg L⁻¹).

One barrier to the development of large-scale fermentation for plant cells is the fragile nature of plant cells when coupled with hydrodynamic shear stress necessary for adequate mixing. Shear stress is one of the important factors that alters secondary metabolism of plant cells. Shear stress may affect plant cell growth and cellular metabolism negatively or positively, depending on the level of applied shear stress, the properties of the cell line, and its physiological state (Dunlop et al., 1994). Shear stress at 2.1 s⁻¹ was found positive to paclitaxel production, compared to zero shear stress using T. cuspidata (Sun and Linden, 1999); however, greater shear stress levels resulted in physiological changes of the cells that, at the extreme, led to cell death.

From the comparison of cultures in the spinner flasks, growth yield (Y_{px}) in the semicontinuous culture with cell recycle was less than 50% of the batch culture. On the other hand, total sugar uptake and Y_{px} in the semicontinuous culture with cell recycle increased about 3-fold compared those in the batch culture. No significant difference of Y_{px} was observed between batch and semicontinuous cultures in the spinner flasks. During cultivation in the spinner flasks, dark pigmentation in the medium increased progressively, particularly after elicitation, that stopped growth and resulted in the stationary phase. Older brown-colored cell suspensions in previous studies were also observed when cells produced high levels of taxanes. This observation was also reported in T. cuspidata by Fett-Neto and DiCosmo (1996) and in callus cultures of T. baccata, T. cuspidata, and T. media (Wickremesinhe and Arteca, 1993). Darkening of the medium may be due to the release and oxidation of phenolic compounds produced by the cell cultures (Fett-Neto et al., 1994).

When coupled with elicitation, the paclitaxel yield could also be enhanced by supplementation of precursors that lead to synthesis of the paclitaxel side chain. The examination of taxane profiles in some cell lines (a larger amount of baccatin-III and 10-deacetylbaccatin-III (10-DAB) compared to paclitaxel and cephalomannine) indicated the occurrence of a metabolic block at the point of side-chain addition (Fett-Neto and DiCosmo, 1996). This was also characteristic of our T. cuspidensis (C93AD) cultures that were not elicited with MJ (data not shown). Hirasauna et al. (1996) reported that addition of 1 and 10 mM acetate in nonproducing T. brevifolia (PC2) cultures increased the level of paclitaxel from 0.08 to 0.12 and 0.17 mg L⁻¹, respectively; addition of 50 mM acetate was detrimental to the cells. Two groups independently demonstrated the ability of extracts of crude cell homogenates of T. cuspidata to convert 10-DAB into baccatin-III using acetyl-CoA (Fett-Neto and DiCosmo, 1996; Zocher et al. 1996). In this work, supplementation of acetyl-CoA and MJ to the culture proved to be another way to improve paclitaxel yields.

Conclusions

Although the kinetic profiles of sugar uptake in the spinner flask and shake flask cultures were almost the same, rates of total paclitaxel production and cell growth differed. The lower growth and production rate observed in the spinner flask cultures may be related to the differences in hydrodynamic shear. In batch cultures, growth yield (Y_{px}) and production yield (Y_{px}) of the cultures in the spinner flask dropped approximately 40% relative to those in shake flasks.

Table 1. Growth and Productivity Parameters from Batch and Semicontinuous with Total Cell Recycle Cultures of Taxus canadensis Grown with a Headspace Gas Composition of 7 ppm Ethylene, 15% O_2, and 0.5% CO_2

<table>
<thead>
<tr>
<th>treatments</th>
<th>cell dry wt (g L⁻¹)</th>
<th>sugar uptake (g L⁻¹)</th>
<th>Y_{px} (x 10⁻³)</th>
<th>Y_{px} (x 10⁻³)</th>
<th>Y_{xs}</th>
<th>paclitaxel productivity (mg L⁻¹ d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>batch in shake flasks, MJ elicitation at day 9</td>
<td>4.21</td>
<td>9.22</td>
<td>0.49</td>
<td>0.22</td>
<td>0.46</td>
<td>2.06/19 = 0.11</td>
</tr>
<tr>
<td>batch in spinner flask, MJ elicitation at day 16</td>
<td>2.68</td>
<td>9.67</td>
<td>0.28</td>
<td>0.08</td>
<td>0.28</td>
<td>0.75/19 = 0.04</td>
</tr>
<tr>
<td>semicontinuous in spinner flask, MJ elicitation at day 16</td>
<td>3.11</td>
<td>28.42</td>
<td>1.16</td>
<td>0.13</td>
<td>0.11</td>
<td>3.00/19 = 0.16</td>
</tr>
</tbody>
</table>

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Figure 6. Comparison of the paclitaxel accumulation profiles of T. canadensis in batch culture (●) and semicontinuous culture (■) with cell recycle spinner flask cultures.
Considerably improved paclitaxel production was observed when using semicontinuous culture with total cell recycle. Paclitaxel accumulation increased by a factor of 4.0 relative to that in the batch culture during 35 days of cultivation. Production yield (Ypx) and total sugar uptake in the semicontinuous culture with cell recycle increased about 3-fold relative to those in the batch culture. No significant difference of Ypx was observed between the batch culture and the semicontinuous culture with cell recycle.

Given the profiles above, higher levels of paclitaxel could be obtained by prolonging incubation using the same process strategy. The preliminary kinetic experimental results suggest that feeding precursors with fresh medium and MJ in semicontinuous culture with cell recycle or perfusion culture could be an alternative way to enhance paclitaxel production.

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References and Notes