Appl Microbiol Biotechnol (2001) 55:404–410
DOI 10.1007/s002530000567

ORIGINAL PAPER

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Enhancement of Taxol production and excretion in *Taxus chinensis* cell culture by fungal elicitation and medium renewal

Received: 12 April 2000 / Received revision: 24 October 2000 / Accepted: 3 November 2000 / Published online: 6 March 2001

Abstract An endophytic fungus, *Aspergillus niger*, isolated from the inner bark of a *Taxus chinensis* tree, was used as an elicitor to stimulate the Taxol (paclitaxel) production in a *Taxus chinensis* cell suspension culture. Different elicitor doses and elicitation times were tested in a batch culture; and the highest volumetric Taxol yield was achieved when 40 mg of the fungal elicitor (carbohydrate equivalent) l^{-1} was added to the culture during the late exponential-growth phase. The elicitation resulted in a more than two-fold increase in the Taxol yield and about a six-fold increase in total secretion. The Taxol yield was further improved substantially by applying medium renewal and re-elicitation to the culture. In particular, with repeated medium renewal (in a way similar to medium perfusion) and a second elicitation of the culture, the volumetric Taxol yield was increased to 67.1±7.5 mg l^{-1}, which was about seven times the amount obtained in the non-elicited batch culture. The Taxol productivity of the perfusion-like culture with repeated fungal elicitation was 1.5 mg l^{-1} day^{-1}, which was about 40% higher than that of the elicitor-treated batch culture and three times the productivity of the non-elicited batch culture.

Introduction

Paclitaxel (Taxol), a plant-derived diterpene amide, is one of the most prominent anticancer drugs from a plant source. It has been approved for the treatment of ovarian and breast cancers and has also shown clinical activity against a number of other notorious diseases (Woo et al. 1994). However, its natural supply from plant sources, *Taxus brevifolia* or other *Taxus* spp, is very limited (Bringi et al. 1995). The chemical synthesis of Taxol also seems to be an economically unfavorable process, due to the complexity of its chemical structure. The plant cell culture of various *Taxus* spp has been sought as a viable alternative for the mass production of Taxol and its taxane relatives. There have been considerable research efforts over the past few years contributing to understanding the limiting factors for Taxol production and finding the means to improve the Taxol yields in *Taxus* cell cultures.

Taxol and its taxane relatives are the secondary metabolites of the *Taxus* plant. Like most other plant secondary metabolites, they are usually produced in extremely small quantities in normal and intact plants. Their synthesis is often further depressed or even completely turned off in plant cell cultures. Although the depressed secondary metabolite production is still not fully understood, it is suspected that the genes coding for the enzymes catalyzing the biosynthetic reactions of the desired products are repressed in the cultured cells (Nishi 1994). Therefore, a suitable mechanism is needed to switch on the controlling genes and to initiate the secondary metabolite production. The process that induces the genetic expression of the enzymes for the secondary metabolite synthesis in plant cell culture is known as elicitation (Roberts and Shuler 1997). The elicitation of secondary metabolite production in plant cells is usually achieved by the contact of plant cells with various biotic and abiotic elicitors. The term “elicitor” was originally used in the field of plant pathology for agents stimulating any type of plant defense responses (Ebel and Mithöfer 1998). Plants are able to produce antimicrobial secondary metabolites, such as phytoalexins, in response to microbial attack (Nishi 1994). Therefore, microorganisms (particularly fungi and their constituents) have been widely used as elicitors of secondary metabolite production in plant cell cultures (DiCosmo and Misawa 1985; Chang and Sim 1994). The cell extracts and filtrates of

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four species of fungi, for example, were used for the elicitation of cell cultures of *Taxus* sp. for the synthesis of Taxol and its relatives (Ciddi et al. 1995).

Since the synthesis of Taxol and its relatives by *Taxus* spp is believed to be associated with the plant's defense reactions against fungal attack, the fungal microorganisms found in the tree may be a possible source of elicitors for Taxol synthesis in *Taxus* cell cultures. In the past few years, our group has isolated several fungal strains from various parts of the *T. chinensis* tree and has screened for the most effective elicitors to stimulate Taxol synthesis in cell cultures of *T. chinensis* (Lu et al. 1998).

In this study, one of the endophytic fungus strains isolated from the *Taxus* tree was selected as the elicitor to stimulate Taxol biosynthesis in a suspension culture of *T. chinensis* cells. The first objective of our work was to examine the effects on cell growth of elicitor dosage and the timing of elicitor addition, to examine Taxol production and release, and to determine the optimal elicitor dose and addition time for improving the Taxol production. Although the addition of an elicitor could stimulate the secondary metabolite synthesis, it could also cause growth inhibition and rapid loss of cell viability. Without a proper measure to fortify the biological activity of the culture, the stimulating effect on Taxol synthesis of an elicitor would be short-lived and very limited. In order to minimize the negative effects of the elicitor on the cells and to maximize the product yield, we applied a repeated medium-renewal strategy, which was similar to medium perfusion, for the elicitor-treated culture. This perfusion-like mode of operation, combined with re-elicitation, led to a dramatic increase in Taxol productivity.

Materials and methods

Cell line initiation and culture conditions

A Chinese yew (*T. chinensis*) from the forests of Hubei Province in China was used as the plant material for callus induction. Calli were induced from the young stems of the *Taxus* tree, using MS basal medium (Murashige and Skoog 1962) supplemented with (per liter): 0.1 mg 2, 4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg α-naphthelene acetic acid, 0.5 mg benzyl adenine, 500 mg casein hydrolysate, 30 g sucrose and 8 g agar. The medium was adjusted to pH 5.8 and then sterilized by autoclaving at 121 °C for 20 min. Suspension culture was initiated from a ninth-generation callus and cultured in a liquid medium similar to that for the callus culture, but excluding the 2,4-D and agar. The suspension culture was kept in 250-ml Erlenmeyer flasks with 90 ml of liquid in each, placed on an orbital shaker at 110 rpm and 25 °C in the dark. The shake-flask suspension culture was subcultured every 14 days, by inoculating 10 g of fresh cells into 90 ml of fresh medium.

All the elicitation and medium renewal experiments in this study were carried out in the shake-flask suspension culture. Each data point shown in the results, including the biomass density, cell viability and Taxol yield, represents the mean value of two or three independent estimates.

Elicitor preparation

The fungal elicitor was an endophytic strain of *Aspergillus niger*, which was screened out from several microorganisms isolated from the inner bark of the *T. chinensis* tree. Details of the elicitor isolation and screening processes have been given elsewhere (Lu et al. 1998). Strain identification of the fungus was mainly based on the morphological characteristics of the colony, mycelium and conidial spores formed in a solid culture. The fungus was incubated in a liquid potato-dextrose medium in Erlenmeyer flasks shaken at 120 rpm and 25 °C. After 7 days of incubation, the fungi were harvested from the shake-flasks, separated from the liquid by filtration and washed several times with distilled water. Each 5 g of the fresh fungal biomass was resuspended in 100 ml distilled water, homogenized by intense mechanical agitation and then autoclaved at 120 °C for 2 h. The sterilized fungal homogenate was used as the elicitor in the following experiments. The elicitor dose was measured by the total carbohydrate content of the fungal homogenate, which was determined by the phenol-sulfuric acid method using glucose as the standard (Dubois et al. 1956).

Batch culture elicitation experiment

In the batch culture experiment, various amounts (carbohydrate equivalents) of fungal homogenate were added to the shake-flask culture on day 15, to compare their effect on cell growth and Taxol production. Each flask, containing 90 ml of medium, was inoculated with 9 g fresh weight (fw) of cells from a normal shake-flask culture. The culture was harvested on day 21, i.e., 6 days after the elicitation. An optimal elicitor dose for Taxol production was chosen from this experiment and applied to the following elicitation experiments. The timing of the elicitor treatment was then examined by adding a chosen amount of the fungal elicitor to the culture on various days over a total culture period of 30 days.

Medium renewal and re-elicitation experiment

The experiment started with a shake-flask culture, as described above, which was treated with 40 mg of the fungal elicitor l^{-1} on day 15. On day 24 of the culture, 9 g fw of the cells collected from the flasks was inoculated into 41 ml of fresh medium in a 250-ml flask and cultured for the second cycle. During the second culture cycle, four different modes of operation were applied to the culture: the first with no further medium renewal and elicitation (1E+MR), the second with repeated medium renewal every 3 days (1E+MMR), the third with a second elicitor treatment by 40 mg of the fungal carbohydrate l^{-1} on day 6 (MR+2 E), and the fourth with both repeated medium renewal and a second elicitor treatment (MMR+2 E). For the medium-renewal culture mode, 30 ml of the spent medium (culture supernatant) was removed from each flask aseptically with a pippet and replaced with an equal volume of fresh medium. There were altogether six medium-renewals over the 24-day culture period in this mode; and the spent medium collected at each time was analyzed for secreted Taxol.

Measurement of cell density and viability

The fw was obtained by filtration and the dry cell weight (dw) by drying the fresh cell mass at 30 °C under vacuum for about 2 days, to constant weight. Cell viability (percentage of viable cells in a cell population) was determined by the reduction of 2,3,5-triphenyltetrazolium chloride (Towill and Mazur 1975).

Taxol extraction and analysis

Intracellular Taxol was extracted from the cells using a procedure similar to that reported by Seki et al. (1995), using methanol as the solvent. Taxol in the medium was extracted by dichloromethane. The solvent was then removed by evaporation under vacuum and the solid residual was re-dissolved in methanol. The Taxol content in the methanol sample solution was analyzed by reverse-phase HPLC with UV detection at a wavelength of 227 nm. The HPLC

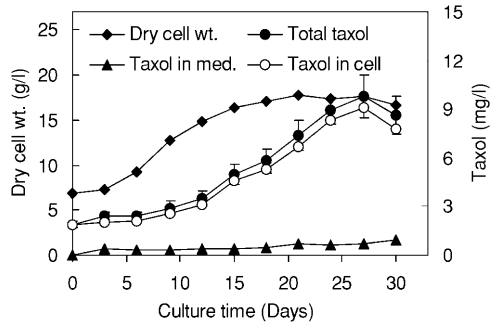

Fig. 1 Time courses of growth and Taxol accumulation in *Taxus chinensis* cell suspension culture without elicitor treatment. (*Error bars* represent standard error, based on triplicate measurements)

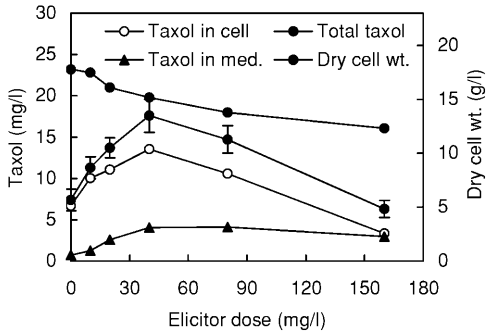

Fig. 2 Biomass and Taxol yields of *T. chinensis* cell culture treated with various doses of fungal elicitor (added on day 15 and measurement taken on day 21)

column was a 250 mm×4.6 mm Kromasil C18 with 5 µm packing. All sample solutions for HPLC analysis were filtered through a 0.2-µm membrane before injection. The mobile phase consisted of methanol:acetonitrile:water in a 25:35:45 ratio. The flow rate was 1 ml min^{-1}. The quantification of Taxol was based on an external standard of genuine paclitaxel (Sigma, St. Louis, Mo.).

Results

Cell growth and Taxol production in normal, non-elicited culture

Figure 1 shows the typical trends of cell growth and Taxol production in normal culture of *T. chinensis* cell suspensions. Cell growth reached the stationary phase around day 21. The biomass productivity or average growth rate over the growth period is given by $P_r=(X_{max}-X_o)/t$ (X_{max} = maximum cell density, X_o = initial cell density, and t = the time for maximum cell density; Wu and Ho 1999) and was about 0.85 g l^{-1} day^{-1}. The biomass growth index (ratio of maximum to initial cell density, X_{max}/X_o) was about 2.6.

The maximum Taxol concentration, 9.8 mg l^{-1}, was obtained on day 27 and was found mainly in the cells, with less than 10% secreted into the medium. The maximum Taxol productivity was 0.37 mg l^{-1} day^{-1}, estimated from the volumetric yield on day 24. The Taxol synthesis showed a lag behind the growth and increased most rapidly when the cell growth was in the deceleration and stationary phases. The inverse relationship between cell growth and Taxol accumulation has been observed previously in four other *Taxus* callus and cell cultures (Fett-Netto et al. 1993; Wickremensinhe and Arteca 1993). It indicates that secondary metabolite synthesis flourishes when the primary metabolism (biomass synthesis) is inactive.

Effects of elicitor dosage on cell growth and Taxol production

As shown in Fig. 2, the Taxol yield was increased at elicitor concentrations between 10 mg l^{-1} and 80 mg l^{-1}, while the biomass yield was depressed by the elicitor treatment at all concentrations except the lowest (10 mg l^{-1}). The culture treated with 40 mg fungal elicitor l^{-1}, for example, had an average growth rate of 0.72 g dw l^{-1} day^{-1}, about 15% lower than that of the control (0.85 g dw l^{-1} day^{-1}). The elicitor concentrations between 20 mg l^{-1} and 80 mg l^{-1} induced a pronounced increase in Taxol accumulation with a moderate depression of biomass yield. The highest volumetric Taxol yield (17.6 mg l^{-1}) was obtained at an elicitor concentration of 40 mg l^{-1}, which was about 1.8 times that obtained in the non-elicited control culture. On a per cell basis, the Taxol yield of the culture treated with 40 mg fungal elicitor l^{-1} was 1.16 mg g^{-1} cell, about 2.8 times that of the control culture, 0.42 mg g^{-1} cell. In addition, the extent of Taxol secretion was enhanced with elicitor treatment and increasing elicitor dose, e.g., Taxol release was 23% at 40 mg l^{-1} and 28% at 80 mg l^{-1}, compared to 9.5% in the non-elicited control. The increased extracellular Taxol concentration may also be attributed in part to cell lysis caused by the addition of the elicitor.

Effects of timing of elicitor addition on cell growth and Taxol production

Figure 3 shows the Taxol yield and cell viability of cultures subjected to elicitor treatment at different growth stages. As shown by Fig. 3a, adding elicitor to the culture in the early growth phase (day 9) or late stationary-growth phase (day 21) resulted in relatively little enhancement of the Taxol accumulation. The addition of fungal elicitor to the culture on day 12 or 15, which corresponded to the late exponential-growth phase, was more effective in stimulating Taxol synthesis. In all the cases tested, Taxol production in the culture showed an immediate increase upon the addition of elicitor. The highest Taxol yield was obtained in the culture treated with elicitor on day 15, with a total volumetric yield of 25.4 mg l^{-1} on day 24, which was 2.6 times that in the non-elicited control. The Taxol productivity at this elicitor dose was 1.06 mg l^{-1} day^{-1}, about 2.8 times that in the control.

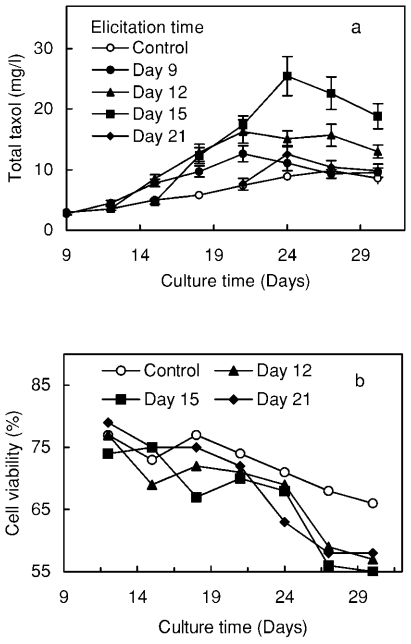

Fig. 3 Effects of elicitor addition at different culture stages on Taxol yield (**a**) and cell viability (**b**)

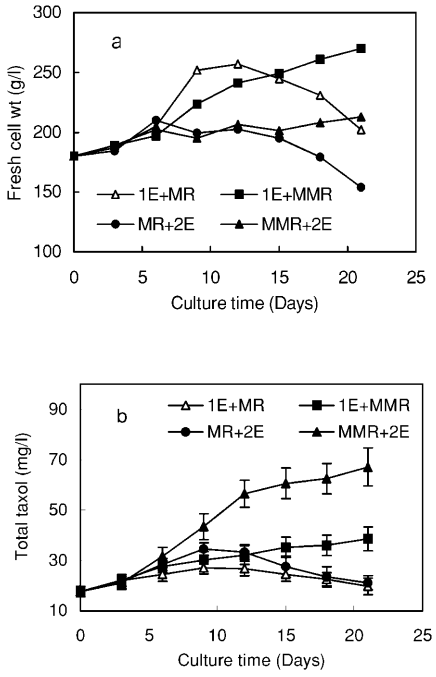

Fig. 4 Time courses of cell growth (**a**) and Taxol production (**b**) by elicitor-treated cells in a second culture cycle, with or without further medium renewal and elicitation (See Materials and methods for process codes). The Taxol yield in the 1E+MMR and MMR+2 E includes both the Taxol in the flask and that in the spent medium removed at each renewal, while the Taxol concentration is based on the liquid volume in the flask

The addition of elicitor, regardless of the timing, also caused a more rapid decrease in cell viability in the later days of the culture period (Fig. 3b). The viability usually dropped immediately upon the addition of elicitor and then recovered slightly 6–7 days later. From visual observation of the culture, we found that the culture turned brownish after the addition of elicitor and the brown color became lightened several days later, corresponding to the recovery of cell viability. Based on our experience, cells in the healthy and actively growing state were bright yellow; and cells in the late stationary and death phases usually turned darker and brownish. The later recovery of cell viability may be due to the adjustment of the cells to the presence of elicitor.

Although elicitor treatment of the cells increased the Taxol biosynthesis in the batch culture, the Taxol accumulation after the elicitation could only last for a short period of time, about 9 days (up to day 24); and it then dropped rapidly (Fig. 3a). This cessation of Taxol synthesis was usually accompanied by a rapid decline in cell viability (Fig. 3b). Besides elicitor toxicity, the loss of cell viability was most probably due to the depletion of nutrients and the accumulation of toxic metabolites in the medium.

Enhancement of Taxol production with medium renewal and re-elicitation

Figure 4 shows the time courses of cell growth and Taxol production in the elicited cultures after medium renewal. In the 1E+MR mode, i.e., no further medium exchange and elicitor treatment, the cell growth continued for almost 10 days, reaching a significantly higher biomass concentration (Fig. 4a), while the Taxol yield only showed a small rise (Fig. 4b). The addition of another elicitor dose to the culture (in the MR+2 E mode) stimulated Taxol synthesis further but inhibited biomass growth. The culture subjected to repeated medium renewal but no further elicitation (1E+MMR) showed a steady increase in biomass concentration, and a steady but slow increase in the total Taxol yield. A more significant increase in Taxol yield was achieved with the MMR+2 E culture (repeated medium renewal and re-elicitation), giving a total volumetric Taxol yield of 67.1 mg l^{-1}. Compared with the 1E+MMR culture, an additional elicitor treatment stimulated Taxol synthesis considerably but also curbed biomass growth.

In the MMR+2 E culture, the most rapid accumulation of Taxol occurred in the first 12 days, with a productivity of about 3.2 mg l^{-1} day^{-1}, which was much higher than the Taxol productivity, 2.1 mg l^{-1} day^{-1}, over the whole of the 24-day second culture cycle. In the late culture period after day 12, the Taxol curve of the MMR+2 E culture was nearly parallel to that of the 1E+MMR mode. It seems that the stimulating effect of the second elicitation lasted for about 6 days in the second culture cycle, which was shorter than the 9-day period after the elicitation in the first culture cycle (Fig. 3a).

Table 1 summarizes the Taxol yields and productivities obtained in all types of culture processes tested in

Table 1 Taxol production and distribution in various culture processes. See Materials and methods for process codes. Taxol yield and total were measured on the day when maximum total yield was achieved (*in parentheses*). The Taxol yield in 1E+MMR and MMR+2 E includes both the Taxol in the flask and that in the spent medium removed at each renewal, while the Taxol concentration is based on the liquid volume in the flask. The total yield is the mean ± standard error of triplicate results

Process mode	Taxol yield (mg l^{-1})			Specific yield (mg g^{-1} cell)	Productivity (mg l^{-1} day^{-1})
	In cell	In medium	Total		
Control	9.1	0.7	9.8±1.3(day 27)	0.57	0.36
Batch, elicited	13.6	4	17.6±2.0(day 21)	1.16	0.84
1E+MR	23.4	3.7	27.1±2.4(day 33)	1.45	0.82
1E+MMR	17.5	19.1	38.6±4.7(day 45)	1.93	0.86
MR+2 E	26.2	8.3	34.5±2.4(day 33)	2.33	1.05
MMR+2 E	17.2	49.9	67.1±7.5(day 45)	4.25	1.49

this work. The Taxol productivity of the culture with medium renewal and double elicitation, 1.49 mg l^{-1} day^{-1}, was more than four times that of the control, i.e., the non-elicited batch culture. The specific Taxol yield (on a per cell basis) was increased dramatically with the addition of elicitor, from 0.57 to 4.25 mg g^{-1} cell, a more than six-fold increase. One-off or repeated medium renewal did not increase the Taxol productivity significantly, unless the culture was re-elicited. Repeated medium renewal increased Taxol excretion from the cells into the medium most significantly; and, particularly in the MMR+2 E mode, nearly 75% of the total Taxol was collected in the medium. In addition, the repeated medium renewal also reduced the intracellular Taxol content significantly. Cell lysis was unlikely to have been the major cause of the significant increase in Taxol release observed in the MMR+2 E culture mode, because the fw showed no obvious decline (Fig. 4). However, a much smaller fraction of Taxol was released into the medium in the MR+2 E culture (Table 1), even though there was a decline in the fw (Fig. 4).

Discussion

The homogenate of an *A. niger* fungal strain isolated from the bark of the *T. chinensis* tree has been shown to be an effective elicitor for Taxol biosynthesis in the cell culture of *T. chinensis*. The use of an elicitor to stimulate Taxol synthesis in the culture was not only significant but also rather instantaneous, being exhibited very soon after elicitation. To achieve the maximum stimulation of Taxol synthesis, both the elicitor dose and the age of culture to be elicited needed to be optimized. This conclusion agrees with that reached for several other metabolite and plant cell systems, e.g., shikonin production by *Lithospermum erythrorhizon* (Chang and Sim 1994), shikonin by *Arnebia euchroma* (Fu and Lu 1999), and taxoids by *T. cuspidate* (Ketchum et al. 1999). While the optimal time for elicitor addition may be specific to the elicitor and the culture system, in general it was in the mid- to late exponential-growth phase, as found in our present work. The existence of an optimal dose may suggest that, at elicitor doses smaller than the optimum, the elicitor-binding sites in the cells were still not fully utilized for activating the secondary metabolite synthesis, while excessive doses caused a deleterious effect on the cells' biosynthetic activity.

Various fungal elicitors, including cell wall fragments, polysaccharides, oligosaccharides, and glycoproteins, have also been used in secondary metabolite production with many other plant cell species (DiCosmo and Misawa 1985; Chang and Sim 1994). However, it is still not well understood how these elicitors mediated the secondary metabolite biosynthesis in plant cells. Some recent studies on one class of putative elicitors, oligoglucosides, suggested that elicitors first bind to certain proteins (the elicitor-binding proteins) in the cell membrane, which might function as signal transduction receptors to initiate a series of subsequent defense-related responses (Ebel and Mithöfer 1998). These responses may include the synthesis and incorporation of hydroxyproline-rich glycoproteins, cellulose callose and polymers, the production of phytoalexins, and the enhanced expression of genes encoding enzymes such as phenylalanine ammonia-lyase. The relevance of these responses to the enhancement of secondary metabolite synthesis remains to be discovered.

While stimulating secondary metabolite accumulation, the fungal elicitor also caused a negative effect on biomass growth in the *T. chinensis* cell culture. As a matter of fact, elicitor inhibition of plant cell growth has also been observed in other elicitor-cell systems, such as fungal powder on *Arnebia euchroma* (Fu and Lu 1999), yeast extract on *Escherichia california* (Byun and Pedersen 1994), and alginate on *Catharanthus roseus* (Akimoto et al. 1999). The elicitor may play the role of depressing the primary metabolism and switching on the secondary metabolism. In view of the inverse relationship between biomass growth rate and secondary metabolite accumulation, the cell growth depression arising from elicitation may be a favor for the secondary metabolite synthesis.

However, Taxol secretion was also enhanced by elicitation. Fungal elicitor-induced secretion of secondary metabolites has also been reported with other plant cells, e.g., alkaloids in *C. roseus* cell suspension culture (Nef et al. 1991). The presence of fungal elicitor may accelerate the physiological or molecular processes leading to

the release of secondary metabolites. It may also result from excretion by cells which have reached their highest level of Taxol accumulation. The secretion of secondary metabolite by the plant cells may be reversible and the concentration measured in the medium may be only a net excretion. So, the actual efflux could be much higher if there is re-absorption or degradation in the medium. When the desired product is not the end-product of a biosynthetic chain but is further metabolized, the problem of degradation should not be overlooked. For example, the decline of Taxol yield after its peak in the late culture period (Figs. 1, 3a) may be partially attributed to increased product degradation. This problem can be solved by continuous product removal from the production system, such as the repeated medium renewal exercised in our work. It can be seen that Taxol accumulation in these culture modes (1E+MMR and MMR+2 E) showed no decline throughout the culture cycle (Fig. 4b). Therefore, the increase in the Taxol yield by medium renewal may be attributed not only to its beneficial effect on cell growth and biosynthetic activity, but also to the prevention of product degradation in the culture system.

Since the productivity of Taxol is the product of biomass concentration and the specific Taxol yield on a per cell basis, measures to maintain a high and active biomass concentration of elicitor-treated culture are essential to achieve high productivities of the secondary metabolites. This objective has been accomplished in our work by repeated medium renewal, or a perfusion-like culture mode. The repeated medium renewal also made it possible to exploit re-elicitation of the culture to ultimately stimulate the Taxol yield. The increase in the specific Taxol yield on a per cell basis by medium renewal and re-elicitation, as seen from our experiments (Table 1), was an augmentation of the cells' biosynthetic capacity for the desired secondary metabolite. Although medium renewal or elicitation alone could increase the specific Taxol yield, combining the two treatments was far more effective. Such a process strategy may be a useful example for enhancing the production of other secreted plant secondary metabolites in the presence of pathogenic or offensive elicitors. It may be applied to large-scale production in a batch or semi-continuous bioreactor, equipped with a medium-perfusion mechanism. However, the optimal elicitation conditions and medium renewal frequency need to be established for each culture system. Medium perfusion has also been used successfully by Seki et al. (1997) for continuous Taxol production by immobilized *T. cuspidata* cells, but without fungal elicitation. Because of the limited life-span of the elicitor-treated culture, a fully continuous mode of operation may be not feasible.

According to the report by Hezari et al. (1999), the maximum Taxol concentrations and productivities attained in cell suspension cultures of various *Taxus* spp are mostly below 10–20 mg l^{-1} and 0.5–1.0 mg l^{-1} day^{-1}, respectively. Only a few research groups have achieved much higher levels of Taxol production, through the selection of productive cell strains, the use of elicitors, and the optimization of culture media and processes. Bringi et al. (1995) reported an accumulation of 153 mg Taxol l^{-1} over a period of 42 days (equal to a productivity of 3.57 mg l^{-1} day^{-1}) in *T. chinensis* cell cultures. Yukimune et al. (1996) obtained 110 mg Taxol l^{-1} over a 2-week period (equal to a productivity of 7.86 mg l^{-1} day^{-1}) in *T. media* cell cultures. More recently, Ketchum et al. (1999) observed a maximum Taxol concentration of 117 mg l^{-1} over a 5-day culture period in *T. canadensis* cell cultures, equal to a Taxol productivity of 23.4 mg l^{-1} day^{-1}. In our experiments, therefore, the maximum Taxol productivity of about 3.2 mg l^{-1} day^{-1} observed in the culture with medium renewal and double elicitation culture is much higher than those obtained in many previous studies, but is still lower than the highest levels achieved so far. To further improve the effect of the fungal elicitor on Taxol accumulation, we may need to use more purified and defined constituents of the fungal homogenate and exclude those which may cause adverse effects. Furthermore, the dosage, timing and frequency of elicitation and the medium renewal strategy should be optimized systematically and simultaneously.

Acknowledgement This work was partially supported by an Internal Grant of the Hong Kong Polytechnic University.

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