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Paclitaxel Production by Immobilized Cell Suspension Cultures of *Taxus cuspidata* var. nana

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Abstract: For the production of taxol (paclitaxel) by immobilized cells cultured in a bioreactor, cells prepared from *Taxus cuspidata* var. nana were entrapped with calcium alginate and the amount of paclitaxel released into F4G4 liquid medium was investigated. Immobilized cell cultures were developed using various calcium alginate gel concentrations (2, 2.5 and 4% w/v) and cell concentrations (3, 5 and 10% w/v). Some of the paclitaxel produced in the immobilized cells was found to be released into F4G4 liquid medium. The amount released depended on the gel and cell concentrations used. The free cells in suspension cultures accumulated paclitaxel in intracellular compartments, while the cells immobilized with the calcium alginate gel released some paclitaxel into the medium. The gel forced some of the paclitaxel produced in the cells to be released into the medium. When the cultures using immobilized cells entrapped at a 3% cell concentration and 2.5% gel concentration with an air-lift bioreactor were conducted for 30 days, the amount of paclitaxel released into the medium increased with time on the addition of an elicitor, chito-heptaose. The release of paclitaxel was enhanced to 2.4 mg L^{-1} of culture medium after the 30-day-incubation. This is the first report that the release of paclitaxel into the medium by immobilized cell cultures of *Taxus* sp. was enhanced by the addition of an elicitor. Eight five percent of the paclitaxel produced in the cells was released by the immobilized cell cultures in 30 days. The results obtained in this study, with immobilized *T. cuspidata* var. nana cells in a 2.5% calcium alginate gel, may pave the way for the production of paclitaxel by immobilized cell cultures in a bioreactor.

Key words: *Taxus cuspidata* var. nana, immobilized cell cultures, taxol (paclitaxel), calcium alginate gel, chito-heptaose

INTRODUCTION

Taxol (Paclitaxel) is currently the best known drug approved for use in the treatment of breast, ovarian and non-small cell lung cancer and AIDS-treated Kaposi's sarcoma (Ojima *et al*., 2002). Trees of the genus *Taxus* grow slowly, yielding relatively little paclitaxel. Also, there are restrictions on the harvesting of yew trees. Therefore, alternative sources of paclitaxel, chemical synthesis, semi synthesis, cell cultures and so on, have been sought. The semi synthesis of paclitaxel from baccatin III isolated from the needles of *Taxes baccata* has provided an immediate and renewable source of the drug (Kingston *et al*., 1994). Alternatively, the production of paclitaxel by *Taxus* cell cultures (e.g., Yukumune *et al*., 1996; Son *et al*., 2000; Navia-Osorio *et al*., 2002; Zhang *et al*., 2007) and hairy root cultures (Furmanowa and Syklowska-Baranek, 2000) has been conducted. However, yields were comparatively low. To enhance the productivity of *Taxus* cell cultures, biogenetic precursors (Muranaka *et al*., 2004), Nutrient feeding (Choi *et al*., 2000), *in situ* extraction (Zhang and Xu, 2001), elicitors (Zhang *et al*., 2000; Linden and Phisalaphong, 2000; Wang *et al*., 2001; Yuan *et al*., 2001; Tachibana *et al*., 2007), methyl jasmonate (Yukumune *et al*., 1996; Ketchum *et al*., 1999; Wang *et al*., 2004), a combination of inducing factors (Khosroushahi *et al*., 2006) and jasmonic acid (Baebler *et al*., 2002), have all been used. On the other hand, production of paclitaxel by immobilized cell cultures of *Taxus* sp. with a bioreactor has been reported by Seki *et al.* (1997), Bentebibel *et al.* (2005) and Cheng *et al.* (2006). The amount of paclitaxel released into the medium ranged from about 0.4 to 4 mg L^{-1}. The production could be enhanced by adding an elicitor or biogenetic precursor. However, the productivity was not as high as that in free cell cultures.

In continuation of our research on the production of paclitaxel by callus and cell cultures of *Taxus cuspidata* var. nana, we reported on the production of paclitaxel on the addition of elicitors, chito-oligosaccharides and chito-

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heptaose, on the addition of a biogenetic precursor, phenylalanine (Yoshida *et al*., 2002; Muranaka *et al*., 2004) and on the addition of chito-heptaose or jasmonic acid and another biogenetic precursor, phenylisoserine, with a combination of two elicitors and with a combination of one elicitor and the biogenetic precursor, in cell suspension cultures (Tachibana *et al*., 2007). We also reported that the amount of paclitaxel produced was increased by supplying air to the cell suspension cultures, though productivity depended on the amount of air supplied (Tachibana *et al*., 2007). Furthermore, we also found that some of the paclitaxel produced in the cultures was released into the medium when cells from *T. cuspidata* var. nana were immobilized with calcium alginate.

This research describes the effect on the release of paclitaxel into the culture medium by immobilized cell cultures of calcium alginate gel and cell concentrations and incubation time. It also describes the amount of paclitaxel released by immobilized cells cultured with a bioreactor and an elicitor.

MATERIALS AND METHODS

Authentic taxol (paclitaxel) was purchased from Sigma Chemical Company. Chito-heptaose seven hydrochloride and entrapment reagents were purchased from Sekagaku Kogyo Ltd. and Wako Pure Chemical Company Ltd., respectively.

Plant materials: Fresh stems of *Taxus cuspidata* var. nana were collected in July 2002 from the garden of a house located in the suburbs of Matsuyama City, Ehime, Japan.

Cell suspension cultures of *T. cuspidata* var. nana and collection of cultured cells for immobilization: Cell suspension cultures in F4G4 medium were conducted under the same conditions described in a previous paper (Muranaka *et al*., 2004). Ten-day-old cultured cells were collected by filtration through a buchner funnel from the suspension cultures and used for immobilization. Paclitaxel content was measured as described previously (Tachibana *et al*., 2007).

Immobilization procedure: The immobilization of cells was conducted by reference to the method of Komaraiah *et al*. (2003). For immobilization of the cells collected by filtration from the culture medium described above, 3, 5 and 10 g of the cells (fresh weight) were added to 50 mL of fresh F4G4 medium and mixed with 50 mL of 4, 5 and 8% (w/v) sodium alginate made up in the culture medium. The mixture was added dropwise to 300 mL of a stirred culture medium containing cold calcium chloride (0.1 M), through a pipette with a tip 2 mm in diameter. Beads formed immediately on contact with the calcium chloride solution. The beads were shaken for 2 h to ensure complete fixation. The beads were washed twice with 250 mL of fresh culture medium by shaking at 120 rpm for 15 min.

Determination of paclitaxel production: Paclitaxel content was measured in the immobilized cells and liquid medium after incubation for 7, 14 and 21 days under the conditions described above. The liberated cells obtained by filtration of each culture (7 day intervals for 21 days) after solubilization of the immobilized matrix with a sodium citrate solution (4%) for 30 min at 25°C were freeze-dried for one week, ground with a pestle in a motor and extracted with 50% methanol for one week at room temperature. The extraction was repeated two times and the solution was concentrated under reduced pressure to obtain extracts. The liquid medium was freeze-dried for one week, ground with a pestle in a motor and extracted with 50% methanol for one week at room temperature. The extraction was repeated two times and the solution was concentrated under reduced pressure to obtain extracts. Both extracts were separated with dichloromethane and water (1:1) as described previously (Muranaka *et al*., 2004) and gave each dichloromethane soluble. The amount of paclitaxel in each soluble was determined as described previously (Tachibana *et al*., 2007). The experiments were repeated once with three replicates. The results shown are the average of three measurements and standard deviation. Statistical analyses were carried out with student's t-test. The deviation of each experimental value was considered significant at $p<0.05$.

Effect of calcium alginate gel concentration on release of paclitaxel into culture medium: The immobilized cells prepared at a 3% cell concentration and at 2, 2.5 and 4% calcium alginate gel concentrations, respectively, were each resuspended in 100 mL of fresh F4G4 culture medium supplemented with NAA (5 mg L^{-1}) in a 300 mL flask and cultured for 14 days. Paclitaxel content was measured in the cells and liquid medium after incubation for 14 days by the method described above. The experiments were repeated once with three replicates. The results shown are the average of three measurements and standard deviation. Statistical analyses were carried out with student's t-test. The deviation of each experimental value was considered significant at $p<0.05$.

Effect of cell concentration on release of paclitaxel into culture medium: The immobilized cells prepared at 2, 2.5 and 4% cell concentrations and at a 2.5% calcium alginate gel concentration, respectively, were resuspended in

100 mL fresh of F4G4 culture medium supplemented with NAA (5 mg L^{-1}) in a 300 mL flask and cultured for 14 days. The amount of paclitaxel in the cells and the medium was measured as described earlier. The experiments were repeated once with three replicates. The results shown are the average of three measurements and standard deviation. Statistical analyses were carried out with student's t-test. The deviation of each experimental value was considered significant at $p<0.05$.

Incubation of immobilized cell cultures with an air-lift type bioreactor: The immobilized cells prepared at a 3% cell concentration and at a 2.5% calcium alginate gel concentration were packed with 500 mL of an air-lift type bioreactor (MBRS-015; Tokyo Rikakikai Co. Ltd., Tokyo, Japan) in 300 mL of liquid medium and incubated for 3- day-intervals until day 30 by supplying air (3 vvm) with or without the addition of an elicitor, chito-heptaose (3 mg L^{-1}), at 25°C in the dark, respectively. Fifteen milliliters of liquid medium was removed every 3 days and extracted with dichloromethane. The dichloromethane solution was concentrated in vacuo to give dichloromethane soluble after drying with anhydrous sodium sulfate. The amount of paclitaxel in each soluble was determined as described previously (Tachibana *et al*., 2007). In addition, the amount of paclitaxel released into the medium by immobilized cell cultures without air induction in an Erlenmeyer flask was also measured and compared to that with the air-lift bioreactor.

The results shown are the average of three measurements and standard deviation. Statistical analyses were carried out with student's t-test. The deviation of each experimental value was considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Effect of calcium alginate gel concentration on release of paclitaxel into medium by immobilized cell cultures of *T. cuspidata* var. nana: To clarify a suitable calcium alginate gel concentration for immobilized cell cultures of *T. cuspidata* var. nana, the effect of the gel concentration on the release of paclitaxel into the medium was investigated. Immobilized cells and medium from immobilized cell cultures were harvested at 14 days for the determination of paclitaxel content (Table 1). No release of paclitaxel into the culture medium was recognized at a gel concentration of 2 or 4%. Paclitaxel was only released when the cells were entrapped with the 2.5% calcium alginate gel and immobilized cell cultures were conducted for 14 days, as shown in the Table 1. The cells entrapped with the 2.5% calcium alginate gel released paclitaxel (0.60 mg L^{-1}). The value shows that 28% of the paclitaxel produced in the cells was released into the medium. In a previous research (Muranaka *et al*., 2004), no release of paclitaxel into the medium was recognized in free cell suspension cultures of *T. cuspidata* var. nana. From the results obtained here, it was found that a suitable gel concentration was necessary for the release of paclitaxel into the medium. The reason for this was unclear. In the case of the 4% calcium alginate gel, it may be hard to release paclitaxel because of the hardness of the incorporated membrane or of restrictions to cell permeability. In the case of the 2% calcium alginate gel, osmotic pressure may be low inside the membrane.

Table 1: Effect of calcium alginate gel concentration on release of paclitaxel into medium by immobilized cell cultures of *Taxus cuspidata* var. nana for 14 days

Gel concentration (%, of medium)	Cell concentration (%, of medium)	Paclitaxel content	
		Medium (mg L^{-1})	Cell (mg L^{-1})[a]
2	3	nr[b]	2.10 (0.02)
2.5	3	0.60 (0.02)	1.51 (0.03)
4	3	nr[b]	2.13 (0.03)

*All numbers are means of three replications. Values in parentheses are standard deviation. [a]: Paclitaxel content in the cells showed the value to the medium (mg L^{-1}). [b]: No release

Matrices generated in gel beads are strongly dependent on several variables like ionic strength and polymer concentrations (Mohamed and Scragg, 1990; Nava Saucedo *et al*., 1996). Thus different matrices, obtained by varying the amount of alginate gel (2, 2.5 and 4%), were used to study the influence of gel concentration on the release of paclitaxel from *T. cuspidata* var. nana cells. Unlike in free cell suspension cultures, the growth of immobilized cells was retarded at all concentrations. The growth of immobilized cells may be better in the 2.5% calcium alginate gel than 2 and 4% calcium alginate gels. Bentebible *et al*. (2005) reported that the growth of cells entrapped in calcium alginate gels was restricted by the gel concentration. However, they also reported that cell viability in free cells and entrapped cells was almost the same as or a little lower (about 5%) than that of free cells until 24 days of incubation, though it depended on the gel concentration. The viability of the cells entrapped in the gel needs to be measured to clarify the relationship between cell growth and release of paclitaxel. From the results obtained here, a gel concentration of 2.5% was best for the immobilized cell cultures.

Effect of cell concentration on release of paclitaxel into the culture medium by immobilized cell cultures of *T. cuspidata* var. nana: The amount of paclitaxel released into the medium depended on the cell concentration (Table 2). The immobilized cells at a 3% concentration and

2.5% gel concentration exhibited a maximum release of paclitaxel (0.60 mg L^{-1}) for 14 days, while those cultured at 5 and 10% cell concentrations and at a 2.5% gel concentration showed lower levels of paclitaxel (0.05 and 0.18 mg L^{-1}), respectively. In the case of the immobilized cells entrapped at a cell concentration of 3, 5 and 10% as shown in Table 1, 28, 2 and 8% of the paclitaxel they produced was released into the medium, respectively. These results showed that the cell concentration of the immobilized cultures depended on the release of paclitaxel into the medium. There is almost no report on the effect on the release of paclitaxel by immobilized cell cultures of the cell concentration. From the results obtained here, a 3% cell concentration was best for the release of paclitaxel into the medium. Therefore, immobilized cells prepared at 3% cell and 2.5% gel concentrations were used for further experiments.

Effect of incubation time on release of pactitaxel into medium in the immobilized cell cultures of *T. cuspidata* var. nana: Paclitaxel was only released into the culture medium after incubation time of between 7 and 14 days. No release of paclitaxel was recognized after 21 days of incubation. The reason for this was not clear, but may be related to the conditions for immobilized cells (Table 3).

Table 2: Effect of cell concentration of release of paclitaxel into medium by immobilized cell cultures of *Taxus cuspidata* var. nana for 14 days

Gel concentration (%, of medium)	Cell concentration (%, of medium)	Paclitaxel content	
		Medium (mg L^{-1})	Cell (mg L^{-1})[a]
2.5	3	0.60 (0.02)	1.51 (0.03)
2.5	3	0.05 (0.01)	2.24 (0.04)
2.5	3	0.18 (0.02)	2.23 (0.04)

*All numbers are means of three replications. Values in parentheses are standard deviation. [a]: Paclitaxel content in the cells showed the value to the medium (mg L^{-1})

The amount of paclitaxel released into the medium was greatest when the cultures were conducted for 14 days at a 3% cell concentration. The maximum amount released was 0.45 mg L^{-1} when the immobilized cultures were conducted for 7 days at a 10% cell concentration. The maximum amount released was 0.60 mg L^{-1} when the immobilized cell cultures were conducted for 14 days at a 3% cell concentration. It was found that the release of paclitaxel into the medium began at 7 days and continued until day 14 at a 3% cell concentration. In the case of immobilized cells at a 5 and 10% concentration, the amount of paclitaxel released decreased with the increase in incubation time. In the case of immobilized cells at a 3% cell concentration, however, the amount released into the medium increased with the increase in incubation time until day 14. From the results obtained here, the amount of paclitaxel released into the medium from the immobilized cells differed with cell concentration and incubation time. It is unclear why the pattern of release differed with the cell concentration. The result may be ascribed to the immobilized membrane's thickness in the cells entrapped with the immobilizing agent, permeability of the cell membrane, or action of the elicitor as the immobilizing agent by itself. However, the mechanism of release by the immobilized cells remains to be clarified.

Release of paclitaxel into medium by immobilized cell cultures of *T. cuspidata* var. nana with an air-lift bioreactor: In the case of immobilized cell cultures in Erlenmeyer flasks under rotation without a supply of air induction, the amount of paclitaxel released into medium every 3 days ranged from 0.18 to 0.37 mg L^{-1} (Table 4). In the case of incubation with an air-lift bioreactor supplying air (3 vvm), the amount released every 3 days was 0.14 to

Table 3: Effect of incubation time on amount of paclitaxel released into medium by immobilized cell cultures of *Taxus cuspidata* var. nana for 3 weeks

Gel concentration (%, of medium)	Cell concentration (%, of medium)	Paclitaxel content released into medium (mg L^{-1})			
		Incubation time (week)			
		0	1	2	3
3	2.5	nr[a]	0.15 (0.02)	0.60 (0.02)	nr[a]
5	2.5	nr[a]	0.16 (0.03)	0.05 (0.02)	nr[a]
10	2.5	nr[a]	0.45 (0.03)	0.18 (0.02)	nr[a]

*All numbers are means of three replications. Values in parentheses are standard deviation. [a]: No release

Table 4: Amount of paclitaxel released into medium by immobilized cell cultures of *Taxus cuspidata* var. nana for one month

	Paclitaxel content of medium (mg L^{-1})										
	Incubation time (day)										
	0	3	6	9	12	15	18	21	24	27	30
Erlenmeyer flask	nr[a]	0.18 (0.01)	0.14 (0.01)	0.21 (0.02)	0.19 (0.02)	0.32 (0.03)	0.22 (0.03)	0.22 (0.02)	0.37 (0.02)	0.36 (0.02)	0.34 (0.03)
Air-lift	nr[a]	0.33 (0.01)	0.28 (0.01)	0.27 (0.03)	0.21 (0.03)	0.24 (0.02)	0.23 (0.03)	0.16 (0.02)	0.15 (0.03)	0.17 (0.02)	0.14 (0.01)
Air-lift+Elicitor[b]	nr[a]	0.99 (0.04)	1.12 (0.03)	1.32 (0.03)	1.65 (0.03)	1.85 (0.04)	2.01 (0.03)	2.11 (0.04)	2.19 (0.05)	2.31 (0.05)	2.39 (0.04)

*All numbers are means of three replications. Values in parentheses are standard deviation. [a]No release, [b]Chito-heptaose (3 mg L^{-1}) as an elicitor was added to the medium

0.33 mg L^{-1}. To enhance the amount released, chito-heptaose (3 mg L^{-1}), the most active elicitor for the production of paclitaxel according to our research (Tachibana *et al*., 2007), was added to the immobilized cell cultures with an air-lift bioreactor supplying air (3 vvm) (Table 4). The amount of paclitaxel released into the medium increased with the incubation time. The maximum amount released was 2.39 mg L^{-1}. The amount released increased gradually day by day as shown in the Table 4. The release of paclitaxel into the medium was found to be 85% in comparison with the amount of paclitaxel in the cells after 30 days of incubation. In a earlier study (Tachibana *et al*., 2007), we reported that the amount of paclitaxel produced by cell suspension cultures of *T. cuspidata* var. nana was 16.6 mg L^{-1}. The amount produced by the cell cultures was greater than that produced by the immobilized cell cultures. In the case of free cell cultures, paclitaxel must be extracted from the cells when the cultures have finished. However, immobilized cell cultures have two advantages over free cell cultures. One is the recovery of paclitaxel from liquid medium without its extraction from the cells. The other is that the immobilized cells can be used repeatedly for production of paclitaxel.

This is the first report that the release of paclitaxel into the medium was enhanced by the addition of an elicitor, chito-heptaose. The results show that the harvesting of paclitaxel from the culture medium of immobilized cells cultured in a bioreactor is possible. The amount of paclitaxel released could be enhanced by increasing the amount produced in the cells via several methods. Seki *et al.* (1997) reported that the paclitaxel concentration in the medium was maintained at about 0.3 to 0.4 mg L^{-1} for over 30 days and up to 40 days using immobilized cells from *T. cuspidata.* The results obtained here with the flask culture and the air-lift bioreactor without an elicitor was almost the same as those of Seki *et al.* (1997). However, the amount of paclitaxel released into the medium could be enhanced about 6 times by adding an elicitor, chito-heptaose. Bentebible *et al.* (2005) reported that the amount of paclitaxel produced by immobilized cell cultures of *T. baccata* supplemented with methyl jasmonate, mevalonate and N-benzoylglycine with an air-lift bioreactor was enhanced about 10 to 17 times compared to than produced by free cells. However, they did not report the amount of paclitaxel released into the medium. The results obtained in this study, with immobilized *T. cuspidata* var. nana cells in a 2.5% calcium alginate gel, may pave the way for the production of paclitaxel by immobilized cell cultures on a large-scale in a bioreactor.

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