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– *Notes* –

Effective Production of Paclitaxel and the Related Taxanes in a Plant Cell Culture by *in situ* Extraction with Sequential Refreshment of Water-Immiscible 1-Butyl-1-methylpyrrolidinium Bis(trifluoromethanesulfonyl)imide

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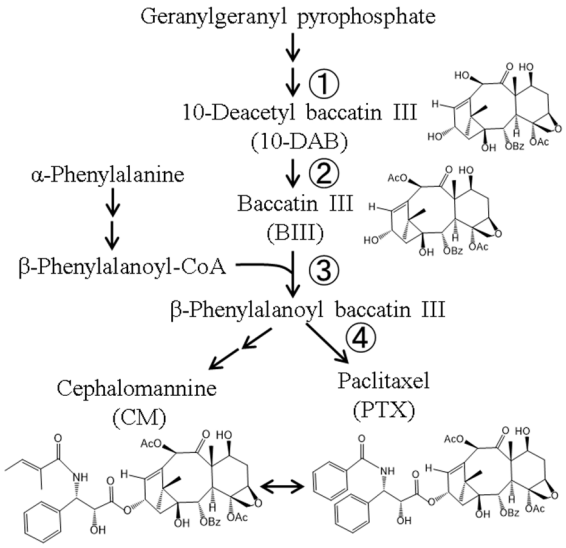
We report here the sequential refreshment of a water-immiscible ionic liquid, 1-butyl-1-methylpyrrolidinium bis(trifluoromethanesulfonyl)imide (P14TFSI) enhanced the production amount of paclitaxel and the related taxanes with *in situ* extraction from an aqueous medium in a plant cell culture. The production amount of the taxanes with refreshment of 2.5 vol% P14TFSI twice every 2 days was three times greater than that without the refreshment and 600 times greater than that of control culture without P14TFSI. The enhanced production amounts of taxanes in the culture with P14TFSI might be due to the deceased feedback inhibition of paclitaxel and abiotic stress responding against elevation of intracellular production of reactive oxygen species (ROS) derived from P14TFSI's abiotic stress.

1. Introduction

Paclitaxel (PTX, Figure 1), which is semi-synthetically produced from precursors of taxanes such as 10-deacetyl baccatin III or baccatin III extracted from yew tree needles, is used as a treatment for a variety of cancers. However, the semi-synthetic production method has many chemical reaction steps including various environmentally-hazardous organic solvents, making the production cost very high [1,2]. A method using plant cell culture is promising and attractive for a cost-effective and safer production process. The productivity of paclitaxel in the cell culture is very low due to its feedback inhibition [3], and a strategy to avoid the feedback inhibition needs to be considered.

Focusing on the fact that taxanes are hydrophobic, two-phase culture system using hydrophobic absorbents and liquids have been



①DBBT: taxane 2a-O-benzoyltransferase
②DBAT: 10-deacetylbaccatin III-10-O-acetyltransferase
③BAPT: baccatin III: 3-amino, 3-phenylpropanoyltransferase
④DBTNBT: 30-N-debenzoyl-2-deoxytaxol-N-benzoyltransferase

Figure 1. Metabolic pathway of paclitaxel and the lated taxanes.

proposed for the *in situ* extraction and adsorption of paclitaxel and the related taxanes from the culture medium [4,5]. We investigated a culture technique to reduce the inhibitory effect of paclitaxel on cell growth by conducting a two-phase culture system using water-immiscible liquids by means of hydrophobic interactions [5-8]. Recently, ionic liquids have getting attention as alternatives to conventional organic solvents for the extraction of biologically active compounds from biomass [9-12]. Ionic liquids, which remain non-volatile liquid at room temperature and have chemical stability, are attracting attention from the viewpoint of green chemistry as substances that are safer than organic solvents. Bioactive compounds such as 3-indole-butyric acid from aqueous extract of *Kappaphycus alvarezii* could be extracted and separated by ionic liquids [13]. Extraction of natural medicinal compounds from *Stereocaulon glareosum* with ionic liquids is described by E. Calla-Quispe *et al.* [14] and Fan *et al.* extracted a high-value of anthocyanin delphinidin-3-rutinoside with high-purity by ionic liquids [15]. It is reported amino acid ester based phenolic ionic liquids can be a potential solvent for poorly soluble bioactive compound luteolin [16]. We previously reported the enhanced production amount of paclitaxel in plant cell culture with the *in situ* extraction by sequential refreshment of a hydrophobic ionic liquid, 1-butyl-1-methylpyrrolidinium bis(trifluoromethane-sulfonyl)imide (P14TFSI) [17].

In this study, we aimed to enhance production amount of paclitaxel and taxanes (10-Deacetyl baccatin III; 10-DAB, Baccatin III; BIII, Cephalomannine; CM) by exchanging ionic liquids with fresh ones during culture. Significantly enhanced productivity of paclitaxel in the culture including P14TFSI was observed in the previous study, which might be the result from the stress response by the P14TFSI's abiotic stress in addition to the decreased feedback inhibition by the produced paclitaxel. Rapid and transient production of reactive oxygen species (ROS) is an event in the defense response [18]. Relationship between the production amount of taxanes with the refreshment frequency of P14TFSI and production of ROS content is examined, too.

2. Experimental

2.1 Cell, medium and reagents

The cells induced from the needles of *Taxus cuspidata* according to the procedures as described previously [5] were used in the present research. A modified Gamborg's B5 medium including 20 g/L sucrose, 0.5 mg/L 1-naphthaleneacetic acid and 0.05 mg/L benzyl adenine was utilized for the cell culture [5]. The induced cells were subcultured on a solid medium containing the modified Gamborg's B5 components. Precultured cells which were cut into pieces using a knife were prepared for the culture.

A hydrophobic ionic liquid, 1-butyl-1-methylpyrrolidinium bis(trifluoromethanesulfonyl)imide (P14TFSI) purchased from Tokyo Chemical Industry Co. (Tokyo, Japan), whose chemical formula is shown in Figure 2, was used in the present research. The density and solubility in aqueous medium of P14TFSI are 1400 kg/m^3 and 16.1 mM [19], respectively. Commercially available taxanes of 10-deacetyl baccatin III (10-DAB), baccatin III ((BIII), cephalomannine (CM) and paclitaxel (PTX) were used as standard reagents for HPLC analysis.

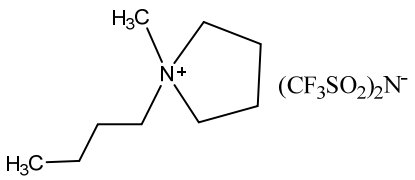


Figure 2. Chemical formula of P14TFSI.

Partition coefficients of taxanes in the P14TFSI-

medium two-phase system, which were measured in our experiments, are shown in Table 1. These coefficients are greater values, which means that P14TFSI is an excellent solvent for the taxanes.

Table 1. Partition coefficients of taxanes in the P14TFSI-medium two-phase system.

	10-DAB	BIII	PTX	CM
Partition coefficient of taxane [-]	5.2	51.6	45.4	37.3

2.2 Cell culture in a two-phase culture system

Suspension culture inoculated by the precultured cells was carried out in a 100 cm^3 Erlenmeyer flask covered with a porous silicone cap, whose schematic representaion is shown in Figure 3, on a rotary shaker (NR-150, Taitec, Saitama, Japan) at 110 rpm in the dark at 26 ˚C. The flask contained 20 cm^3 of the modified B5 medium and 0.5 mL of P14TFSI, which was calculated as 2.5 vol% based on the medium volume. The amounts of fresh cells and taxanes in the flask after a 7 d culture period were measured. Experiments per culture condition as described below were carried out in duplicate.

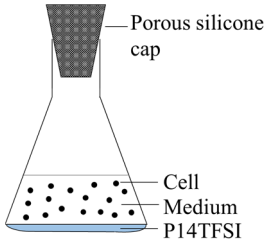


Figure 3. Two-phase culture system including modified B5 medium and P14TFSI.

2.3 Culture conditions with sequential refreshment of P14TFSI

To investigate the effectiveness of P14TFSI refreshment frequency on the cell growth and production amounts of the taxanes in the two-phase culture system, the following four refreshment frequencies during a 7d culture period were carried out [17]. The refreshment of P14TFSI was conducted to exchange the P14TFSI used in the culture for fresh using a micropipette after the defined culture period. The culture in the absence of P14TFSI was the control.

a. No refreshment of P14TFSI (This culture procedure is designated "0".)
b. P14TFSI refreshed once at 4 d (This culture is designated "1".)
c. P14TFSI refreshed twice at every 3 d (This culture is designated "2".)
d. P14TFSI refreshed three times at every 2 d (This culture is designated "3".)

For examining the effectiveness of P14TFSI refreshment frequency, the enhancement factor of cell growth, E_{FCW}, and that of the total production of taxanes (10-DAB, BIII, CM and PTX), E_P, were defined and used as follows.

$$E_{FCW} = FCW_{P14TFSI} / FCW_C \quad (1)$$

$$E_P = P_{P14TFSI} / P_C \quad (2)$$

where FCW_C, $FCW_{P14TFSI}$, P_C and $P_{P14TFSI}$ are the fresh cell weight in the control, the fresh cell weight in the culture with P14TFSI, the total sum of the taxanes in the control and the total sum of taxanes in the culture with P14TFSI after the 7 d culture periods.

2.4 Analysis

Cells were collected from each culture, washed with pure water, blotted on filter paper to remove excess water, and then weighed to determine the fresh cells weight. The amounts of the paclitaxel and the related taxanes in the medium phase, the P14TFSI phase and the cells in all samples were analyzed by using a reversed-phase HPLC system according to the analytical procedures described previously [6].

Intracellular reactive oxygen species (ROS) content with the aid of 2',7'-dichlorofluorescein diacetate (DCFH-DA, Nacalai tesque, Japan) by modifying Kellett's method [20] was measured [17]. Briefly, a black plate, where 20 mmol/L DCFH-DA in phosphate buffered saline (pH7.4) and the homogenized 50 mg cells collected from each culture condition were mixed, was excited at 484 nm in the Fluorometer (Fluoroskan Ascent, Thermo Scientific, Japan) and the emitted fluorescence intensity at 527 nm was measured over time as the ROS content. The greater the intensity, the greater the ROS content in the cells was.

3. Results and Discussion

3.1 Effect of refreshment frequency of P14TFSI on the cell growth

Figure 4 shows the effect of refreshment frequency of P14TFSI in the two-phase culture system on enhancement factor of cell growth after the 7 d culture periods. The values of E_{FCW} in the cultures including P14TFSI regardless of the refreshment frequency of P14TFSI were greater than that of the control culture where P14TFSI was absent. This resulted from the decreased paclitaxel's feedback inhibition in the culture because most of the produced paclitaxel was extracted with P14TFSI. The lowered value of E_{FCW} in the culture with 3 times refreshment of P14TFSI rather than that with twice refreshment, might be due to the removal of conditioning factors which are unclear metabolites related to the cellular growth and metabolism [21] by excessive refreshment frequency of P14TFSI. The cellular shapes in the culture with P14TFSI were almost similar to those in the control culture, indicating that the P14TFSI does no damage to the cells.

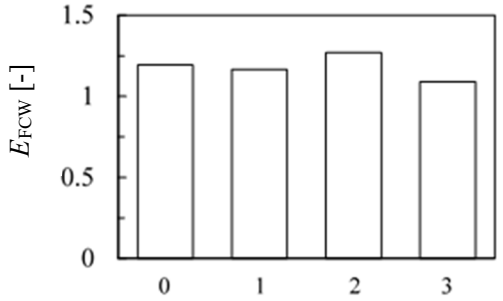


Figure 4. Effect of refreshment frequency of P14TFSI on enhancement factor of cell growth E_{FCW}. Experiments per culture condition were carried out in duplicate (n=2).

3.2 Effect of refreshment frequency of P14TFSI on the production amount of taxanes

Figure 5 shows the effect of refreshment frequency of P14TFSI on the production amount of the taxanes after the 7 d culture periods. The values of enhancement factor of the total sum of the produced taxanes, E_P, in the cultures with increase of the refreshment frequency of P14TFSI from 0 times to twice was observed. This enhancement resulted from partition of the produced paclitaxel and the related taxanes into the P14TFSI phase by means of hydrophobic interaction. The production amount of the taxanes when P14TFSI was refreshed twice was three times than that without the refreshment and 600 times greater than that of control culture. Most of the taxanes produced by the cells in the cultures with the

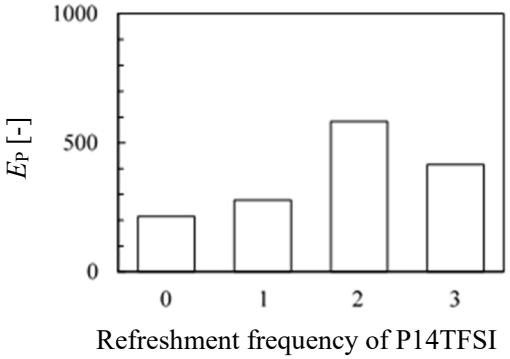


Figure 5. Effect of refreshment frequency of P14TFSI on enhancement factor of production amount of the taxanes of E_P. Experiments per culture condition were carried out in duplicate (n = 2).

refreshment of the P14TFSI were secreted and partitioned into the aqueous medium due to the higher values of partition coefficients as shown in Table 1, providing the decreased feedback inhibition of paclitaxel and subsequently increased production of the taxanes.

In previous research, increasing the amount of P14TFSI from 2.5 to 10 vol% in the two-phase culture system to reduce the PTX's feedback inhibition improved the cell growth rate and enhanced the production amount of the PTX by factor of 1.5 [19]. The productivity of PTX in the culture with twice refreshment of 2.5 vol% P14TFSI in this research is 7 times that without the refreshment (Figure 5). The data indicated that the refreshment of P14TFSI was more effective for the productivity of PTX than the increased amount of P14TFSI. The reason for the effectiveness of the refreshment of P14TFSI might be due to the concentration of PTX in the aqueous medium in the culture with the refreshment of P14TFSI being almost 0, which means little feedback inhibition of PTX, while that with increased amount of P14TFSI was 0.04 mg/L [19].

The significant greater enhancement of the taxanes could not be explained by the decreased feedback inhibition of paclitaxel. It is supposed that there is a possibility that P14TFSI's stimulating factor enhanced the production amount of the taxanes. Since the P14TFSI has non-cytotoxic to the cells judged from the data in Figure 4, it might be an abiotic stress against the present cells, stimulating production of secondary metabolites such as paclitaxel and the related taxanes as a defense response. Sykłowska-Baranek *et al.* reported that a water-immiscible agent, perfluorodecalin, enhanced the taxanes production in hairy root cultures [22]. The decrease in amount of the taxanes in the culture when P14TFSI was refreshed three times was due to the removal of the conditioning factors partitioned in P14TFSI from the culture medium as described above. Figure 6 shows the amount of paclitaxel and the related taxanes in each culture condition. The production amount of CM and BIII in the culture with twice refreshment of P14TFSI was greatest and nearly zero among the refreshment frequency of P14TFSI, respectively. These results suggest that the twice refreshment of P14TFSI promotes the rapid conversion of BIII to PTX and CM (Figure 1), however, this should be further examined. The reason for the greater production amount of CM in the culture with the twice refreshment of P14TFSI is under investigation.

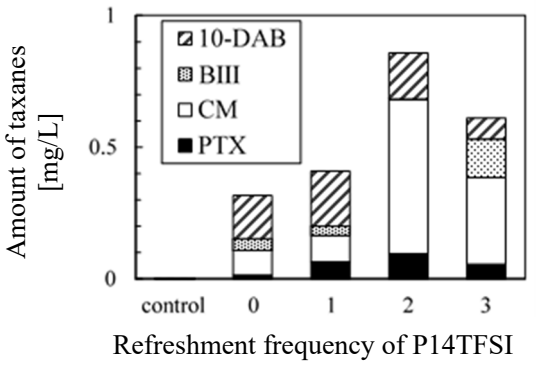


Figure 6. Effect of refreshment frequency of P14TFSI on production amount of the taxanes. Experiments per culture condition were carried out in duplicate (n=2).

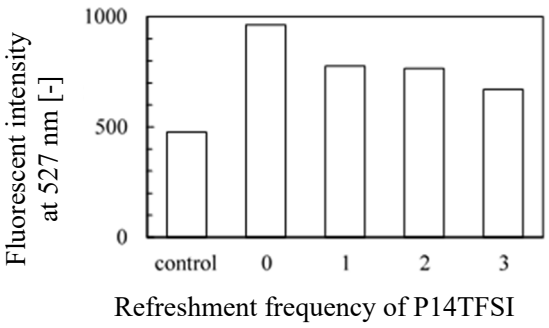


Figure 7. Relationship between fluorescent intensity and refreshment frequency of P14TFSI.

The secondary metabolites play a role of a plant defense strategy in response to entry of herbivores, pathogens, and a variety of environmental stresses. The defense strategy in the host plant cells is activated

by elicitors. The oxidative burst, characterized by rapid and transient production of ROS, is an early event in the defense response [18]. As discussed in the previous research where the production of paclitaxel in the cultures with P14TFSI was investigated, the trend of the ROS content was similar to that of the production amount of paclitaxel and the ROS content in the cultures including P14TFSI was greater than that in the control culture [17]. Figure 7 shows the relationship between fluorescent intensity reflecting ROS content and refreshment frequency of P14TFSI. Although the consistency of trend of the ROS content and the produced taxanes in the cultures including P14TFSI was not observed, the ROS content in the cultures including P14TFSI regardless to the refreshment frequency was greater than that in the control. This means that P14TFSI might be an abiotic stress to the present cells, promoting the production of paclitaxel and the related taxanes as a stress response like an elicitor.

A recovery method by back-extracting PTX from P14TFSI for its actual production process is under examination. Although an adjustment of the medium pH was effective back-extraction of hydrophobic biomolecules from 1-hexyl-3-methylimidazolium hexafluorophosphate [23], attempts to back-extract PTX by adjusting pH in the culture medium have failed. HPLC can be an effective method for recovery of PTX from the P14TFSI in the two-phase culture system.

4. Conclusion

For enhancing the cell growth and the production amount of paclitaxel and the related taxanes, the refreshment frequency of hydrophobic ionic liquid, P14TFSI, in the two-phase culture system was conducted. The refreshment frequency contributed to enhancing the cell growth because of decrease of the feedback inhibition of the produced paclitaxel. Similarly, the greater production amount of paclitaxel and the related taxanes with increase in the refreshment frequency of P14TFSI was due to the *in situ* extraction with P14TFSI from the culture and stress response against ROS derived from P14TFSI's abiotic stress. The culture condition where P14TFSI was twice refreshed during 7 days culture was optimal for the purpose.

Acknowledgement

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