Enhancement of Paclitaxel Production in Plant Cell Culture Including Increased Amount of Water-immiscible 1-Butyl-1-methylpyrrolidinium Bis(trifluoromethanesulfonyl)imide

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We report here the effect of increased amount of a water-immiscible ionic liquid (IL), 1-butyl-1-methylpyrrolidinium bis(trifluoromethanesulfonyl)imide (P14-TFSI), enhancing the production amount of paclitaxel with in situ extraction from an aqueous medium in a plant cell culture. The productivity of paclitaxel in the culture including the IL of 10 vol% was 1.5 times greater than that of 2.5 vol%. Moreover, addition of an elicitor, methyl jasmonate (MJ) enhanced the production amount of paclitaxel in the culture including 10% P14-TFSI. The amount of paclitaxel in the culture including 1 µM MJ and 10 vol% P14-TFSI was more than 78 times greater than those in the control culture without both P14-TFSI and MJ, and two times greater than those in the culture including 2.5 vol% P14-TFSI alone.

1. Introduction

Paclitaxel is an excellent anticancer drug against a variety of cancers by stabilizing their depolymerization of microtubules during mitosis. It is very expensive because its manufactured production is carried out by semi-synthetic method which requires many reaction steps with numerous harmful organic solvents from precursors such as baccatin III (BIII) or 10-deacetyl baccatin III (10-DAB) extracted from the needles of Taxus species [1,2]. A plant cell culture is one promising method for a cost-effective and safer production process with reduced amounts of the plant resource and harmful solvents. Since the productivity of paclitaxel decreases by its feedback-inhibition in the cell culture [3], a strategy for improving the productivity should be explored.

Two-phase culture system using hydrophobic solid absorbents and water-immiscible liquids has been proposed for the in situ extraction and absorption of hydrophobic paclitaxel from the culture medium, respectively [4-6]. We reported the effectiveness of hydrophobic organic solvents such as lauryl alcohol for the production of paclitaxel in the plant cell culture [7,8]. Recently, ionic liquids (ILs) have been paid attention to as substitutes of conventional organic solvents for the extraction of bioactive compounds from biomass. [9-11]. Bioactive compounds such as hydrophobic 3-indole-butyril acid, ferulic acid and caffeine acid synthesized in plants could be extracted and separated by ILs [12-18]. Surface-active ILs have greater extraction efficiency for triterpenic acids from apple peels [9]. We found that the enhancement of paclitaxel in the plant cell culture with the in situ extraction of the compounds of the taxanes by hydrophobic ILs and 1-butyl-1-methylpyrrolidinium bis(trifluoromethanesulfonyl)imide (P14-TFSI), which has more
hydrophobic than 1-hexyl-3-methylimidazolium hexafluorophosphate (HMIM-PF₆) [19], enhanced the productivity of paclitaxel, BIII, 10-DAB, and cepharomannine (CM). Moreover, methyl jasmonate (MJ), a well-known elicitor which can stimulate stress responses in plants leading to enhancement of synthesis of secondary metabolites such as paclitaxel, has been used to increase the productivity of paclitaxel [8,20].

In our previous study, the addition of P14-TFSI into the culture medium compared with the IL-free medium (control) was effective to produce paclitaxel because P14-TFSI stimulated the production of paclitaxel and the amount of the paclitaxel produced in the culture was lowered by in situ extraction with the IL, decreasing the feedback-inhibition [19]. It is assumed that the increased amount of the IL in the cell culture contributes to more decreasing the inhibition and subsequently increasing the productivity of paclitaxel in the cell culture. In the present research we investigated the effect of the increased amount of P14-TFSI on the enhancement of the paclitaxel production amount in the two phase culture system. The effect of MJ addition on the production amount of paclitaxel of in the culture was examined too.

2. Experimental

2.1 Cell, medium and reagents

Induction of callus from the needles of Taxus cuspidata was carried out according to the procedures as described previously [5]. 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 used for the plant cell culture [5]. The induced callus was subcultured on a solid medium containing the modified Gamborg's B5 components. Precultured callus, which was cut into pieces using a knife to avoid the limited supply of the nutrients and oxygen into the callus, was prepared for the culture.

A hydrophobic IL, 1-butyl-1-methylpyrrolidinium bis(trifluoromethanesulfonyl)imide (P14-TFSI) purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) was utilized in the present research. The chemical formula and partition coefficient of paclitaxel in the IL-medium two phase culture system are shown in Table 1. Methyl jasmonate (MJ, Figure 1), an elicitor promoting paclitaxel synthesis [20], was used in the medium including the IL. Paclitaxel purchased from Wako Pure Chem. Co. (Osaka, Japan) was used as a standard reagent for HPLC analysis.

![Figure 1. Chemical structure of methyl jasmonate (MJ).](image)

Table 1. Chemical formula and physicochemical properties of P14-TFSI used in this research [19].

| Chemical formula | Solubility in aqueous medium [mM] | Partition coefficient of paclitaxel in the IL-medium two phase system [-] |
|------------------|----------------------------------|-------------------------------------------------|
| ![Chemical structure](image) | 16.1 | 45.4 |
2.2 Plant cell culture

Suspension culture inoculated by the precultured cells was carried out in a 100 cm$^3$ Erlenmeyer flask containing 20 cm$^3$ of the modified B5 medium and the defined amount of the IL on a rotary shaker (NR-150, Taitec, Saitama, Japan) at 110 rpm in the dark at 26 °C. The amounts of P14-TFSI, 0.5 and 2 mL which were calculated as 2.5 and 10 vol% based on the medium volume of 20 cm$^3$, were used in the culture. A schematic representation of culture system used in this research is shown in Figure 2. The concentration of MJ, 1 µM, was selected form the previous study where the MJ concentration was optimal for the production of paclitaxel in the IL-medium two phase culture system [19] and used for the present experiments. The amounts of fresh cells and paclitaxel in the culture flask after a 7 d culture period were measured. Experiments per culture condition were carried out in duplicates.

2.3 Effectiveness of IL and MJ on productivity of paclitaxel

For examining the effectiveness of P14-TFSI and MJ, the enhancement factor of the paclitaxel productivity, $E_P$, was defined as follows,

$$E_P \ [-\] = \frac{P_{P14-TFSI,\ MJ}}{P_{control}}$$

(1)

where $P_{P14-TFSI,\ MJ}$ [mg/L] and $P_{control}$ [mg/L] are total amount of the produced paclitaxel which is total sum of paclitaxel in the cells, medium and P14-TFSI in the cultures including the defined amount of P14-TFSI and/or MJ, and the amount of the produced paclitaxel in the control, respectively. These values were estimated from two individual experiments.

2.4 Analysis

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

3. Results and Discussion

3.1 Effect of amount of P14-TFSI on the production of paclitaxel

Figure 3 shows the effect of the amount of P14-TFSI on the production amount of paclitaxel after a 7 d culture period. Enhancement of the total amount of the produced paclitaxel, which is the total sum of paclitaxel in the cells, medium, and P14-TFSI in the cultures with the increased amount of the IL from 2.5 to 10 vol% was observed. This enhancement was caused by partition of the produced paclitaxel into the IL by means of the hydrophobic P14-TFSI. In addition, there is a possibility of P14-TFSI's stimulating factor enhanced the productivity of paclitaxel. Though the IL was judged non-cytotoxic liquid from the previous experiments [16], it might function as abiotic stress against the present plant cells to produce the secondary metabolites such as paclitaxel. A similar observation on enhanced production rate of the taxane in the culture using hairy root of *Taxus* species by perfluorodecalin has been reported [21].
3.2 Effect of amount of P14-TFSI on the cell growth

The effect of increased amount of P14-TFSI on cell growth is shown in Figure 4. The fresh cell weight in the culture with 2.5 vol% P14-TFSI was smaller than that in the P14-TFSI-free (control) culture, while that with 10 vol% P14-TFSI was greater than that in the control culture. The smaller weight of fresh cells in the culture including 2.5 vol% P14-TFSI might be due to the higher concentration of paclitaxel in the medium by excess production amount of paclitaxel in spite of the in situ extraction with the P14-TFSI (Figure 5). The higher concentration of paclitaxel gave feedback-inhibition against the cell to decrease the cell growth of the plant cell, which is a well-known phenomenon in plant cell culture [3]. The greater fresh cell weight in the culture including 10 vol% P14-TFSI might be due to the decreased concentration of paclitaxel in the medium and subsequent decrease of the paclitaxel’s feedback-inhibition by the in situ extraction with the increased amount of P14-TFSI from 2.5 to 10 vol% (Figure 5). Shapes of cells in the culture including P14-TFSI were almost similar as those in the control culture.

3.3 Effect of MJ addition on paclitaxel production in the two phase culture system

MJ, a well-known elicitor, enhances productivity of secondary metabolites such as paclitaxel in plant cell culture. Effect of MJ addition on the productivity of paclitaxel in the two phase culture system was examined. The effectiveness of P14-TFSI amount and MJ in the two phase culture system, $E_p$ defined by Equation (1), was evaluated. As shown in Figure 6, the $E_p$ values of P14-TFSI alone at 2.5 and 10 vol% were 39 and 60, respectively, which means the productivity of paclitaxel in the culture of 10 vol% was 1.5 times greater than that of 2.5 vol%. These data suggest that the increasing amount of P14-TFSI is useful for more production of paclitaxel due to increasing capacity of the in situ extraction with P14-TFSI. The $E_p$ values in the MJ added-culture including 2.5 and 10 vol% were 47 and 78, respectively. These results might be due to synergistic effects of P14-TFSI with decreasing the paclitaxel’s feedback-inhibition and MJ with enhancing the productivity of the paclitaxel on the paclitaxel production. The larger amount of P14-TFSI
and the higher concentration of MJ, which must be more effective culture conditions for the purpose, are under consideration.

A method for back-extracting paclitaxel from P14-TFSI for its actual production process is under examination. Since an adjustment of the medium pH was efficient back-extraction of hydrophobic bioactive compounds from HMIN-PF₆ [14]. Attempts on the back-extraction of the paclitaxel from the P14-TFSI by adjusting pH in the culture medium have not succeeded. We are now examining how to back-extract paclitaxel in the P14-TFSI with organic solvents which is volatile and immiscible with P14-TFSI.

4. Conclusion

Since the addition of hydrophobic P14-TFSI into the culture medium enhanced the productivity of paclitaxel, the increase of its amount and the use of an elicitor, MJ, were carried out. It is shown that the increased amount of the IL from 2.5 to 10 vol% and the addition of 1 µM MJ gave significant enhancement of the production amount of paclitaxel. These results suggest that P14-TFSI could be an excellent extractant and the two phase culture system using P14-TFSI in the presence of MJ could be effective for enhancement of paclitaxel production.

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