Effects of organic solvents on membrane of *Taxus cuspidata* cells in two-liquid-phase cultures

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Received 11 August 2003; accepted in revised form 16 March 2004

**Key words:** membrane permeability, organic solvents, *Taxus cuspidata*, two-liquid-phase

**Abstract**

The effects of organic solvents (oleic acid and dibutyl phthalate) on viability and membrane integrity of *Taxus cuspidata* cells were investigated in two-liquid-phase suspension cultures. It has been found that the cell viability, electrical conductivity and concentration of malonyl dialdehyde did not change obviously when the content of oleic acid or dibutyl phthalate was 2% (v/v), but varied markedly when the contents of oleic acid or dibutyl phthalate were raised to 6% (v/v) or more, indicating that the organic solvents at higher concentrations severely affected the cell membrane permeability.

**Abbreviations:** 6BA – 6-benzyl aminopurine; EC – electrical conductivity; MDA – malonyl dialdehyde; TBA – thiobarbituric acid; TCA – trichloroacetic acid; TTC – 2,3,5-triphenyltetrazolium chloride

**Introduction**

Taxol, a novel diterpenoid secondary product formed by yew species, has been approved as an efficient anti-cancer drug. Suspension cell cultures have been viewed as a promising alternative for obtaining taxol and related taxane compounds (Jaziri et al., 1996) and many strategies have been proposed to enhance taxol production (Yukimune et al., 1996; Yuan et al., 1998; Ketchum et al., 1999; Wu et al., 2000), among them the two-liquid-phase suspension cultures has been proved to be one of the most effective ones (Wu et al., 2000). In two-liquid-phase cultures, the extracellular products are generally partitioned into the non-polar phase. Not only does this ease the purification of the secondary metabolites by *in situ* removal, but it may also increase taxol production (Collins-Pavao et al., 1996). It has been shown that taxol was mainly intracellular in suspension cultures of *T. cuspidata* and partitioned significantly into the organic phase of two-liquid-phase cultures. Other advantages of two-liquid-phase cultures include the decrease of product feedback inhibition and increase of product yields (Collins-Pavao et al., 1996).

Previous studies mainly focused on enhancing the production of secondary metabolites in two liquid-phase cultures. Wang et al. (2001) showed that adding an extractive organic solvent into suspension cultures of *Taxus chinensis* not only stimulated taxol release and facilitated taxol recovery but also enhanced the yields of secondary metabolites.

Organic solvents used in two-liquid-phase suspension cultures, however, may be toxic to cells thus affecting their viability and metabolic activity. Two points need to be considered in selecting suitable organic solvents for two-liquid-phase cultures. First, the organic solvents should be biocompatible with cultured plant cells. Second, the partition coefficient of the desired secondary
metabolites should be high enough. It is generally accepted that the toxicity of organic solvents increases with decreasing log p (Harrop et al., 1992; Nikolova et al., 1993). Solvents of log p > 4 are generally considered to be hydrophobic and biocompatible with cells. For example, the log p values of oleic acid and dibutyl phthalate are 7.7 and 5.4 and the partition coefficients of taxol in them are 154 and 236, respectively. The addition of organic solvents partitions taxol into the organic phase, favoring taxol production. Whether and how the organic solvents affect the cell membrane permeability and change the secondly metabolism in two-liquid-phase cultures is an issue worth investigating. As the cell membrane is an interface for cells to exchange with the outer environment, the external stimuli may change some cellular functions (Goodman et al., 1995). In this work, the effects of organic solvents on membrane of two-liquid-phase suspension cultures of T. cuspidata were investigated in detail so as to have a better understanding of the molecular mechanism of taxol biosynthesis.

Materials and methods

Chemicals

2,3,5-triphenyltetrazolium chloride (TTC) and Evens Blue were purchased from Sigma. Oleic acid, dibutyl phthalate and other chemicals were of analytical grade and obtained commercially (sangon, China).

Culture conditions

The cell line was derived from young stems of T. cuspidata (Wu et al., 2000) and sub-cultured in modified B5 solid medium containing sucrose (25 gl l−1), α-naphthylacetic acid (2 mg l−1) and 6-BA (0.15 mg l−1). The medium pH was adjusted to 5.8 prior to autoclaving. The cells were cultured for five generations in liquid medium at 25 °C by shaking at 110 rpm in the dark. Cells (3.0 g) of the 5th generation were inoculated in 50 ml fresh modified B5 medium in a 250 ml Erlenmeyer flask.

Organic solvents and two-liquid-phase cultures

Lanne et al. (1987) reported that generally organic solvents with log p > 4 were biocompatible with cells. p is the partition coefficient of a compound over a standard i-octanol/water two-phase system. The log p of organic solvents can be found in relevant literature (Lanne et al., 1987; Vermue et al., 1993). Table 1 shows the physical properties of the organic solvents used in this work. The partition coefficients of taxol in the two-phase systems (organic solvent/aqueous medium) were measured at 25 °C as reported previously (Wu et al., 2000). Organic solvents were preconditioned by mixing with aqueous medium (10:90, v/v). The pre-balanced solvents were autoclaved at 120 °C for 30 min prior to addition into the cultures. For the two-phase culture experiments, appropriate volume of the sterilized solvent was added to each flask containing 50 ml culture medium on the 10th day of cell culture and the shake-flask cultivation continued.

Cell viability

Cell viability was assayed using 2,3,5-triphenyltetrazolium chloride (TTC) following the method of Iborra et al. (1992). Fresh cells (200 mg) were collected in a tube and 8 ml TTC solution (0.8% TTC in 0.05 M phosphate buffer, pH 7.4, and 0.5 ml l−1 Tween 20) were added and the mixture was infiltrated for 5 min at reduced pressure. The cell samples were incubated in the TTC solution for 24 h at 25 °C in the dark, then cells were removed and rinsed with distilled water, placed in separate quartz tubes containing 3 ml of 95% ethanol in each, and submerged for 24 h at 25 °C in the dark. The absorbance was measured at 485 nm. Cell viability was expressed as the

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<td>Oleic acid</td>
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<td>Dibutyl phthalate</td>
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