Biotransformation of Monoterpenes by Polyurethane Foam-Immobilized Cells of *Petroselinum crispum* (Mill) Nyman

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Cells of *Petroselinum crispum cv.* "Paramount" and "Plain-leaved" immobilized in polyurethane foam retained the biotransformational characteristics of their freely suspended cells for certain monoterpenic substrates. Immobilized cells of both cultivars were more efficient than their free cells in isomerizing geraniol into nerol, but less efficient (<60%) in reducing the aldehydes, citral and citronellal. Maintenance of the immobilized cells under the condition of growth limitation did not enhance the efficiency of the biochemical reactions. Under the same condition, the freely suspended cells of cult. "Paramount" exhibited higher efficiency (up to 1.6 times) of biotransformation of citral when compared to those in sucrose-supplemented media. This investigation lends support to the view that the biochemical productivity of cultured plant cells for especially monoterpenoids may not necessarily be improved upon immobilization.

Introduction

Among the methods for improving the synthetic potential of plant cultures is the immobilization technique which has received some attention in recent years [1, 2]. Usually, cells are immobilized by entrapment in calcium alginate and agar [3, 4], but more recently, the use and beneficial effects of porous (reticulate) polyurethane foam matrices for immobilization has been described [4–7].

We have recently studied the biosynthetic potential of undifferentiated cultures of *Petroselinum crispum* [8]. As a continuation of this investigation, we hereby report on the biotransformational characteristics of *P. crispum cv.* "Paramount" and "Plain-leaved" cultures immobilized in polyurethane foam, and maintained in both sucrose-supplemented and sucrose-free media.

Results and Discussion

Parsley cells (PCV 53%) used in this study were completely entrapped in the porous polyurethane foam cubes within two weeks of culture. Cultures were sampled for the conversion products at 4, 8 and 12 h respectively for citral, citronellal and geraniol, since maximal conversions were observed at these times for both the free and immobilized cells from a previous investigation (unpublished results). As indicated in Fig. 1, immobilized cells of *P. crispum* exhibited 15–31% (cult. "Plain-leaved") and 14–54% (cult. "Paramount") of the reductive bioconversion capacities of their freely suspended cells for the aldehydic substrates. However, efficiency of bioconversion of geraniol was several times greater for the immobilized cells of the two cultivars.

There is some literature evidence [9, 10] which indicated that immobilized cell systems are not necessarily superior to free cells in biosynthetic and biotransformational capacities. In another in-

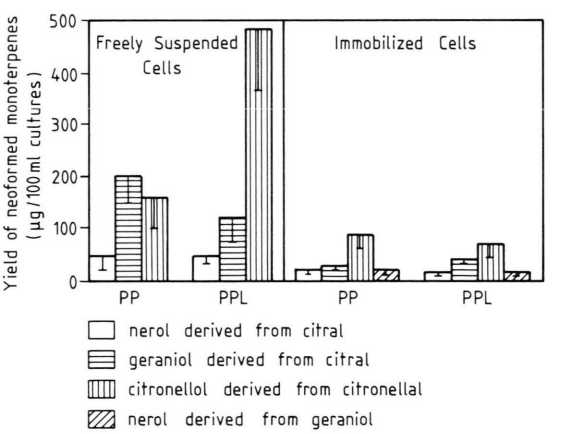

Fig. 1. Biotransformation of monoterpenes by freely suspended and immobilized cells of parsley maintained in sucrose-supplemented media. PP = cult. "Paramount"; PPL = cult. "Plain-leaved". Mean ± SD, $n = 3$.

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vestigation involving hydroxylation of digitoxigenin to periplogenin using *Daucus carota* cells, Jones and Veliky [11] attributed the reduced efficiency of the immobilized cells to diffusional barriers of the substrate. Since entrapment of cells in polyurethane foam appears not to have any deleterious effects [5] on the synthetic potential of immobilized plant cells, it is conceivable to expect factors other than diffusional barriers to be responsible for the reduced efficiency of immobilized parsley cells in biotransforming monoterpene aldehydes. One of the characteristics of immobilized plant cells is the release of the desired products into the medium. However, it was soon evident that metabolites usually stored in the cells were also accumulated within the entrapped cells [12]. In the present study, neoformed monoterpenic products were extracted from both the entrapped cells and media in a proportion that did not differ significantly from that observed for the free cell suspensions. Thus, immobilization seemed to be restricted to systems where the desired product was spontaneously released into the culture medium [4, 5].

Lindsey and Yeoman [5] have described an optimization of capsaicin synthesis by pepper cells when cultured in growth-limiting medium. In order to determine the effect of this factor on the bioconversion of some monoterpenes, appropriate callus tissues of parsley were subcultured into suspension culture liquid media devoid of sucrose, and maintained by repeated dilution for two generations before use. We observed reduced efficiency (9–16%, cult. "Paramount"; 16–27%, cult. "Plain-leaved") of biotransformation of aldehydes by immobilized cells of the two cultivars of parsley (Fig. 2) when compared to those freely suspended in sucrose-supplemented media (Fig. 1). Under this condition, the cells were more rapidly entrapped in the polyurethane foam, but showed no appreciable growth (PCV 18%). When compared to immobilized cells grown in sucrose-supplemented media (Fig. 1), the efficiency of the bioconversion was no greater (usually 5–6% and 56–82% respectively for citronellal and citral). Cells of cult. "Paramount" freely suspended in sucrose-free media demonstrated higher bioconversion efficiency for citral (2- to 3-fold) when compared to similar cells of cult. "Plain-leaved" (Fig. 2). However, isomerization of geraniol to nerol by both the free

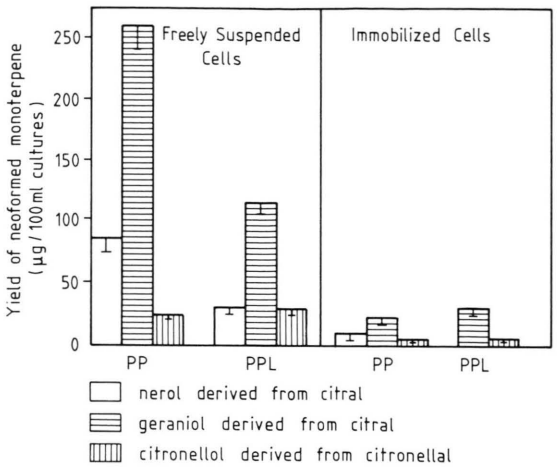

Fig. 2. Influence of nutrient limitation on the bioconversion of monoterpenes by freely suspended and immobilized cells of parsley maintained in sucrose-free media. Mean ± SD, $n = 3$.

and immobilized cells of the two cultures was not favoured appreciably under the growth-limiting condition.

The reported beneficial effect [5] of growth limitation was however evident in the biotransformation abilities of cult. "Paramount" free cells which exhibited higher efficiency (up to 1.6 times) for the bioconversion of citral when compared to those in sucrose-supplemented media. This was not the case with cultures of cult. "Plain-leaved" whose free cells in sucrose were more efficient in reducing citral (into nerol, 2-fold) and citronellal (19-fold) than those under the growth-limiting condition. Similarly, free cells of cult. "Paramount" in sucrose showed higher (6-fold) biotransformation ability for citronellal than those under growth-limiting condition. Elimination of sucrose from the culture media (leading to reduced culture growth) and the concomitant lower efficiency of biotransformation of added monoterpenes contradict the general view [13] that culture growth is inversely related to secondary product synthesis. Aviv and Galun [14] have reported that the biotransformation ability of *Mentha* cells is directly related to cell density. The effect of lower cell density (PCV 18%) of immobilized parsley cells under growth-limiting condition appears to outweigh other advantages of such a system, and hence preferentially accounted for the reduced efficiency of the immobilized cells

in performing certain biotransformational reactions, especially reductions.

In view of the results presented in this report, it is conceivable that immobilized systems may be more suited to performing certain chemical reactions *e.g.* isomerization of geraniol to nerol than others *e.g.* certain hydroxylations [10, 11]. In fact, Galun *et al.* [15] have reported that *Mentha* free cells were not different from those immobilized in cross-linked polyacrylamide-hydrazide in their efficiency of reducing menthone and pulegone. Previous reports have shown that immobilized cell systems grow more slowly than freely suspended cells, are partially differentiated and hence accumulate higher levels of secondary metabolites [2, 4–6]. Although immobilized parsley cells differentiated roots, there was no greater biotransformational abilities for the monoterpenic aldehydic substrates. Thus, immobilized cells may not necessarily show higher biosynthetic and biotransformational capabilities than corresponding freely suspended cells.

This investigation further lends support to the view [8] that morphological differentiation is not an absolute requirement for secondary product synthesis and accumulation in cultures plant cells. The biochemical processes in polyurethane foam-immobilized cultured plant cells may require further investigation.

Experimental

Establishment of cell suspensions

Cell cultures were initiated from the tenth and seventeenth generations respectively of actively growing callus tissues by cult. "Plain-leaved" and "Paramount", and maintained as previously described [8].

Immobilization of cells

The polyurethane foam material used for immobilization of plant cells was supplied by Delcon (Corby, Northants, U.K.), Fifteen foam materials (1 cm × 1 cm × 1.5 cm, 10 pores/inch) were threaded onto stainless steel wire according to Ishida [6] and submerged in 250 ml Erlenmeyer flasks containing distilled water and autoclaved prior to use. Autoclaved flasks were drained, rinsed with acetone, drained again and dried in an air circulating oven at 100 °C for 48 h. The third generation of appropriate cell suspensions were subcultured into immobilization flasks containing 50 ml sterile liquid medium, and agitated at 150 rpm until the cells were completely entrapped.

Precursor feeding and extraction

250 ml Erlenmeyer flasks containing 100 ml free or immobilized cell suspensions (three weeks in culture) were separately fed with 30 ppm levels of filter-sterilized ethanolic solutions of monoterpenic substrates in triplicate, and incubated [8] as described for the maintenance of cell suspensions. At analysis times, cultures were sampled, prechilled for 30 min and vacuum-filtered. Prechilled cells were freeze-dried for 12 h using Edwards Modulyo EF4 freeze dryer, and extracted in redistilled dichloromethane. The filtrates were similarly extracted for 2 h by shaking. The dichloromethane extracts for each analysis were reduced under nitrogen to 200 µl prior to GC analyses.

Analysis of components by GC and GC/MS

Authentic samples of the different substrates, their conversion products and an appropriate internal standard, citronellal or pentadecane were distinctly separated under the conditions previously described [8]. Quantitative estimations of the products were performed using the appropriate internal standard and an average of triplicate determinations ± SD were recorded.

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