ENTRAPMENT OF PLANT CELLS IN DIFFERENT MATRICES

A comparative study

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1. Introduction

During recent years the interest for plant tissue cultures as producers of secondary metabolites of commercial value has increased considerably [1–3]. Both callus and suspension cultures might be used in principle, but the latter type is preferred. Low productivity of natural products in such tissue cultures is, however, a recognized problem and many attempts are being made to manipulate the overall metabolism of the cells in order to increase the total yield of the product in question.

We reported the immobilization of whole plant cells by entrapment in calcium alginate gels [4] and believe that this technique is a valuable addition to the general techniques used in plant tissue cultures. The production of secondary products by the immobilized cells was higher than that for freely suspended cells under the same conditions. Thus, immobilized cells of *Morinda citrifolia* produced up to 10-times as much anthraquinones as freely suspended cells [5].

Immobilized plant cells were later studied for biotransformations of natural products [6–8]. The immobilization technique used in [6–8] was also entrapment in calcium alginate gels. Such gels require Ca^{2+} as stabilizer, which can lead to problems in phosphate-containing media. Therefore in [7] a special medium lacking phosphate ions was designed.

In our initial studies on immobilized plant cells we chose to use entrapment in alginate because there are also distinct advantages with this immobilization technique, including reversibility of the immobilization which enables the investigation of the cells in free suspension subsequent to their confinement in the immobilized state.

Here, we describe studies on alternative methods for the immobilization of plant cells. All experiments have been carried out with cells of *Catharanthus roseus*, obtained from suspension cultures. After immobilization by entrapment in various gels the viability of the gels (tested by plasmolysis, respiration and cell growth) as well as the ability of the immobilized cells to synthesize an indole alkaloid (ajmalicine) both from precursors (tryptamine and secologanin) and de novo with sucrose as sole carbon source has been investigated.

2. Materials and methods

2.1. *Materials*

Agar was supplied by Difco Labs. (Detroit, MI) and agarose (type VII) and κ-carrageenan (type III) by Sigma (St Louis, MO). Alginate (Manucol DH, batch no. 430041 E 6896) was obtained from Alginate Industries (Girven) and gelatin (commercial grade) from Kebo (Sweden). [2-^{14}C]Tryptamine was from NEN (Boston, MA) and secologanine and ajmalicine were kind gifts from Dr M. H. Zenk. All other chemicals and biochemicals were purchased from commercial sources.

2.2. *Cultivation of* Catharanthus roseus

The cell suspensions of *Catharanthus roseus* were cultivated in the medium of [9] supplemented with 10^{-6} M 2,4-dichlorophenoxyacetic acid and 10^{-6} M naphtylacetic acid (pH 6.0) on a rotary shaker at 100 rev./min and at 26°C.

2.3. *Immobilization of* C. roseus *cells*

The immobilization of *C. roseus* cells in various gels was carried out under sterile conditions in the following way:

Entrapment in calcium alginate was done as in [4] by the method in [10]. The cells (5.0 g wet wt) were suspended in 5% alginate (5.0 g) and the suspension was added dropwise to 50 mM $CaCl_2$. The alginate beads formed were left for 1 h in this solution and then washed.

Immobilization in a copolymer of alginate and gelatin was carried out in a similar way. The cells (5.0 g wet wt) were suspended in a mixture of 5% alginate (3.75 g) and 20% gelatin (1.25 g) and beads were made as above. The beads were then treated with 2% glutaraldehyde (10 ml) for 30 min and then washed again.

Agarose-entrapped cells were prepared by the method in [11]. The cells (5.0 g wet wt) were suspended in 5% agarose (5.0 g) at 40°C. As quickly as possible this suspension was poured over a Teflon plate tightly covered with holes (diam. 3 mm). Another plate was used as support and the two plates were held together by clamps. After the agarose had solidified the form was taken apart and the 'cylindrical beads' were taken out and washed.

Cells were entrapped in a copolymer of agarose and gelatin in a similar manner. The cells (5.0 g wet wt) were suspended in a mixture of 5% agarose (3.75 g) and 20% gelatin (1.25 g) and cylindrical beads were made as above. The washed beads were treated with 2% glutaraldehyde (10 ml) for 30 min and then washed again.

Gelatin-entrapped cells were obtained as follows: The cells (5.0 g wet wt) were suspended in 20% gelatin (5.0 g) and 25% glutaraldehyde (0.4 g) was added. Cylindrical beads were moulded as above and after 30 min the beads were taken out and washed.

Cells were immobilized in carrageenan as follows: The cells (5.0 g wet wt) were suspended in 3% carrageenan (5.0 g) at 50°C and the suspension was as soon as possible added dropwise to 0.3 M KCl. The carrageenan beads formed were left for 1 h in this solution, then washed.

Cells were immobilized in agar by suspending them (5.0 g wet wt) in 4% agar (5.0 g) at 50°C with subsequent moulding of cylindrical beads.

Finally, cells were entrapped in polyacrylamide as follows: The cells (5.0 g wet wt) were suspended in a solution of acrylamide (1.45 g) and *N,N'*-methylendiacrylamide (0.07 g) in 0.05 M Tris–HCl buffer (pH 7.5) (5.0 ml). To this suspension was added TEMED (0.02 g in 0.2 ml H_2O) and ammoniumpersulfate (0.02 g in 0.4 ml H_2O) and beads were moulded in the Teflon-device.

2.4. *Cell growth of immobilized* C. roseus

Cultivation of immobilized *C. roseus* cells in the medium of [9], supplemented with hormones, was initiated by inoculating beads (1.0 g) of the various preparations in samples of the medium (10 ml). The flasks were shaken at 100 rev./min and at 26°C. Cell growth was estimated by observing the number of freely suspended cells in the medium.

2.5. *Biosynthetic studies*

The synthesis of ajmalicine from tryptamine and secologanin by free and immobilized cells was studied in the following way: Free cells (0.5 g wet wt) or immobilized cells (1.0 g beads) were added to medium [9] (10 ml) with no hormones added, containing tryptamine (1.25 μmol) labelled with ^{14}C (400 000 dpm/μmol) and secologanin (1.25 μmol). After 2 and 5 days samples were analyzed for radioactive ajmalicine.

The de novo synthesis of ajmalicine was determined in a similar manner. Thus, free cells (0.5 g wet wt) or immobilized cells (1.0 g beads) were innoculated in the hormone-free medium (10 ml). After 2 weeks samples were taken and analyzed for ajmalicine content.

2.6. *Analytical procedures*

Plasmolysis of cells with glycerol was followed under microscope with phenosaffranin as indicator. Before the immobilized cells were studied the beads were cut into thin slices.

Respiration of the cells was monitored with an oxygen electrode (Rank Brothers). Free cells (0.1 g wet wt) or immobilized cells (0.2 g beads, chopped into small pieces) were added to the growth medium (2.0 ml) and the consumption of oxygen was followed on a recorder.

Ajmalicine extracted as follows: The medium was extracted with methylene chloride (3 × 5 ml). The organic phases were combined and evaporated to dryness under vacuum on a rotavapor. The residue was dissolved in either chloroform (1.0 ml) for the experiments with precursors or in the HPLC-eluent (1.0 ml) for the de novo experiments.

The cells/beads were placed in a Soxhlet-extractor and methanol (25 ml) was used for the extraction which was done overnight in a water bath. The methanol was evaporated and the residue was dissolved in either chloroform of HPLC-medium as above.

The amount of radiolabelled ajmalicine was esti-

mated after TLC-chromatography on silica plates (DC-Alufolien, Kieselgel 60 F254, E, Merck) with acetone–petroleum ether–diethylamine (2:7:1) as eluent. Samples (50 μl) from the extractions were applied and 'cold' ajmalicine was added to each sample in order to simplify detection of the ajmalicine-spots (R_F 0.58), which were cut out and analyzed for radioactivity.

The samples from the de novo synthesis experiments were analyzed by HPLC under the following conditions:

Column size:	200 X 4 mm
Column packing:	Nucleosile-C_{18}, 5 μm
Eluent:	0.01 M $(NH_4)_2CO_2$–acetonitril (3:7)
Flow rate:	1.0 ml/min
Sample size:	10 μl
Detection:	UV, 242 nm

The amount of ajmalicine (retention time 5.4 min) was determined by measuring the peak heights and comparing them to standards. The detection limit is ~0.5 μg/ajmalicine/ml.

3. Results and discussion

Cells from suspension cultures of *Catharanthus roseus* were immobilized by entrapment in various gels (see table 1). Some characteristics of the immobilized plant cells were studied and a comparison of the different immobilization methods was done as described below.

3.1. *The plasma membrane*

An intact plasma membrane is of great importance when a retained viability of the cells is desired after the immobilization. Plasmolysis was used as an indication of the intactness of the plasma membrane of the cells. The immobilized cells were thus exposed to a plasmolysing agent, e.g., glycerol, and studied under microscope. The dye phenosaffranin, which does not penetrate an intact membrane, was used as an additional indicator. Furthermore, such a dye simplifies the detection of plasmolysis.

Table 1
Comparison of various preparations of immobilized *C. roseus* cells

Preparation	Plasmolysis	Respiration	Cell growth
Alginate	+	+	+
Agarose	+	+	+
Agar	+	+	+
Carrageenan	+	+	+
Alginate + gelatin	+	–	–
Agarose + gelatin	+	–	–
Gelatin	+	–	–
Polyacrylamide	–	–	–

As can be seen in table 1 all the immobilized cells except for those entrapped in polyacrylamide were plasmolysed and thus appeared to have intact plasma membranes. Obviously, the chemicals used in the polyacrylamide entrapment are harmful to the cell membrane. The plasmolysis experiments were carried out directly after the immobilization as well as after 20 h. No significant difference was observed between these two sets of experiments, indicating a relatively good stability of the immobilized cells. For the fully viable cells (see under cell growth below) the plasma membrane appeared to stay intact over an extended period of time (up to 2 weeks; longer incubation times not tested).

3.2. *Respiration*

A second indication of viability is retained respiration of the cells after immobilization. Respiration of the cells was measured with an oxygen electrode. To reduce diffusion barriers the beads containing the plant cells were chopped into relatively small pieces before the measurements. As can be seen in table 1 the cells within the gels treated with glutaraldehyde (i.e., the gels containing gelatin) lost their respiration capacity. The respiratory chain of these cells appears to be sensitive to glutaraldehyde. It may, however, be possible to crosslink with glutaraldehyde under milder conditions [12], which may leave the respiratory chain intact. Furthermore, it can be pointed out that this study on respiration is only qualitative since some diffusion barriers still exist after the beads have been chopped into pieces.

The cross-linking with glutaraldehyde was attempted in order to stabilize the gels. In the case of alginate such a stabilization by cross-linking could obviate the requirement for high calcium concentrations in the medium and thereby eliminate problems with the administration of phosphate to the immobilized plant cells.

3.2. *Cell growth*

The ultimate criterion on viability is, of course, the growth and division of cells. As can be seen in

table 1 the cells entrapped in polyacrylamide and in the gels treated with glutaraldehyde did not, as expected, grow at all and therefore these preparations were not tested further. On the other hand, when the cells immobilized in agar, agarose or alginate were placed in a complete medium they grew and divided and after 1 week the number of cells had increased to such an extent that the beads started to burst. After a few additional days most of the cells were found in a free suspended form. On a semi-quantitative basis it was concluded that the cells entrapped in agar and agarose grew better than the cells immobilized in alginate. The cells immobilized in carrageenan did not grow to such an extent but after 1 week a number of cells could be found in the medium. Obviously, the elevated temperatures (up to 50°C) required for the entrapment in agar, agarose and carrageenan did not influence the viability of the cells to any great extent.

In the biosynthetic studies (see below) the immobilized cells were placed in a growth limiting medium, i.e., no hormones were added. The number of cells did not increase to any great extent in such a medium and after 2 weeks incubation no free cells were observed in the medium.

3.4. *Synthesis of ajmalicine from precursors*

Cells of *C. roseus* entrapped in alginate can synthesize ajmalicine from the distant precursors tryptamine and secologanin [4]. The various preparations of immobilized cells were incubated with these precursors and the production of ajmalicine was determined. The results are given in table 2 and it can be seen that relatively high incorporations of [^{14}C]tryptamine were observed for all the preparations studied. The increase in incorporation between day 2 and day 5 was relatively small but relative to freely suspended cells the increase for the immobilized cells was larger. Furthermore, it can be concluded that the cells entrapped in agarose or alginate were more efficient than free cells under the conditions used. Cells entrapped in agar and carrageenan were on the other hand somewhat less effective.

3.5. *De novo synthesis of ajmalicine*

We have also studied the de novo synthesis of ajmalicine with sucrose as the sole carbon source by immobilized cells of *C. roseus*. As can be seen in table 2 all the preparations of immobilized cells were capable of synthesizing ajmalicine at a rate 60–140% of that observed with freely suspended cells. There are several reports on the de novo synthesis of ajmalicine by *C. roseus* cells in suspension cultures [13–15]. Obviously, the ability to synthesize ajmalicine is preserved in the immobilized cells and even enhanced in the case of alginate entrapped cells. The synthesis of ajmalicine can be increased considerably for both free and immobilized cells by using the alkaloid production medium in [13]. The reason for the increased synthesis observed with alginate entrapped cells is at present under investigation and will be reported elsewhere [16].

4. Conclusions

These data clearly demonstrate that plant cells can be immobilized by various methods with preserved metabolism. The secondary product formation (from precursors or de novo) is in the cases studied highest for alginate entrapped cells but also agar, agarose and carrageenan appear to be suitable polymers for entrapment of plant cells. Furthermore, it can be concluded that plant cells are sensitive to reactive chemicals (e.g., glutaraldehyde) which are often used for the immo-

Table 2
Synthesis of ajmalicine by free and immobilized cells of *C. roseus*

Cell prepn.	From precursors				De novo	
	2 days		5 days		2 weeks	
	% Incorp.	Rel. incorp.	% Incorp.	Rel. incorp.	μg/sample	Rel. prod.
Free cells	7.2	100	7.9	100	4.2	100
Agar	6.4	89	7.5	95	3.7	88
Agarose	7.8	108	9.0	114	4.2	100
Alginate	9.1	126	13.9	176	5.9	140
Carrageenan	5.5	76	6.5	82	2.6	62

bilization of biocatalysts to various supports. Consequently, entrapment seems to be the most suitable method for immobilization of plant cells.

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