IMMOBILIZED PLANT CELLS FOR THE PRODUCTION AND TRANSFORMATION OF NATURAL PRODUCTS

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Received 19 April 1979

1. Introduction

There has been considerable interest in the past few years in the area of immobilized entities, such as enzymes and microbial cells [1]. Preparations of this kind have proven to be of value not only for studies of a fundamental nature but also for a number of practical applications including the production and transformation of food stuff additives and drugs. The advantages of using such preparations include, in particular, a lower cost in biotechnological applications because of the possibility of reusing the 'biocatalyst' and there is no need for separation of the product from the catalyst. Furthermore, immobilized preparations are ideally suited for flow-through processes; these advantages of immobilized systems have been amply reviewed [2,3].

In this study we report on an extension of the use of the immobilization technique to include also, for the first time, plant cells. Isolated growing plant cells have been discussed as a potential source for the production and biotransformation of natural products (e.g., [4]). The same advantages mentioned above should also apply to immobilized plant cells. In addition, it appears likely that the chemical potential of normally slow growing plant cells (under batch conditions) could be more efficiently exploited by immobilization.

Three examples of the use of immobilized plant cells are given here. First the de novo synthesis of anthraquinones by entrapped cells of *Morinda* is demonstrated, second the formation of indole alkaloids from distant precursors by *Catharanthus* cells and third, the position and stereospecific hydroxylation of the cardiac glucoside digitoxin to digoxin by *Digitalis* cells is presented.

2. Materials and methods

2.1. *Materials*

[2-^{14}C]Tryptamine was from NEN (Boston, MA), the sodium salt of alginic acid was obtained from Sigma (St. Louis, MO), secologanin and digitoxin from Boehringer (Mannheim). All other chemicals and biochemicals were purchased from commercial sources.

2.2. *Methods*

The cell suspensions were cultivated in the following media:

Morinda citrifolia, medium of Gamborg et al. [5] containing 10^{-5} M naphtylacetic acid (pH 5.5) (medium A). This medium was modified for the experiments with immobilized *Morinda* cells in that no hormone was added.

Catharanthus roseus: medium of Linsmaier and Skoog [6] with 10^{-6} M 2,4-dichlorophenoxyacetic acid and 10^{-6} M naphtylacetic acid (pH 6.0) (medium B).

Digitalis lanata (strain Gö 20 W): medium of Nitsch and Nitsch [7] (pH 5.5) (medium C).

The immobilization of *M. citrifolia* and *D. lanata* cells were carried out under sterile conditions. This was not necessary for the *C. roseus* because of the shorter duration of the experiments.

Immobilized *Morinda* cells were prepared using the alginate method [8] as follows: The cells (15 ml

packed cells) were suspended in 3% alginate (25 ml) and medium A (5 ml), without hormones was added. The suspension was next added dropwise to medium A containing 50 mM $CaCl_2$. The alginate beads formed (mean diam. 3.5 mm) were left for 4 h in this solution and then washed extensively with the appropriate medium.

Catharanthus cells were immobilized in a similar way. Beads were made by dripping a suspension of cells (10 g wet wt) in 2% alginate (10 g) into medium B containing 50 mM $CaCl_2$. The beads were left overnight and then washed with medium B containing 5 mM $CaCl_2$.

Digitalis cells were filtered through a net (850 μm) before immobilization in order to remove larger cell aggregates. Subsequently the cells (3 ml of packed cells) were suspended in 3% alginate (10 ml) and made into beads as described above. After 4 h the beads were washed 3 times with medium.

Cultivation of immobilized *Morinda* cells in medium A without hormones was initiated by innoculating 30 beads in 25 ml samples of the medium; the flasks were shaken at 100 rev./min and at 23°C. During cultivation samples were taken and analyzed for anthraquinone content as well as for the number of cells within the beads as follows: The beads from one flask were separated from the medium and washed with water. Beads (20) were extracted in 80% ethanol by refluxing for 30 min. Afterwards the beads were removed and then the extract was made up to 25 ml. The anthraquinone content was measured spectrophotometrically at 434 nm [9]. The number of cells was estimated by dissolving 1 bead in 1 ml of 1 M potassium phosphate buffer (pH 6.5). Samples (10) of the cell suspension obtained were counted under the microscope in a haemocytometer.

For cultivation of freely suspended *Morinda* cells, under the same conditions as the immobilized cells, 1 ml packed cells was innoculated in 25 ml batches of the hormone free medium. For estimation of anthraquinone 1 g cells (fresh wt) was extracted and analyzed as before. A sample of the culture was appropriately diluted (5–25-fold) and the number of cells counted.

The beads with immobilized *Catharanthus* cells (10 g wet beads) were packed in a column and these were used to study the formation of alkaloids of the ajmalicine group. A typical experiment was carried out as follows: A solution of the precursors tryptamine (25 μmol) labelled with ^{14}C (233 000 dpm/μmol) and secologanin (25 μmol) in medium B containing 5 mM $CaCl_2$ (10 ml) was recirculated through the column at a flow rate of 25 ml/h. Lipophilic products were continuously extracted from the aqueous phase by bubbling the reaction mixture through chloroform (10 ml). Samples (10 μl) were withdrawn from the two phases and analyzed for radioactivity. Samples (10 μl) were also applied to thin-layer chromatographic plates (silica) and were developed in acetone/petroleum ether/diethylamine (2:7:1). The tryptamine spot (R_F 0.23) as well as the alkaloid spot (R_F 0.58) were cut out analyzed for radioactivity and alkaloid quality [10].

Digitoxin (500 μg in 0.5 ml of 96% ethanol) was added under sterile conditions to medium C (25 ml). Beads (30) containing *Digitalis* cells were used in each flask and the flasks were shaken (100 rev./min) at 23°C. Samples (0.2 ml) of the medium were taken under sterile conditions and stored frozen until analyzed by radioimmunoassay according to [11].

3. Results and discussion

Cells of *Morinda citrifolia* were entrapped in alginate beads to investigate whether such an immobilization procedure would adversely effect the metabolism of the cell. To this end we chose to follow the de novo synthesis of anthraquinones normally produced by this strain in suspension cultures [9]. As can be seen in fig.1 the anthraquinone content of the immobilized cells increased for an extended period of time under conditions where sucrose served as the only carbon source. Furthermore, it can be seen that under the same conditions the anthraquinone content of freely suspended cells decreased with time. It should be pointed out that the cultivations were carried out in medium containing no hormone. Under such conditions of hormone limitation the production of anthraquinones per cell is increased (G. Wilson, personal commun.) as compared with cells multiplying freely in media with growth hormones. The drastic difference observed for immobilized and freely suspended cells cannot at present be explained satisfactorily. Whether this is an effect of immobilization as such or due to some microenvironmental effects within the beads has still to be investigated.

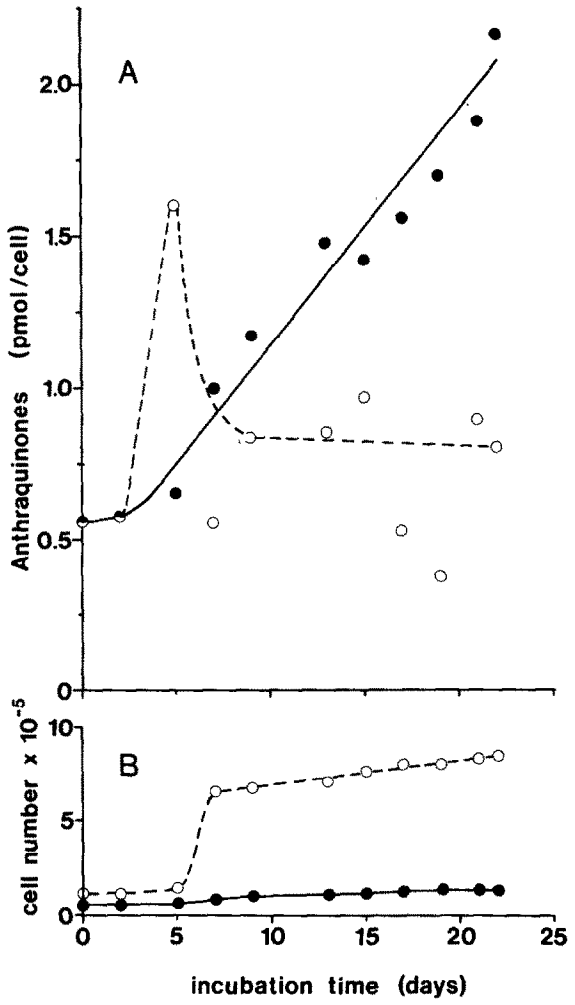

Fig.1. (A) The de novo synthesis with time of anthraquinones by *Morinda citrifolia* cells. (B) Increase in number of cells with time per ml for free and per bead for immobilized cells, respectively. (o-o-o) Free cells; (•-•-•) immobilized cells.

A similar pattern in the kinetics of anthraquinone production was observed when media with a higher sucrose content (6.5%) were used. It is noteworthy that under conditions of cell entrapment, the de novo synthesis of secondary metabolites (anthraquinones) from a carbohydrate source continues up to at least 22 days. Under these conditions, increase in cell number is only slight and it seems that the biochemical potential of the cells is diverted away from the growth and cell division processes to secondary product formation. It should be also stressed that the immobilized cells even after 22 days at 23°C were alive as shown by plasmolysis and respiration measurements.

The formation of indole alkaloids of the ajmalicine group was studied using immobilized *Catharanthus roseus* cells. The immobilized cells were fed a solution containing the two central precursors tryptamine (^{14}C-labelled) and secologanin. As can be seen in fig.2 tryptamine was continuously consumed while chloroform-extractable compounds were produced. About 30% of the tryptamine had been converted to such lipophilic substances after 90 h and at this point one-third of these products consisted of a mixture of the 3 alkaloid isomers ajmalicine, 19-epiajmalicine and tetrahydroalstonin (in a ratio 15:4:6). It has been shown that cell free extracts of *C. roseus* can produce the same mixture of ajmalicine isomers from tryptamine and secologanin [10]. In this case, however, NADPH had to be added in order to obtain the reducing equivalents required for the transformation of the intermediate cathenamine to ajmalicine [12].

The relatively high yield of product with immobilized cells (10% overall yield) indicates that the endogenous NADPH is recycled within the entrapped cells. Furthermore, it is well known from cell sus-

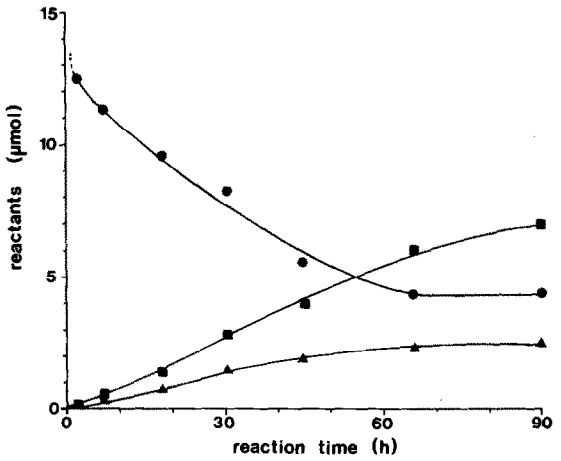

Fig.2. Synthesis of indole alkaloids of the ajmalicine group by immobilized cells of *Catharanthus roseus* as in section 2. (•-•-•) Unreacted tryptamine in aqueous phase; (■-■-■) metabolized tryptamine in the form of chloroform-extractable compounds; (▲-▲-▲) ajmalicine isomers in the chloroform phase.

pension experiments [13] that the products are not excreted into the medium but are stored within the vacuoles of the cells. Under conditions of immobilization, however, the alkaloids formed seem to be released into the medium since 85% of the radioactivity administered was recovered. This might be owing to a change in the membrane permeability caused by traces of chloroform, which is used in the continuous extraction of product, being present in the reaction mixture. It should be pointed out that as this 'release effect' may be of considerable importance for the utilization of immobilized plant cells in the production of natural products, further studies will be carried out on this effect.

The biotransformation of the steroid digitoxin to digoxin involving hydroxylation at position 12β has been investigated using immobilized *Digitalis lanata* cells. As seen from fig.3 the immobilization procedure does not adversely affect the transformation capacity of these plant cells as judged from the increasing amount of digoxin found in the medium over 33 days. The amount of digoxin formed under conditions of entrapment corresponds approximately to that reported for freely suspended cells of the same strain used in the Bochum laboratory [14]. As a control the immobilized cells were also incubated with medium lacking digitoxin. From fig.3 it can be seen that no digoxin (or any derivative of it) was formed proving that de novo synthesis did not account for the

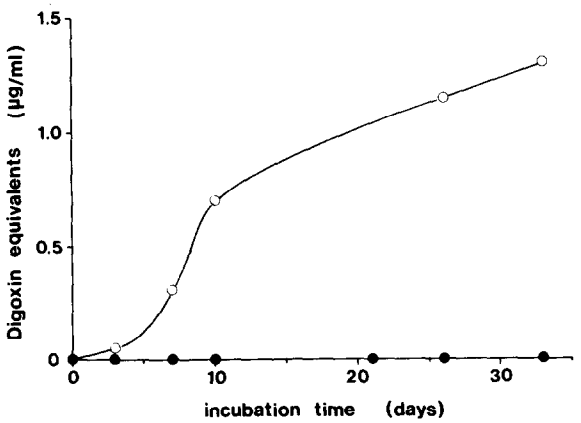

Fig.3. Biotransformation of digitoxin to digoxin by immobilized cells of *Digitalis lanata* as a function of incubation time. (o-o-o) With addition of digitoxin; (•-•-•) without addition of digitoxin.

accumulation of digoxin. The result obtained is similar to that reported on 11β hydroxylation of steroids by entrapped bacteria [15]. It should be added that no optimization of the hydroxylation step has been attempted in this preliminary study but based on the results it appears likely that other biotransformations so far reported [16] including acetylation, glucosylation or demethylation should be possible with immobilized *D. lanata* preparations. It should be added that immobilization under sterile conditions posed no problem.

In our opinion the immobilization of plant cells and protoplasts (with or without regenerated cell walls [17]) shows promising potential for the production of natural compounds either by de novo synthesis, by synthesis from distant precursors or by simple biotransformation. As shown in this paper, immobilized plant cells – although they are much bigger in volume, contain large vacuoles and have a cellulose cell wall – do not differ in their basic behaviour from that of immobilized microorganisms. The metabolic capacity of these entrapped cells seems to stay intact for surprisingly long periods of time and the products formed are, under certain conditions, released into the medium. Also of importance is that recycling and regeneration of the coenzymes involved in the biosynthetic processes take place under conditions of entrapment.

Future experiments will concentrate on testing different immobilization techniques as well as optimizing product formation using whole plant cells or organelles from them as biocatalysts.

Acknowledgements

Research at Bochum was supported by a grant from the Minister für Forschung und Technologie, Bonn, and at Lund in part by the Swedish Board for Technical Development. We thank Dr F. Kaiser, Boehringer, Mannheim, for a gift of secologanin and Dr T. Griffin for linguistic advice.

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