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Immobilized Plant Cells

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I. Introduction

A. IMMOBILIZED BIOCATALYSTS

There has been considerable interest during the last decade in the immobilization of enzymes and cells (Mosbach, 1976; Brodelius, 1978). Immobilization is usually carried out by one of the following procedures: (1) covalent binding, (2) adsorption, (3) entrapment or microencapsulation, or (4) aggregation of enzymes using bifunctional agents leading to insoluble aggregates (Fig. 1). Naturally occurring polysaccharides such as agarose, alginate, various acrylate polymers, or controlled pore glass are usually used as support material. The support (i.e., carrier or matrix) is generally in the beaded form, although enzymes and/or cells bound to or within membranes are also being applied. Such immobilized preparations have found use in three major areas: (1) for the production of biotechnologically interesting substances, (2) in analysis, and (3) in medicine. The advantages that are gained using immobilized preparations are considerable. For instance, in the medical area, missing enzymes can be held in "extracorporal shunts" while immobilized within small polymer beads. On passage of the blood through such beads, toxic components can be removed (Chang, 1977). The alternative approach of injecting soluble enzymes leads to immunogenic reactions and other complications. In the analytic field, immobilization of enzymes

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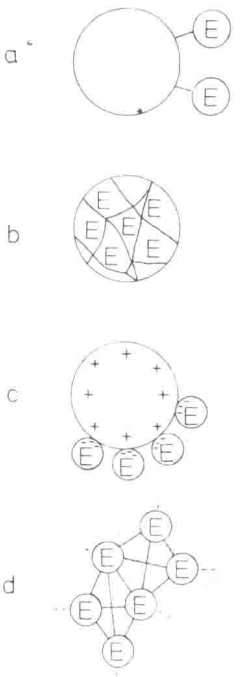

FIG. 1. Alternative principle methods to immobilize enzymes and other biocatalysts. (a) Covalent coupling; (b) entrapment; (c) adsorption; (d) cross-linking.

directly on or close to the transducer (e.g., an electrode or thermistor) gives rise to quicker and more sensitive analyses (Guilbault, 1976; Mosbach and Danielsson, 1981). Finally, for production purposes, immobilization allows reuse of the biocatalyst, often leads to increased stability, and permits continuous processing. A cost analysis comparing the use of an immobilized cell system producing aspartic acid with that of the conventional batch process using intact cells revealed a cost reduction of 40% due mainly to decreased costs of catalysts and labor (the additional price of the support material does not adversely change the overall balance) (Chibata, 1980). The procedure of choice for the immobilization of cells is usually entrapment, whereby the size of the polymer network surrounding the cells is chosen to allow in and/or out diffusion of substrate and/or product and at the same time keep the cells physically encaged. Apart from early isolated attempts to entrap cells in polyacrylamide (Mosbach and Mosbach, 1966), a major breakthrough first came about in the early seventies. Today, some processes employing immobilized cells are already in commercial use including the formation of L-malic acid and L-aspartic acid (Linko and Larinkari, 1980). The immobilized cells hitherto studied and put to use have been predominantly of microbial origin. Originally, single enzyme activities, such as aspartase for

the conversion of fumarate + NH_4^+ to asparate, were utilized. Prior to entrapment, the cells are usually treated with organic solvents, metals, or heat to minimize side reactions which usually cause them to become nonviable. However, during a series of studies on steroid transformation it was found that single step steroid transformations, such as 11β-hydroxylation leading from Reichstein's compound S to cortisol and subsequently to prednisolon by $\Delta^{1,2}$-dehydrogenation, could also be carried out using living and growing cells (within the polymer beads) without the formation of disturbing side products (Mosbach and Larsson, 1970). In fact, the use of immobilized living cells is gaining in importance because it is possible to utilize these cells for more complex coenzyme-requiring multistep enzymic reactions, such as those involved in ethanol production from glucose or penicillin biosynthesis. Since cells can grow within these beads, the original biotransformation or *de novo* synthetic capacity of the immobilized species can often be increased severalfold (Larsson *et al.*, 1976). More recently, even spores have been entrapped and allowed to grow within the beads yielding a uniformly distributed biocatalyst. The technique of immobilization has been refined during the last 10 years so that now any of the members of the sequence enzyme–multienzyme–organelle–cell can be immobilized with retention of activity.

B. Plant Cell Cultures

Before we describe the immobilization of plant cells and the properties of such preparations, we would like to discuss briefly the potential applications of plant cell cultures.

A large number of natural products isolated from higher plants are today utilized in the food, cosmetic, and pharmaceutical industries. For instance, about 25% of all prescribed drugs contain compounds isolated from higher plants; the most common of these substances are listed in Table I. As can be seen, most of them are various kinds of alkaloids. In recent years the supply of some of these plants has become difficult to maintain, and it is likely that the number of such species will increase in the future. Therefore, there has been a search for alternative ways to obtain these valuable substances, and plant tissue cultures appear to be the most, if not the only, promising alternative. A large number of different substances have been obtained in culture, but low productivity is a recurring problem that can, however, be overcome by selection of high-producing cell lines. It was not until very recently that such selection techniques were developed, and the number of high-producing cell lines reported is therefore rather limited. In Table II some examples of cultures producing the compound of interest in equal or higher amounts than the parent plant are listed. Many of the listed com-

TABLE I
THE MOST COMMON AND ESSENTIAL DRUG COMPOUNDS DERIVED FROM HIGHER PLANTS[a]

1. Steroids (from diosgenin)
2. Codeine
3. Atropine
4. Reserpine
5. Hyocyamine
6. Digoxin
7. Scopolamine
8. Digitoxin
9. Pilocarpine
10. Quinidine
11. Colcinine
12. Emetine
13. Morphine
14. Quinine
15. Sennosides
16. Tubocurarine
17. Vincristine

[a]Zenk (1978).

pounds are of commercial interest. Plant tissue cultures thus have a potential for the production of secondary plant products, notably high-cost substances such as drug and fragrant compounds.

Biotransformation is another area where plant tissue cultures have been considered as a potential catalyst. The most studied system, i.e., the stereospecific hydroxylation of digitoxin to digoxin, has reached a state close to commercialization (Reinhard, 1979). Another biotransformation that may prove possible with plant cell cultures is the conversion of thebain to codein, a very important drug, even though no reports have been published yet on this subject.

The potential use of plant tissue cultures in the synthesis and transformation of natural compounds has been more extensively discussed in a number of recent reviews (Zenk, 1978; Kurz and Constabel, 1979; Reinhard and Alfermann, 1980; Staba, 1980).

II. Immobilized Plant Cells

The area of immobilized cells was recently extended with the report on the immobilization of plant cells grown in suspension (Brodelius *et al.*, 1979a). These investigations were initiated to study plant cell behavior in a milieu simulating that prevailing in normal tissues, as well as to establish possible advantages of using immobilized plant cells for the production or biotrans-

TABLE II

CELL CULTURES WHICH PRODUCE PRODUCTS IN AMOUNTS EQUAL TO OR LARGER THAN THE PARENT PLANT[a]

Product	Plant species	Culture condition	Cell culture (% dry weight)	Yield (gm/liter)	Plant (% dry weight)	Ratio cell culture/ parent plant
Ginsengoside	*Panax ginseng*	C	27	—	4.5	6
Anthraquinones	*Morinda citrifolia*	S	18	2.5	2.2	8
Rosmarinic acid	*Coleus blumei*	S	15	3.6	3	5
Shikonin	*Lithospermum erythrorhizon*	C	12	—	1.5	8
Anthraquinones	*Cassia tora*	C	6	—	0.6	10
Diosgenin	*Dioscorea deltoides*	S	2	—	2	1
Biscoclaurine	*Stephania cepharantha*	C	2.3	—	0.8	3
Caffein	*Coffea arabica*	C	1.6	—	1.6	1
Ajmalicine	*Catharanthus roseua*	S	1.0	0.26	0.3	3
Paniculide B	*Andrographis paniculate*	C	0.9	—	0	∞
Serpentine	*Catharanthus roseus*	S	0.8	0.16	0.5	1.6
Serpentine	*Catharanthus roseus*	C	0.5	—	0.5	1
Protopine	*Macleaya microcarpa*	C	0.4	—	0.32	1.25
Visnagin	*Ammi visnaga*	C	0.31	—	0.1	3
Glutathione	*Nicotiana tabacum*	S	—	0.22	0.1	10
Ubiquinone-10	*Nicotiana tabacum*	S	0.036	0.045	0.003	12

[a]C, Callus culture; S, cell suspension culture (from Zenk, 1978).

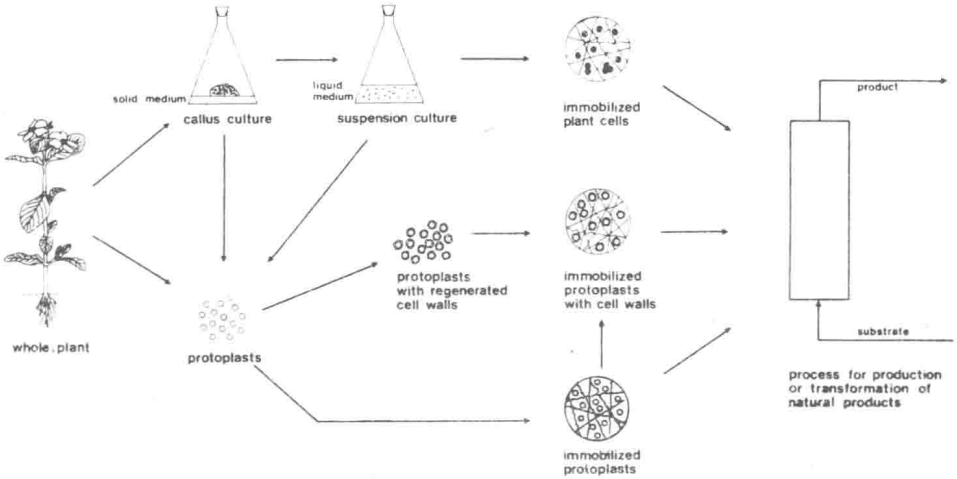

FIG. 2. Alternative approaches to prepare and immobilize plant cells. (From Brodelius *et al.*, 1980.)

formation of valuable plant cell products. As to the latter, special emphasis was laid on the question of whether plant cells in the immobilized state show higher production of secondary metabolites as well as changes in the excretion pattern, i.e., release of product into the medium. The combination of the plant cell culture technique with that of the immobilization technique is schematically illustrated in Fig. 2. As can be seen, after the establishment of a culture the cells can be immobilized and used in a process. Alternatively, protoplasts can be made and subsequently immobilized and utilized in a similar way.

A. IMMOBILIZATION TECHNIQUES

In our initial studies on the immobilization of whole plant cells (Brodelius *et al.*, 1979a,b), we chose to entrap the cells in Ca^{2+}-alginate (Kierstan and Bucke, 1977) since in previous studies on the immobilization of microbial cells, we found this method to be simple, mild, and giving a high yield of active biocatalyst.

Alginate is a polysaccharide composed of guluronic acid and mannuronic acid and thus containing one carboxyl group on each sugar monomer. In the presence of Ca^{2+} or other multivalent ions, alginate forms a gel through the formation of ionic bridges. This gel can be solubilized by addition of a Ca^{2+}-complexing agent such as phosphate ion, citrate, or EDTA. The possibility to reverse the immobilization, i.e., to obtain the immobilized biomaterial back

in free solution and/or suspension, was an additional major advantage of this immobilization technique in our initial studies. The immobilized plant cells could thus be released and studied in various ways. Furthermore, it is relatively simple to immobilize cells under sterile conditions, which is essential when working with slowly growing plant cells.

The immobilization in alginate is simply to carry out as mentioned previously, and large quantities of immobilized cells are readily prepared. Filtered cells [up to 50% cells (wet weight) can easily be entrapped] are added to the alginate solution (2–5% w/w of the sodium salt), and the suspension is dripped into a medium containing at least 50 m*M* $CaCl_2$. The beads formed are left for 30–60 minutes in this medium and then washed and transferred to the appropriate medium, which should contain at least 5 m*M* $CaCl_2$ to stabilize the beads.

Small batches are easily prepared by the utilization of sterile plastic syringes with or without a needle. If needles are used in order to obtain small beads, care must be taken to avoid clogging of the needle by larger cell aggregates. If possible, the cell suspension can be filtered through a nylon net of appropriate mesh to avoid problems of this kind.

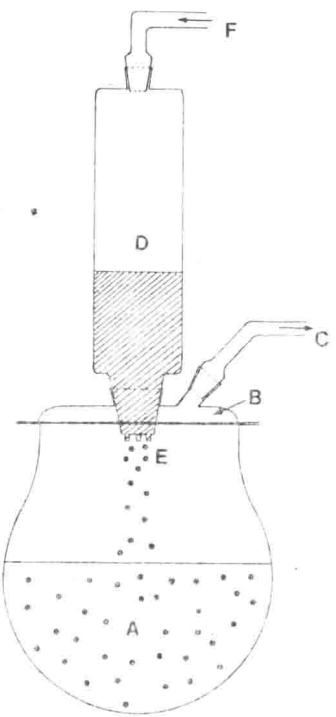

FIG. 3. Device for large-scale preparation of alginate-entrapped plant cells. (A) Medium container; (B) lid; (C) air outlet; (D) reservoir for alginate-cell suspension; (E) six nipples (ϕ = 1 mm) for bead formation; (F) sterile air inlet.

For larger batches of alginate-entrapped cells, we have designed a special device for the immobilization procedure. This device is shown in Fig. 3. The alginate suspension, contained in a reservoir (D), is forced through six nipples [inner diameter (ϕ) = 1 mm] (E) by a light pressure of sterile air (F). Up to 300 gm of beads can be made within a few minutes, but there should be no problem in scaling up. The whole device is autoclaved with the Ca^{2+}-containing medium in the medium container (A), and the cells and alginate are mixed and subsequently poured into the reservoir (D). Beads of uniform size are formed (Fig. 4). Also, in this case it is advantageous to remove larger cell aggregates from the suspension before immobilization.

Recently, we investigated some other gel-forming polymers for the entrapment of whole plant cells (Brodelius and Nilsson, 1980). Some of the polymers tested form a gel on cooling from elevated temperatures (40–50°C). Beads can be molded by pouring a warm suspension of the polymer and the plant cells into a form. We use two teflon plates, one of which is tightly covered with holes (ϕ = 1–2 mm), for the molding. The plates, shown in Fig. 5, are held together by clamps. After the gel has solidified, the plates are taken apart and the "cylindrical beads" (Fig. 6) are taken out, washed, and placed in an appropriate medium. This molding technique was originally used for the immobilization of animal cells (Nilsson and Mosbach, 1980). It can be pointed out that there are no major problems to working under sterile conditions with this technique, which, however, is less suitable for the preparation of large quantities of immobilized cells. The method has been used for the immobilization of plant cells in agarose (2.5% w/w final concentration), agar (2% w/w final concentration), and κ-carrageenan (1.5% w/w final concentration).

Carrageenan is a polysulfonated polysaccharide that forms a relatively strong gel in the presence of potassium ions. After molding, the carrageenan beads are, therefore, placed in a medium containing 0.3 *M* KCl for 30–60 minutes to improve the stability of the gel. Beads of carrageenan can also be made by dripping the warm suspension of carrageenan and cells into a medium containing 0.3 *M* KCl. The beads formed are, however, not of uniform size and they are also irregular.

The molding form has also been used to immobilize cells in polyacrylamide and gelatin (10% w/w final concentration) cross-linked with glutaraldehyde.

Finally, we have immobilized plant cells in copolymers of alginate or agarose and gelatin. After bead formation, treatment with glutaraldehyde was carried out to cross-link the gelatin and thereby make the beads more stable. As can be seen in Table III, however, the glutaraldehyde treatment adversely affected cell respiration and cell growth; consequently, these immobilization methods have been abandoned.

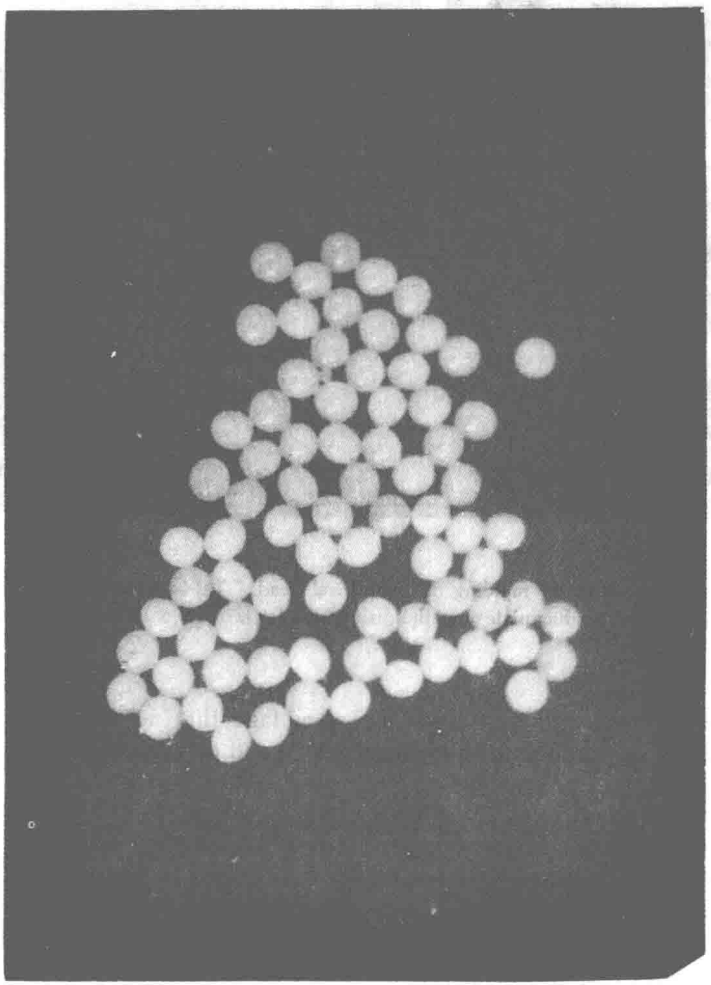

FIG. 4. Alginate beads containing cells of *Catharanthus roseus* made with the device schematically shown in Fig. 3.

So far we have tested a few methods for the immobilization of plant cells, but there are still many other methods which deserve to be investigated. The most convenient technique of those we have tested, especially for the preparation of large quantities of immobilized cells, is entrapment in Ca^{2+}-