Cell Immobilization

Introduction

Cell immobilization is a technique to fix plant cells in a suitable matrix. Cell immobilization is different from cell entrapment in that immobilized cells can be entrapped cells but also the cells are absorbed onto support materials (Pras and Woenderbag, 1999).

Immobilization is now a well-established technique with the history of enzyme immobilization going back over 25 years and including many industrial applications. The immobilization of microorganism is less well developed in terms of large-scale application, but it is widely used in the laboratory. With this background it was inevitable that immobilization techniques should be applied to plant cell cultures and much work has been carried out to establish methods for plant cell immobilization and suitable bioreactors for use with the immobilized cultures (Williams and Mavituna, 1992).

Plant cells grow much more slowly, they produce targetted compounds more slowly, they are more easily disrupted by physical stress and their behaviour (growth and synthesis) is influenced by chemical signals by neigbouring. Then by immobilization, the plant cells are protected from liquid shear forces. Moreover, immobilization facilitates the importance of cellular cross talk, which can establish inter-cellular communication by the action of signalling molecules. This should enhance the biosynthetic of plant cells (Haigh and Linden, 1989; Pras and Woenderbag, 1999).

Freely suspended plant cells mostly accumulate their secondary metabolistes in the stationary phase of their growth cycle, at the point of time their growth stop. Entrapment of plant cells is one the means to create non-growth condition under which the production of secondary metabolites may be improved (Pras and Woenderbag, 1999).

Immobilized plant cells have been employed to perform biotransformations and reported to have higher production rates than freely suspended cells. For example, immobilized Capsicum cultures treated with precursors accumulated more quantities of biotrasnformed compounds than freely suspended cultures (Johnson et al., 1996; Rao and Ravishankar, 2000).

Advantage and Disadvantage of Cell Immobilization

In an immobilized system growth and production phases can be decoupled and controlled by chemical and physical stress conditions. This allows cells to be retained in the bioreactor for extended periods, with alternating rejuvanation/growth and secondary metabolite production cycles (Williams and Mavituna, 1992).

Process engineering problems may develop from the tendency of plant cells to aggregate, which can lead to blockages in pipes and openings and to the culture rapidly sedimenting, if it is not continually agitated. However the shear sensitivity of the culture means that mechanical agitation may be detrimental to cells and that cultures can not be transported using conventional pumps without significant loss of viability. Again immobilization may be a solution to these problems and may offer a microenviroment protected from sustained shear (Williams and Mavituna, 1992).

The physical separation of the cells and medium in the immobilized system also makes for easy exchange of medium for purposes of metabolic control or nutrient replenishment. The composition of the culture medium can be readily and continuously monitored via an external loop, and the concentrations of O_2, sugar, etc. adjusted as required. Similarly, extracellular products can be harvested continuously by adsorption on a suitable resin, or by other means (Tyler et al., 1995).

In the horse radish immobilized cells, the acid invertase activities were lowered therefore the availability of intracellular sucrose for glucosylation was high, thus glucosinolates were produced earlier than that of suspended cell cultures (Mevy et al., 1999)

The main disadvantage of immobilization is that it is only of use with cell lines, which excrete the product of interest into the culture medium. Attempt to induce the release of products which are normally retained within the cells by such techniques as permeabilisation have generally decreased cell viability to an undesirable extend, although reversible permeabilisation of Catharanthus roseus using dimethyl sulfoxide (DMSO) was promising (Williams and Mavituna, 1992).

Function of Immobilization

Immobilized plant cells, just like that of freely suspended cells can be used for the purposes of bioconversion, the novo synthesis and sysnthesis from precursors.

Methods of Immobilization

As mentioned earliear that entrapment is part of immobilization. Entrapment methods that have been used with plant cell cultures can be categorised into:

1. Gel entrapment by ionic network formation.

Entrapment by ionic network formation, especially in the form of alginate beads, is the most widely used method. Alginate is a polysaccharide that forms a stable

gel in the presence of cations, with calcium the most frequently used. Beads of alginate-containing cells, are formed by dripping a cell suspension-sodium alginate solution mixture into a stirred calcium chloride solution. κ-Carragenan can also be used in similar manner instead of alginate, using either calcium or potassium. Advantage of this method is that the gel can be reversible by adding EDTA. Moreover, synerisis can happen in the presence of other Calcium chelating agent such as phosphates (Williams and Mavituna, 1992).

Cultivation of *Morinda citrifolia, Catharanthus roseus* and *Digitalis lanata* were successfully done by this method of immobilization, with significant increase of metabolite and the stability of metabolic capacity was also extended for long periods of time (Brodelius et al., 1979). Moreover the immobilized cells release the metabolite to the medium as also the case with maize plant cells (Zayed, 1997).

2. Gel entrapment by precipitation.

Preparations of agar and agarose can be used to trap plant cells by precipitation. The polysaccahrides form gel when a heated aquoues solution is cooled. The gel can be dispersed into particles in the warm liquid state by mixing in a hydrophobic phase, e. g. olive oil. When particels of the desired size are obtained the entire mixture is cooled and this results in solidification (Williams and Mavituna, 1992).

In the case for *Catharanthus roseus,* 5 gram of wet weight cells was suspended in 5% agarose at 40 degree Celcius. As quickly as possible the suspension was poured over a teflon plate covered with holes (diameter 3 mm). Another plate was used as support, and the two plates were holded together by clamps. After the agarose had solidified, the two plates were taken apart to release the cylindrical beads (Brodelius and Nilsson, 1980). Felix et al. (1981) found out that enzyme isolation was obviated and the enzymes were more stable in immobilized Catharanthus roseus cells (Felix et al., 1981).

3. Gel entrapment by polymerisation.

Gel entrapment by polimerisation is most commonly carried out using polyacrylamide. However, the toxicity of the initiator and cross-lingking agents used in the polymerisation has in some cases caused a loss of cell viability. Brodelius and Nilsson (1980) showed that Catharanthus roseus cells entraped by polyacrylamide gel did not grow at all and hence respiration and plasmolysis were not detected. On the other hand, by suspending plant cells in aqueous solution of prepolymerised linear polyacrylamide partially substituted with acrylhydrazide groups, Galun and co-workers (1984) were successful to maintain the vitality of entraped microbial cells.

4. Entrapment in the preformed structures.

Entrapment in preformed structures involves some form of open network through which nutrient medium may pass, but which entraps plant cells or cell aggregates. Such structures can be facilitated by using cotton fibre, fibreglass mats, reticulate polyurethane foam, and in a cloth nonwoven polyester short fibres.

The polyurethane foam has some merit as a matrix; no reagent which might not toxic to plant cells, and no complicated operation causing microbial contamination are required. Cells were immobilized via their invasion into pre-formed polyurethane foam cut into blocks. Lindsey et al. (1983) immobilized *Capsicum frutescence* cells in reticulate polyurethane foam, and showed that the immobilized cells produced more capsaicin than the free cells.

The glass fibre material were shown to be an ideal substratum for immobilising cultured *Catharanthus roseus* cells because of its high surface free energy and large surface area to give maximum adhesion. This type of inert support eliminates undefined physiological perturbartion in gel systems caused by the high calcium content, the low phosphate level necessary to reduce calcium ion chelation and the polysaccharide gel (Facchini and DiCosmo, 1991)

Factors Affecting Immobilization

Cell-Matrix interaction

It is important to note when using reticulated polyurethane foam, in order for any immobilized cells to grow well, the volumetric fraction of the foam has to be sufficient enough for all the cells and the reticulated pores of the foam is large enough to contain the cells. Moreover, Hu and Yuan (1995) emphasize the importance of initial interaction of cells with the surface of the polyurethane particles, intrusion of cells into the foam, simultaneous growth and coalescence of cells in the foam and retention of cells in the foam.

Mass Transfer

The transfer of compounds through immobilized cell matrix is usually assumed to be by molecular diffusion as in gel entrapment. Nevertheless, the microstructure of the immobilization matrix may bring other types into action such as capilary and active transports. These types of transport mechanism are true for reticulated foam, membrane and fibre mats matrices (Williams and Mavituna, 1992). Naturally, the resistance towards mass transfer that results in a decrease in the transport of nutrients, can be an advantage in creating a stress factor for secondary metabolite synthesis.

Further, Tyler et al. (1995) stated that mass transfer restriction within the biomass can be minimised by surface immobilization in a layer form where there is maximum contact between the surface of the immobilized cultures and the liquid phase.

Aeration of Immobilized Cells

Relationship between metabolism and dissolved oxygen concentration is complex, a conclusion can not be reached about the effect of reduced availability of oxygen in immobilized plant cell system on secondary metabolite production and growth. Alginate entrapped cells of *Thalictrum minus* were found to turn black owing to the insufficient supply of oxygen and they failed to produce berberine (Kobayashi, et. al., 1987).

On the contrary, Wilkinson et al. (1988) discovered that a reduction in the dissolved oxygen concentration of the medium resulted in the production of capsaicin by *Capsicum frutescens* entrapped in polyurethane foam particles.

Light

Metabolism of cultures can be affected by periodic exposure to light, and the quality and intensity of the light are significant. Only the outer cell layers of the cultures in the immobilized matrix may receive some light. This may be advantageous in the case where some precursors are form in light and some in dark condition, such as *Catharanthus* alkaloids. The supply of light to the interior of the immobilized cell matrix may be possible by the use of optical fibres.

Harvesting in Immobilized System

Immobilized plant cell cultures notably allows ease of continuous harvesting in the large scale, provided that metabolites are excreted into the medium.

Some plant cells are able to release their secondary metabolites spontaneously such as indoles, pyridines, quinolines, benzyl isoquinolines, quinolizidines, anthraquinones, capsaicins, opines, phenolics and terpenoids. In certain cases, immobilisation appears to induce spontaneous release of the products that are normally stored within the cells in suspension (Williams and Mavituna, 1992).

The release of metabolites into the medium can be improved by the methods of permeabilization and in-situ extraction. Two most popular approaches in permeabilization involve surface-active chemicals such DMSO, phenetyl alcohol, chroloform, triton X-100 and hexadesyltrymethylamonium bromide and electroporation. Other permeabilization methods include ultrasonication and ionophoretic release eventhough the use of these methods is discouraged due possibility of cell damage (Williams and Mavituna, 1992).

In-situ adsorption and extraction can be performed by adding inert hydrophobic chemicals (liquid or solid) especially having high adsorption capacity for the hydrophobic plant products into the cultures (Kim and Chang, 1990).

Scaling-Up (Bioreactor)

It is worthied to note that the time necessary to establish a large-scale volume of plant cells is time-consuming and expensive process. Moreover, any large bioreactors that must be used to compesate for the low volumetric productivity of slow growing plant cell cultures are sensitive to contamination.

Nevertheless, immobilised plant cell bioreactor has been chosen over the conventional stirred or fed batch bioreactor due to protection of cells from liquid shear forcess allowing for increased mixing speeds and more efficient mass transfer. It is also possible to use smaller reactor volumes since the slow growing immobilised biomass can be maintained in a productive state growth for an extended period. Separation of growth and production phases using easily exchangeable growth and production media is feasible in the immobilised plant cell bioreactors (Facchini and DiCosmo, 1991).

Lambie (1990) stated other advantageous of immobilized cell reactors as follows:

1. It leads to high cell concentration within the reactor.
2. It prevents washouts in continuous reactors.
3. It allows batch reactors to be operated on a drain/refill basis.
4. It permits specific spacial arrangement within the reactors.

Type of Bioreactors for Immobilized Plant Cells

A. Packed Bed Reactors.
   This type of reactor have been used with immbolized cells of Pseudomonas spp. in alginate beads for production of L-Cysteine (Ryu et al., 1996), It appeared this system have poor mass transfer characteristics and the beads may suffer from compresion under their own static weights (Lambie, 1990)

B. Mechanically agitated and Airlift Reactors
   Surfaced immobilized cells of *C. roseus* (Archambault et al., 1989; Tyler et al., 1995) and *T. rugosum* (Faccini and DiCosmo, 1990) were placed in an agitated and airlift reactors for production of alkaloids. Viability of immobilized cells of T. rugosum cultivated in Bioreactores was the same as the one in shake flask but the accumulation of the protoberberine was lower than the one in the shake flask. This, however, was due to low ratio of innoculum to fresh medium in order to maintain a constant ratio of biomas to the fibre substratum. Mechanically agitated reactores might damage the plant cells or the beads (if immobilized in alginate) in which the cells are entrapped. In

contrast, airlift reactore minimise damage to cells, but they are unsuitable for particles eg. Gel beads used to immobilized cells.

C. Bubble Column Reactor
Immobilized cells of *Coffea arabica* in alginates were photocultured using a bubble column reactor. The cells were damaged so severely at a low aeration rate that they neither grow nor produce alkaloids because of physical stress from thin film of bursting bubbles (Kurata et al., 1994; Kurata and Furusaki, 1993).

D. Hollow Fibre Membrane Reactors
Cells of *Glycine max, Daucus carota, Petunia hybrida, Coffea arabica* and *Nicotiana tabacum* were once cultivated using this reactor where their concentrated suspension of cells were introduced into the shell side of the reactor and medium aerated in separate researvoir was circulated through the fibres (Williams and Mavituna, 1992). Such reactors have several advantageous including good control of fluid dynamic and flow distribution, and the inherent advantage of using a membrane, of improved protection againsts contamination. However, membrane reactors are also expensive, liable to fouling, having problems with gas transfer and difficult to innoculate (Lambie et al., 1990).

E. Flat Bed Reactors
In this design the sheet of foam were suspended as vertical baffles in a stirred reactor so that the plant cells were incorporated into the network by the stirring to give solid sheets of cells. Furthermore, the immobilised cells are in direct contact with the nutrient medium; there is no permebiality barrier to nutrients and metabolites that could be created by the gel. This system was applied for immobilised cells of Capsicum frutescens (Lindsey et al. 1983; Williams and Mavituna, 1992).

Conclusion

Immobilisation has been used for several cell lines for either production of metabolites or their biocoversion. For Capsicum frutescens and Catharanthus roseus, immobilisation significantly affects the production of capsain and alkaloids, respectively.

Further research is needed to investigate the possibility of immobilised plant cell cultures to produce compound of interest economically. One paramount requirement for large-scale production is that the cell has to be able to excrete the compound of interest into the medium and hence continuous feeding and harvesting can be achieved.

At the same token, there is also a need to obtain an optimised method for cell immobilisation.

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