PRODUCT ISOLATION AND RECOVERY

Secondary metabolite recovery

Many secondary metabolites are hydrophilic, so they are primarily stored in the cell vacuole; however, hydrophobic molecules are often stored in the cell membrane, vesicles, dead cells and extracellular sites, such as the cell wall. Paclitaxel accumulates within the cell wall, with as little as 7-10% of the product released to the extracellular medium. The use of cell wall digesting enzymes resulted in the recovery of up to 90% of total paclitaxel in the extracellular medium. Cell walls can be ruptured using homogenization, sonication, cell wall digesting enzymes or steam explosion, but all biomass is destroyed. To maintain cell viability and allow for further use of biomass, cells can be permeabilized using pH shock or chemical treatments. If the product is secreted to the medium, cell rupture can be avoided and biomass can be separated from the medium prior to downstream processing. Promoting secretion by utilizing aqueous two-phase systems, media exchange and resin adsorbents are beneficial for product purification because unwanted by-products are minimized.

Adsorption, precipitation, distillation, membrane separation and extraction are examples of different techniques used for initial recovery of secondary metabolites from plant cell culture. There are complications associated with each separation technique, such as protein fouling on membranes and low selectivity of extraction procedures due to complex media compositions. The initial extraction of the product from biomass is typically achieved through liquid-liquid extraction or aqueous two phase systems. The extraction solvent must be chosen carefully based on the physicochemical properties of the product of interest. Solvent properties such as pH and polarity affect product stability and separation efficiency. In addition, interactions between the solvent and molecule of interest sometimes lead to undesired structural changes in the product.

Secondary metabolite excretion

The secondary metabolites produced are stored either intracellularly (in the vacuole or in other organelles), or extracellularly (bound to the cell wall or released into the medium). The excretion behavior of plant cell cultures varies from one species to another, and even within one species, from one cell line to another. As an example, protoberberine alkaloids are usually stored within the cells. However, cell line of *Thalictrun minus* excreting their alkaloids spontaneously in the medium. Apart from the selection of cell lines other approaches exist to trigger the efflux of secondary metabolites, mainly chemical and/or physical changes of the environment of the cells. When the cells excrete their product, the product concentration in the fermentation broth is usually very low, which is an extra cost factor for the downstream processing. Concentration of the product stream, for instance in or on a second phase, could therefore be beneficial. Another important consequence of the removal of the product from the aqueous phase can be the decrease of product inhibition effects, which occur even at the low product concentrations that are usually encountered in plant cell cultures (Buitelaar *et al*., 1991).

Natural harvest

One of the main problems associated with the commercial supply of secondary metabolites is limited compound availability. Secondary metabolites typically represent <1% dry weight of the plant, so natural harvest is often impractical. For instance, 340,000 kg of *Taxus* bark or 38,000 trees were required to meet the 25 kg per year demand for the anticancer drug paclitaxel (Taxol®; Bristol- Myers Squibb, New York, NY). Harvesting is also limited by seasonal availability; species abundance and plant growth rate. Despite these challenges, several compounds continue to be harvested from their native plant owing to lack of better commercialization options. Schillberg and Twyman (2004) report that molecules of 20–30 kDa generally pass through plant cell walls and are thereby secreted into the culture medium. As an alternative to natural harvest, secondary metabolites can also be synthesized and supplied through three general approaches: total or partial chemical synthesis, heterologous expression of the biosynthetic pathway in other organisms and *in situ* production via plant cell culture.

In situ product removal

The accumulation of secondary metabolites can be limited by both feedback inhibition of product synthesis and product degradation. *In situ* product removal from the culture media using two-phase solid-liquid or immiscible liquid-liquid systems can overcome these limitations and increase culture productivity, while simplifying product recovery and allowing for semi-continuous operation. Two-phase systems can also help to simplify purification of products from the media. For example, a six-fold increase in paclitaxel production was achieved in *T. chinensis* suspension cultures in aqueous-organic two-phase systems with sucrose feeding, with 63% of the product released to the media. One phase is the aqueous medium; the second phase can be either a water-immiscible organic solvent or a solid compound. Criteria for the selection of suitable second phases are: The material to be used as the second phase should be autoclavable, non-toxic, it should not influence the medium composition, it should bind the desired product, preferably in a specific manner, and finally, the product should easily be recovered from the second phase. The addition of an artificial site for the accumulation of secondary metabolites can be an effective tool for increasing biosynthetic pathways in plant cell cultures. If the formation of a product is subject to feedback inhibition or intracellular degradation, the removal and sequestering of the product in an artificial compartment may increase total metabolite production.

Two-phase systems even accumulate traces of secondary metabolites from the culture medium, thus avoiding any type of feedback inhibition. Another effect may be the enhancement of secondary metabolite release from the cultures or the initiation of a release of compounds normally stored within the cells. Secreted secondary metabolites may be protected from degradation in the culture medium because of excreted catabolic enzymes and acids. Evaporation of the product into the gas phase can be reduced by trapping flavor compounds in artificial accumulation sites. Desired plant products can then be removed

selectively from the culture systems. The product can be concentrated by in situ recovery, and downstream purification may be reduced if product removal from the culture medium and cells is selective. Consequently, recovery and purification are generally simplified, thus reducing production costs.

Membrane Permeabilization

In most cases, products formed by plant cell cultures are stored in vacuoles. In order to release the products from vacuoles of plant cells, two membrane barriers (plasma membrane and tonoplast) have to be penetrated. Cell permeabilization depends on the formation of pores in one or more of the membrane systems of the plant cell, enabling the passage of various molecules into and out of the cell. Attempts have been made to permeabilize the plant cells transiently, to maintain the cell viability, and to have short time periods of increased mass transfer of substrate and metabolites to and from the cell. Permeabilization of plant membranes for the release of secondary metabolites is often connected with the loss of viability of the plant cells treated with permeabilizing agents and methods. Various methods have been used to initiate product release from cultured plant cells. These methods include chemical treatments (e.g., with solution of high ionic strength, change of external pH, permeabilization with dimethylsulfoxide DMSO, chitosan) and physical treatments (e.g., high electric field pulses, ultrasonics, ultra-high pressure).

Chemical Permeabilization

Active uptake mechanisms have also been reported for indole alkaloids in *Catharantus roseus* vacuoles. In terms of product release, it is pertinent to note that in cell cultures, an efflux of alkaloids was observed under certain conditions, indicating equilibrium between the intracellular and extracellular compartments that could be perturbed by medium acidification with subsequent product release. The release of serpentine by *Catharantus roseus* cells was observed when the cells were filtered and resuspended in fresh or conditioned medium, and it was suggested that temporary membrane uncoupling was responsible for it. According to Dornenburg and Knorr (1997), *Chenopodium rubrum* cells could be permeabilized by treatment with chitosan. This polycationic polysaccharide induces pore formation only in the plasmalemma of the plant cell cultures. The leakage caused by chitosan can be considered as leakage from cytosol. Treatment of *Beta vulgaris* cell culture for 15 min with 0.7 mM Triton X-100 induced the release of 30% of betacyanines without loss of cell viability. After this permeabilization treatment, *Beta vulgaris* cultures regrew normally, reaching a maximum biomass concentration of 48% higher than non-permeabilized cultures after 14 days of culture.

Many attempts have been made to permeabilize the plant cell membranes in a reversible way with organic solvents. Especially dimethylsulfoxide (DMSO) has been used in many cases, because it is known to extract sterols from the membranes of eukaryotic cells (Felix, 1987). Of all the cell types tested, only *Catharanthus roseus* survived the treatment with DMSO and could be used in a cyclic process, where product release and growth were alternated. For the other cell types investigated, like *Cinchona ledgeriana*, *Chenopodium rubrum* or *Thalictrum*

rugosum, much higher concentrations of DMSO were necessary for extraction of the products, but lead to cell death. Probably, the reason for this cell death is not the toxicity of DMSO as such, but the loss of cell compartmentation with the concomitant release of toxic compounds and degradative enzymes into the cytoplasm. Apart from DMSO, other chemicals have been used, like chloroform and propanol, mostly without success. The organic solvents used in successful (meaning: with preserved viability) experiments all had a lipophilic character, like hexadecane, perfluorchemicals and Miglyol.

Physical Permeabilization

Physical factors causing membrane permeabilization include high electric field pulses, high hydrostatic pressure, ultrasound, etc. Products were released up to 100%, depending on the voltage applied, but viability decreased substantially. Application of the high electric field pulses is based on the principle of development of membrane pores under external electric fields. Depending on electric field strength or pulse number, the pore formation can be reversible or irreversible. Application of high electric field pulses led to high levels of cell permeabilization in cultures of *Chenopodium rubrum*, but at field strengths beyond 0.75 kV/cm and constant amount of ten pulses, cell viability approached zero values. Treatment with high hydrostatic pressure of 50 MPa increased the production of amarantin and antraquinones in cell cultures of *Chenopodium ruburum* and *Morinda citrifolia*. It was found that pressure higher than 250 MPa causes the loss of cells viability, most likely because of permeabilization of tonoplast.

Temperature

Secondary metabolites can be released with minimal loss of cell viability by the application of mild heat treatment to the cell cultures. Weathers *et al*., (1990) treated hairy roots of *Carthamus tinctorius* and root disks and hairy roots of *Beta vulgaris* with temperatures ranging from 25-55 °C. The release of polyacetylenes (*C. tinctorius*) and betanin (*B. vulgaris*) increased with increasing temperature. The viability of the cells however, decreased rapidly when temperatures higher than 35 °C were used.

Enzymatic separation

Hydrophobic secondary metabolites typically accumulate in membranes, vesicles, dead cells or extracellular sites such as the cell wall. Paclitaxel is hydrophobic and essentially insoluble in aqueous solutions (including cellular cytoplasm) and is suggested to be stored in the cell wall. The application of cell wall digesting enzymes cellulase (1%) and pectolyase (0.1%) to *Taxus canadensis* suspension cultures induced a significant increase in the paclitaxel present in the extracellular medium while maintaining membrane integrity, and more than 90% of the total paclitaxel was recovered in the extracellular medium following treatment with the enzymes. The addition of cell wall digesting enzymes to a cell culture bioprocess may be an effective way of enhancing paclitaxel release to the extracellular medium and hence simplify product recovery. This simple method may be applicable to the localization of other hydrophobic secondary metabolites in suspension cultures. The addition of cell wall digesting

enzymes to cell cultures where secondary metabolites are stored in the cell wall is additionally a simple and effective way of enhancing release into the extracellular medium, and studies are currently underway to optimize this protocol for secondary metabolites production via plant cell suspension culture.

Medium composition

Various physicochemical aspects including media composition, plant growth regulators (PGRs), temperature, light, pH, and aeration influence the culture productivity of plant cells. In some cases excretion of secondary products can be enhanced by changing the composition of the medium. An increased ionic strength of the medium has also proved to enhance the release of intracellular stored products (Tanaka *et al*., 1985). The pH of the medium can also influence the excretion of secondary metabolites. Majerus and Pareilleux (1986) observed a sharp increase of the excretion of alkaloids by *Catharanthus roseus* when the pH of the culture medium was changed from 9.0 to 4.3. The type and concentration of the carbon source have important effects on cell growth and yield of secondary metabolites. Available concentration of nitrogen was also found to affect the contents of proteinaceous or amino acid products in cell suspension cultures. General plant tissue culture medium including MS, LS, or B5 usually have both NO^{3-} and NH^{4+} ions as sources of nitrogen. The ratio of the NH^{4+}/NO^{3-} and the total content of nitrogen are significantly affective for successful plant tissue culture systems. Phosphate level in the medium may have a major impact on the production of secondary metabolites in plant cell cultures. Higher concentrations of phosphate ion can enhance the cell growth with negative influence on secondary product accumulation. The effects of PGRs on secondary metabolite production are variable. Important biochemical changes are induced by some PGRs. The PGRs have remarkable consequences on physiological and biochemical processes and gene regulation as well as plant growth and development. Secondary metabolite production was strongly affected by the presence of 6-benzylaminopurine (BA), kinetin (Kin), or other cytokinines such as 4-chloro-2- diphenylurea in the medium. Production of secondary metabolites in *in vitro* culture of *Mentha piperita* was monitored only by the addition of cytokinin, which resulted in about 40% increase in the total production of essential oils. Gibberellic acid increases secondary metabolite production in *Echinacea purpurea* hairy roots. A considerable rise in the contents of phenols and flavonoids in culture of *Stevia rebaudiana* was proven in response to a combination of BA either with gibberellic acid (GA_3) or indol-3-acetic acid (IAA) compared to singly apply of PGRs indicating synergistic effects of PGRs. The biosynthetic pathway is also controlled by physical conditions of cell culture like temperature and pH. Each plant species may favor a specific temperature for optimum *in vitro* metabolic activity usually in the range of 17–25 ° C. In the case of *Eleutherococcus senticosus*, the low (12 and 18 °C) and high (30 °C) temperatures resulted in significant reduction in fresh weight, dry weight, total phenolics, flavonoids, and total eleutheroside accumulation, while low temperature increased eleutheroside E accumulation in somatic embryos. It has been shown that the pH value of *Stevia rebaudiana's* nod culture is a determinant of leaf metabolite, polyphenolics levels, and their distribution between different tissues. The inoculum density is another determinant for plant cell growth and accumulation of secondary metabolites. The flavonoid accumulation in

cell suspension cultures of *Glycyrrhizin inflata* was shown to be correlated with the inoculum density as well as sucrose and nitrogen concentrations.

IMMOBILIZATION

Background

Cessation of growth, stress situations and the onset of differentiation has been shown to be important factors triggering secondary compound production. While the first two factors may be established in a chemo-stat culture, differentiation is effectively prevented by the predominantly unicellular state in suspension cultures.

- **Sticky Secretions:** In order to maintain a suspension, the tendency of cells to aggregate must be overcome. This tendency is due to secretion of a slime layer rich in carbohydrates, proteins and pectins (arabinogalactan, xyloglucan). For example, *Jasminum officinale, J. odoratissimum* and *J. stephanese* calli cannot be dispersed at all in Gamborg's liquid medium. If growth continues, they agglutinate to smooth, rounded balls with a doubling time of 10-12 days.
- **Van der Waals Forces:** In addition, single cells with their weak negative surface charge tend to aggregate on surfaces with an opposite charge. The effective adsorption forces depend mainly on Van der Waals interactions effective up to a distance of 6 A. Under physiological conditions, this adhesion is controlled by interactions between thermodynamic and electrostatic forces. The degree of adhesion usually increases as the pH diminishes.
- **Two-Step Process:** The process of cell adhesion to a surface consists of two steps: first the physicochemical interactions (Van der Waals forces) leading to adhesion and, second, the cell's physiological response, as manifested in the excretion of sticky secretions.
- **Gradient Formation:** These properties may be used to allow the cells to develop structural units without having to go through the process of morphologically visible differentiation. Such units are characterized by cell layers growing near the surface and those deeper down. The former receive an optimal supply of nutrients and O_2, while the latter are located behind the diffusion barrier of the surface cells. Due to direct cell-to-cell contact, this differentiation leads to development of oxygen and nutrient gradients (Brodelius *et al*., 1980; Brodelius and Mosbach, 1982).

Definitions

- If the controlled process of aggregate formation and adhesion on a matrix proceed under controlled conditions, the process is called immobilization (Hartmeier 1985, 1986). Its purpose is to bring together as many cells as possible in production units to create a continuous process, characterized by decoupling growth and secondary metabolite production without loss of biomass.

- It has been defined as a technique, which confines to a catalytically active enzyme or to a cell within a reactor system and prevents its entry into the mobile phase, which carries the substrate and product.

Need for immobilization

Plant cells are characterized by large size, sensitivity to shear and oxygen and need of a cell to cell contact for metabolic production. The secondary metabolites are triggered by short periods of stress in cultures. Immobilization can overcome many of the limiting factors of suspension cultures with the distinct advantages of easier operation of biocatalyst from the product and also being amenable for biotransformation of low value compounds to high value products.

- They provide protection from degradation and deactivation.
- Cost efficient.
- Enhanced stability.
- Allows development of multi-enzyme.
- Use as controlled release agents.
- Retention of enzyme and enzyme free products.
- Ability to stop the reaction rapidly by removing the enzyme from the reaction.

Different types of immobilization

- Direct intracellular binding due to natural affinity (adsorption, adhesion and agglutination).
- Covalent coupling on otherwise inert matrices.
- Intracellular connection via bi or poly functional reagent (cross-linking).
- Mixing with suitable materials, changing their consistency with temperature (embedding).
- Physical retention within the framework of diverse pore size and permeability (entrapment, micro encapsulation) (Novais 1988; Phillips and Poon 1988).

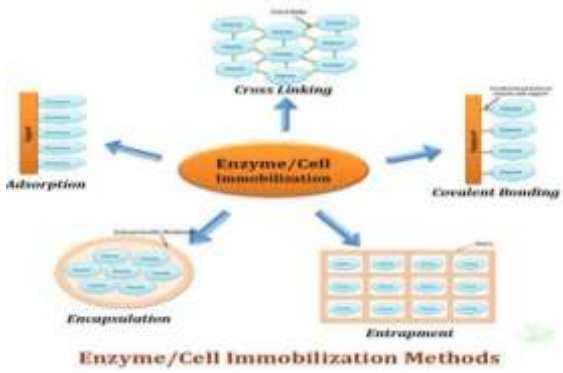
Selection/Choice of immobilization system

The selection of a particular method for immobilization of enzymes is based on a trial and error approach to choose the ideal one. Among the factors that decide a technique, the enzyme catalytic activity, stability, regenerability and cost factor are important.

The choice of a suitable immobilization system is determined by the following requirements.

- The polymer material used for immobilization must be available in large quantities; it must be inert, non-toxic and cheap.
- It must be able to carry large quantities of biomass and its fixing potential must be high.
- The immobilization process must not diminish enzymatic activity of biological catalyst.

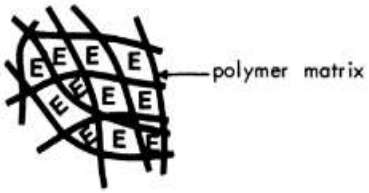
- Manipulation of the biological catalyst must be as simple as possible.



Enzyme/Cell Immobilization Methods

Gel entrapment

The predominant method used for plant cell immobilization is gel entrapment. The most common gel is calcium alginate. A mixture of sodium alginate and cells is extruded drop wise into a $CaCl_2$ solution, where bead hardening starts to take place immediately. Alginate is the preferred gel because of the ease with which it is handled and because of its mildness (neither high temperatures nor aggressive chemicals are needed). Moreover, calcium alginate can have beneficial effects on the production of secondary metabolites. Asada and Shuler (1989) immobilized *Catharanthus roseus* cells in calcium alginate, which resulted in a threefold increase of extracellular ajmalicine. Kim and Chang (1990) obtained a 2.5-fold increase of shikonin production when they immobilized *Lithospermum erythrorhizon* cells in calcium alginate. Some other gels are used for plant cell cultures too, but the reports on them are very few. Noteworthy is the example of Vanek *et al*., (1989), who immobilized *Solanum aviculare* cells in calcium pectate beads.



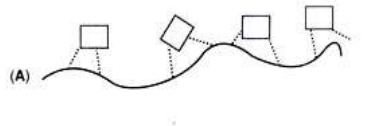
Surface immobilization

Surface immobilization may occur on both natural and other matrices. Examples of natural matrices are deeper callus layers and cellulose, while synthetic one includes nets of steel and nylon. For e.g. cells of *Solanum aviculare* were covalently linked to beds of polyphenylene oxide, which had been achieved using glutaraldehyde. Archambault *et al*., (1986) described the spontaneous and rapid binding of *Chatharanthus roseus* cells to a manmade material.

Adsorption

The simplest and most commonly used technique for immobilizing a biological specimen is based on physical adsorption (physisorption) from solution where a net attractive force is pulling the specimen onto the solid substrate. A variety of forces are involved in this process

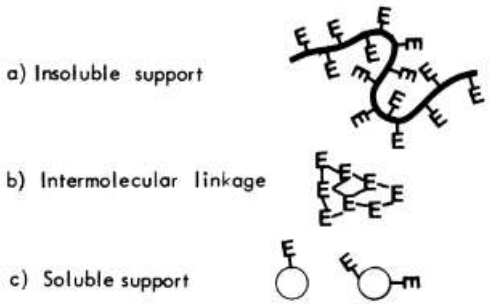
including van der Waals (vdW) forces, electrostatic double-layer (EDL) forces, hydration forces and hydrophobic effects. The adsorption process is very complex and depends on specimen concentration and purity, charge distribution on the surface of the specimen and the substrate, the counterions involved, the size and polarity of the specimen, and the ionic strength and pH of the buffer (Wagner, 1998). Several authors immobilized plant cells by adsorption to various support materials, such as fibreglass, polystyrene, sulfonated polystyrene, fluorinated ethene/propene, polyethene terephthalate, metals, plastics and ceramics. Facchini and Archambault mention the importance of the culture age to obtain good adsorption. When the cells had reached the age of 8-14 days, immobilization was best (Archambault *et al*., 1989), probably due to the higher secretion of polysaccharides by the cells at that age. Adsorption of the cells also stimulated release of the product serpentine into the medium, in contrast to freely suspended cells. The total productivity of the adsorbed cells can surpass that of free cells (Archambault *et al*., 1990 suggest that the adsorbed biomass of *Catharanthus roseus* in their system contains a concentration of indole alkaloids 3-12.5 times as high as the maximum quantity found in suspended biomass in identical culture conditions).



Immobilization of enzymes by adsorption
(A) By van der Waals forces (B) By hydrogen bonding

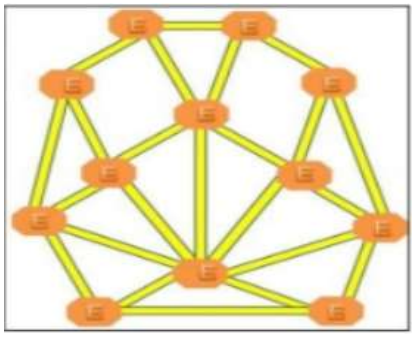
Covalent immobilization

In many cases single molecules and filamentous structures require immobilization where a stable covalent bond is formed between chemical groups of the specimen and functionalities that are exposed at the substrate surface. Covalent immobilization is an important strategy for those applications where displacement or desorption is a critical issue, but also when conditions for adsorption and biological activity are incompatible, or when the molecular objects have to be integrated in complex supramolecular assemblies that include self-assembly processes and require well-defined coupling steps.



Cross-linking

This method is based on the formation of covalent bonds between the enzyme molecules, by means of multifunctional reagents, leading to three dimensional cross linked aggregates. It is used mostly as a means of stabilizing adsorbed enzymes and also for preventing leakage from polyacrylamide gel. The most common reagent used for cross-linking purpose is glutaraldehyde. No matrix or support is required in this.



Microencapsulation

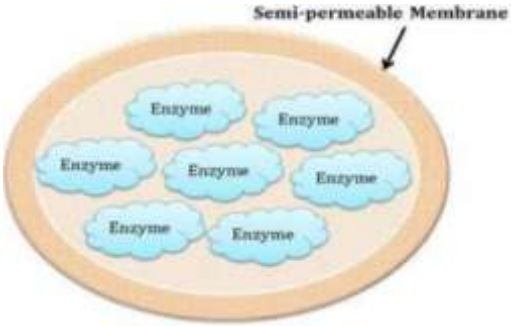
Microencapsulation is a major technique of enzyme immobilization. It refers to the process of spherical particle formation wherein a liquid or suspension is enclosed in a semipermeable membrane. The membrane may be polymeric, lipoidal, lipoprotein-based or non-ionic in nature. There are three distinct ways of microencapsulation.

1. Building of special membrane reactors.

2. Formation of emulsions.

3. Stabilization of emulsions to form microcapsules

Microcapsules are mostly spherical with diameters usually in the micro- to millimeter range and exhibit core-shell structures of variable complexity. Liposomes and vesicles formed from amphiphilic (co)-polymers (polymersomes) are widely used to prepare membrane-coated microcapsules. Hydrogels, sol–gels and other organic–inorganic hybrid materials, or layer-by-layer structures made through controlled assembly of polyelectrolytes are used to prepare microcapsules composed of internal polymeric networks. Advances in the sciences contribute to the development of microcapsules with improved properties such as enhanced morphological stability, reduced enzyme leakage, and designed physicochemical permeability and enzyme biocompatibility. Multienzyme microcapsules are interesting small-scale bioreactors for confined and even compartmentalized cascade biotransformation (Rother and Nidetzky, 2014).



Advantages of Plant Cell Immobilization

- **Retention of biomass** enables its continuous reutilization as a production system, a definite advantage with slow growing plant cells e.g. *Papaver somniferum* have remained stable and active for up to six months.
- **High biomass levels:** The immobilization of cells allows the use of a higher biomass level compared to cell suspension culture, because of the limitation of mass transfer and settling, e.g. bead densities of 110 g dry weight/L have been obtained with calcium alginate entrapped cells when 30 g dry weight/L in suspension cultures. The high cell density allows a reduction in contact in packed bed catalyst leading to an increased volumetric productivity.
- **Separation of cells from medium:** the immobilization separates cells from medium and the product is extra cellular, which will simplify downstream processing compared to extract from tissue.
- **Continuous process:** Immobilization allows a continuous process, which increase volumetric productivity and allows the removal of metabolic inhibitors.
- Decoupling of growth and product formation: Immobilization is compatible with non-growth associated product formation.
- **Reduces problems such as aggregate, growth and foaming:** the immobilization reduces some of the physical problems associated with the cultivation of plant cells such as the formation of aggregates, and susceptibility to mechanical damage (shear stress) are problems which do not affect immobilized system compared to cell culture.

Disadvantage of Plant Cell Immobilization

- Secretion of secondary metabolites requires cellular transport or artificially altered membrane permeability.
- The efficiency of the production process depends on the rate of release of products rather than actual rate of biosynthesis. The immobilization process may reduce biosynthetic capacity. Products must be released from the cell into medium. Release of single cells from cell aggregate may make processing of the product more difficult. The microenvironment optimal production can be unfavorable for released secondary metabolites and cause their degradation or metabolization.
- Immobilization is normally limited to cases where production is decoupled from cell growth

- Initial biomass must be grown in suspension
- Where secretion occurs, there may be problems of extracellular degradation of the products
- When gel entrapment is used, the gel matrix introduces an additional diffusion barrier.

Type of immobilization	Plant species	Product	Fold change	Refs.
Foam	*Capsicum frutescens*	Capsaicin	> 100	Johnson and Ravishankar 1996
Calcium alginate	*Lithospermum erythrorhizon*	Shikonin	2.5	Kim and Chang 1990
Natural glass	*Papaver somniferum*	Saquinarine	2	Dicosmo and Misawa 1995
Gel	*Coffea arabica*	Methylxanthin	13	Brodelius and Pedersen 1993
	Capsicum frutescens	Capsaicin	> 100	Johnson and Ravishankar 1996
	Chenopodium rubrum	Betacyanin		Knorr and Berlin 1987

Effects of immobilization on secondary metabolite production in cell cultures

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