PRODUCT ISOLATION AND RECOVERY IMMOBILIZATION

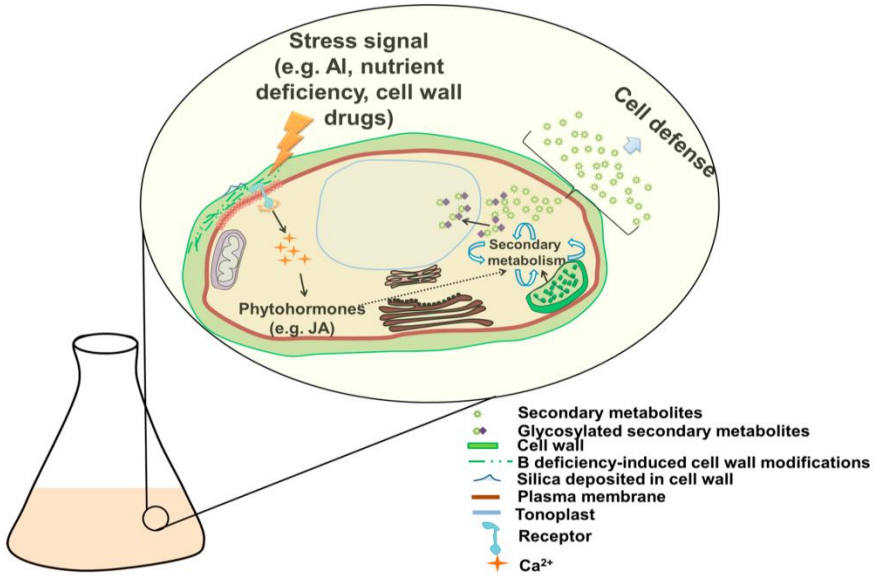
Course Title:

Industrial Production of Secondary Metabolites

PRODUCT ISOLATION AND RECOVERY

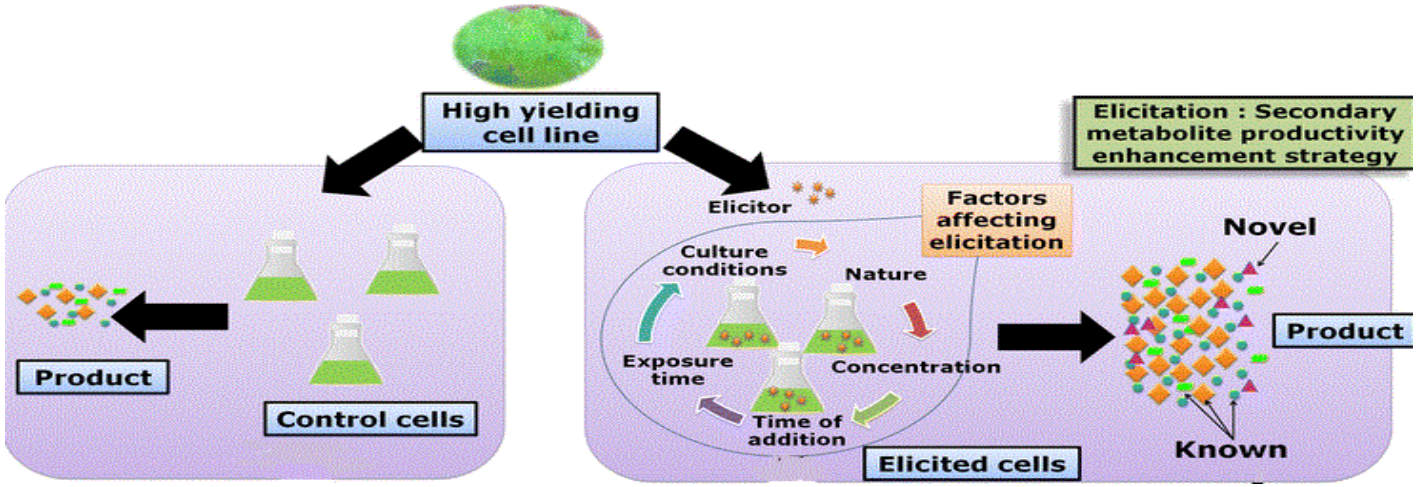
Secondary metabolite recovery

- *Hydrophilic secondary metabolites, stored in the cell vacuole.*
- *Hydrophobic stored in the cell membrane, vesicles, dead cells and extracellular sites, such as the cell wall.*
- *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.*



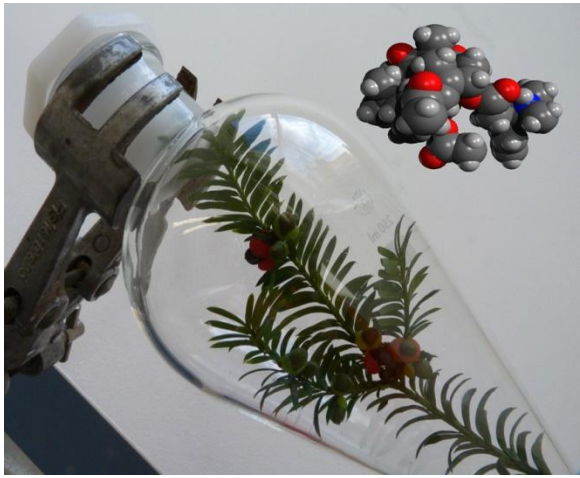
Secondary metabolite excretion

- *The excretion behavior of plant cell cultures varies from one species to another, and even within one species, from one cell line to another.*
- *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.*



Natural harvest

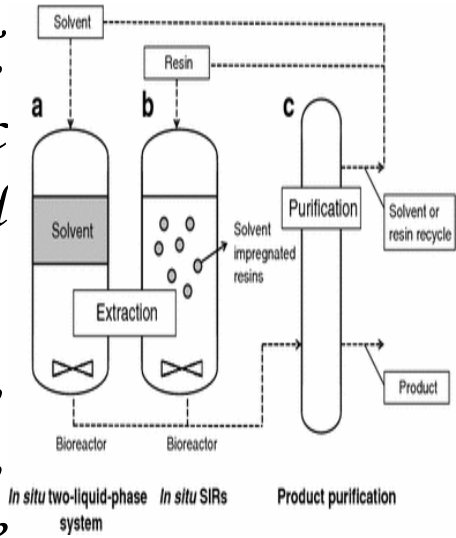
- *Secondary metabolites typically represent <1% dry weight of the plant, so natural harvest is often impractical. For instance, 340,000 kg of Taxus bark or 38,000 trees were required to meet the 25 kg per year demand for the anticancer drug paclitaxel (Taxol®; Bristol-Myers Squibb, New York, NY).*
- *Harvesting is also limited by seasonal availability; species abundance and plant growth rate.*



Methods of recovery and excretion

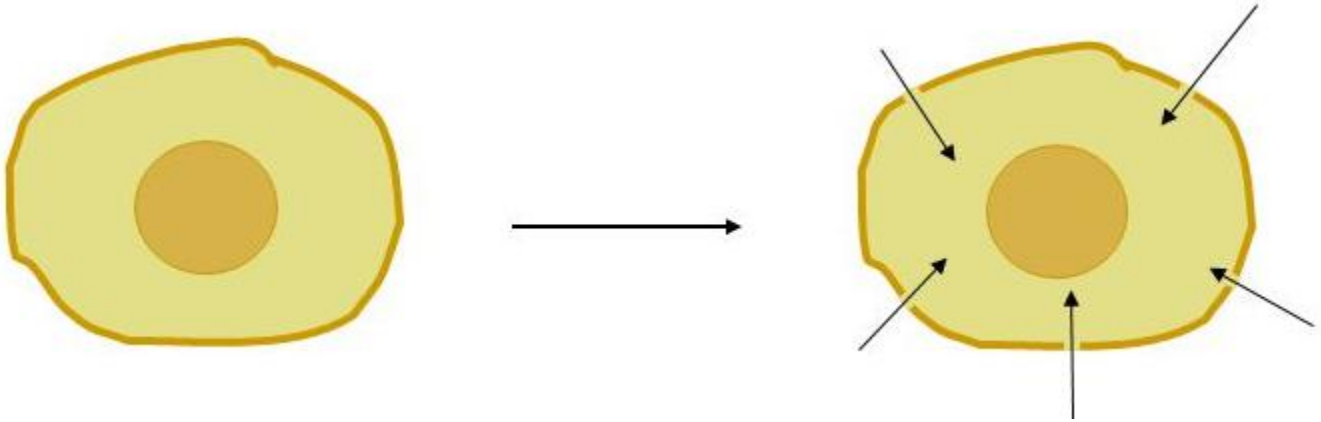
In situ product removal:

- *One phase is the aqueous medium; the second phase can be either a water-immiscible organic solvent or a solid compound.*
- *A six-fold increase in paclitaxel achieved in T. chinensis suspension cultures in aqueous-organic two-phase systems, with 63% of the product released to the media.*
- *Criteria for suitable second phases: autoclavable, non-toxic, not influence the medium composition, bind the desired product, product should easily be recovered from the second phase.*
- *It can enhance secondary metabolite release from the cultures or the initiate a release of compounds normally stored within the cells.*



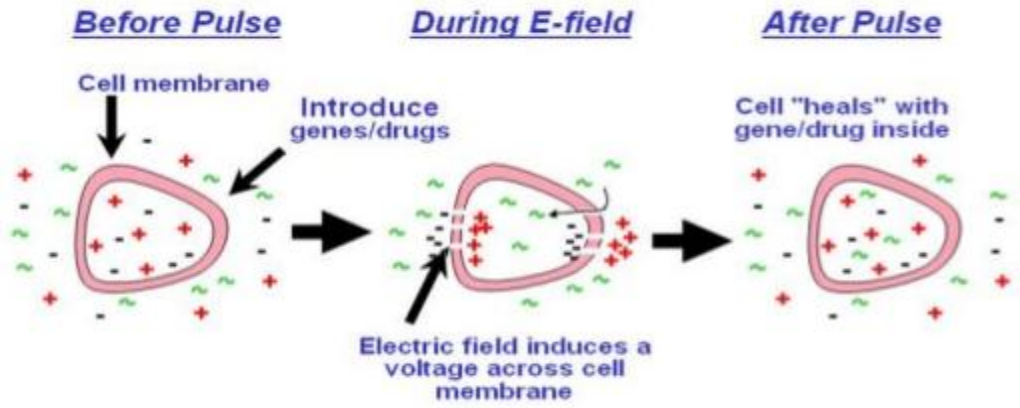
Membrane Permeabilization:

- *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.*
- *Permeabilization often connected with the loss of viability of the plant cells treated with permeabilizing agents and methods.*
- *Various methods: 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).*



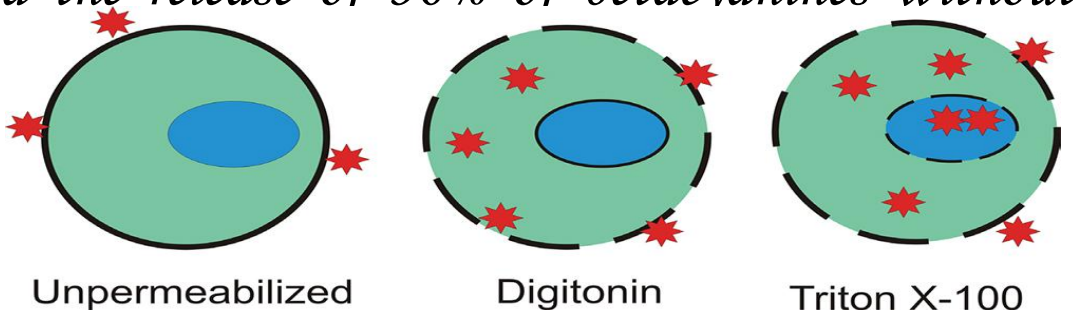
Physical Permeabilization

- *Include high electric field pulses, high hydrostatic pressure, ultrasound, etc.*
- *Products were released up to 100%, depending on the voltage applied, but viability decreased*
- *Based on the principle of development of membrane pores under external electric fields.*
- *Treatment with high hydrostatic pressure of 50 MPa increased the production of amarantin and antraquinones in cell cultures of Chenopodium ruburum and Morinda citrifolia.*



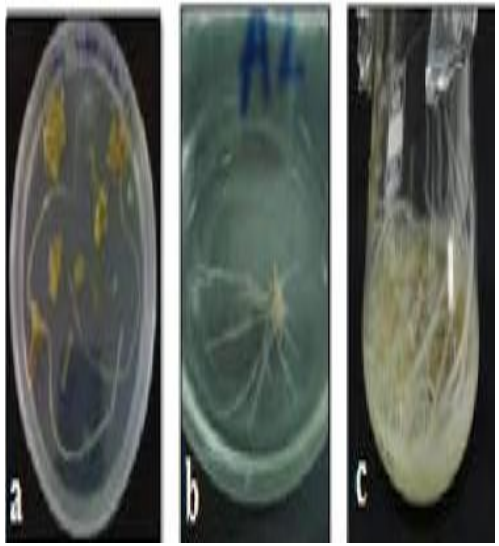
Chemical Permeabilization

- *Dimethylsulfoxide (DMSO) is known to extract sterols from the membranes of eukaryotic cells*
- *Of all the cell types tested, only Catharanthus roseus survived the treatment with DMSO.*
- *Chloroform and Propanol, mostly without success. The organic solvents used in successful are hexadecane, perfluorchemicals and Miglyol.*
- *Chenopodium rubrum cells could be permeabilized by treatment with chitosan. This can induces pore formation only in the plasmalemma of the plant cell cultures.*
- *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.*



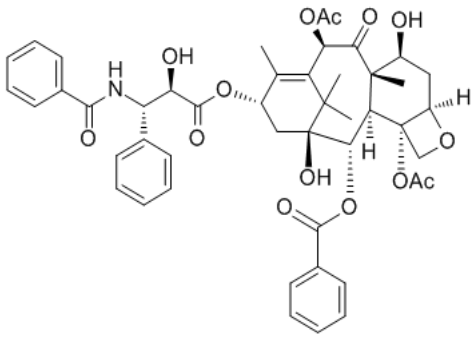
Temperature

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



Enzymatic separation

- 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.
- 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 maintaining membrane integrity, and more than 90% of the total paclitaxel was recovered in the extracellular medium.



Paclitaxel

Medium composition

- *The pH of the medium can influence the excretion of secondary metabolites. E.g. 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.*
- *Higher concentrations of phosphate ion can enhance the cell growth with negative influence on secondary product accumulation.*

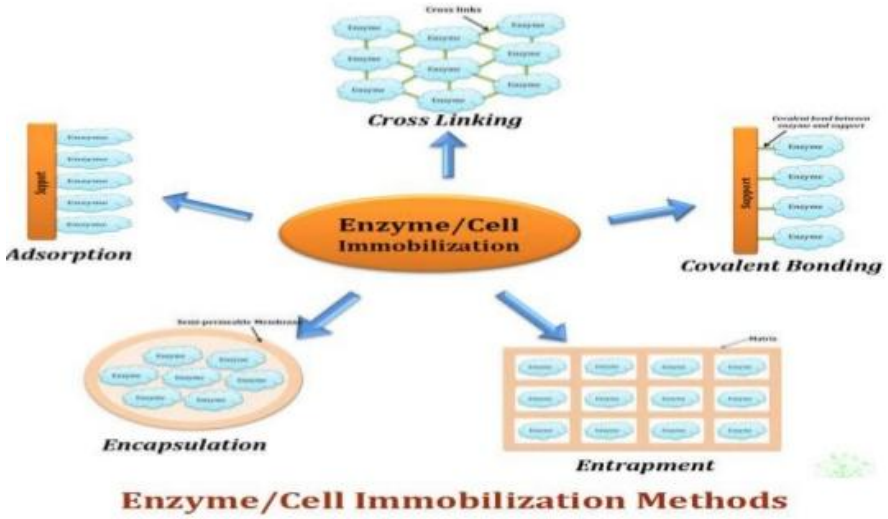
For Example

- *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.*
- *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.*

IMMOBILIZATION

Definitions

- *If the controlled process of aggregate formation and adhesion on a matrix proceed under controlled conditions, the process is called immobilization. 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.*



Enzyme/Cell Immobilization Methods

Immobilized cell or enzyme

- *The **immobilized whole cell** system is an alternative to enzyme immobilization. Unlike enzyme immobilization, where the enzyme is attached to a solid support (such as calcium alginate or activated PVA or activated PEI), in immobilized whole cell systems, the target cell is immobilized.*
- *Such methods may be implemented when the enzymes required are difficult or expensive to extract, an example being intracellular enzymes.*
- *Also, if a series of enzymes are required in the reaction; whole cell immobilization may be used for convenience. This is only done on a commercial basis when the need for the product is more justified.*

Need for immobilization

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.

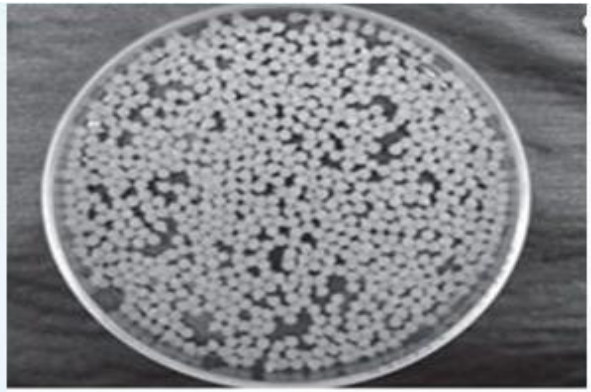
- *They provide protection from degradation and deactivation.*
- *Cost efficient.*
- *Enhanced stability.*
- *Allows development of multi-enzyme.*
- *Use as controlled release agents.*
- *Retention of enzyme and enzyme free products.*
- *Ability to stop the reaction rapidly by removing the enzyme from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

Selection/Choice of immobilization system

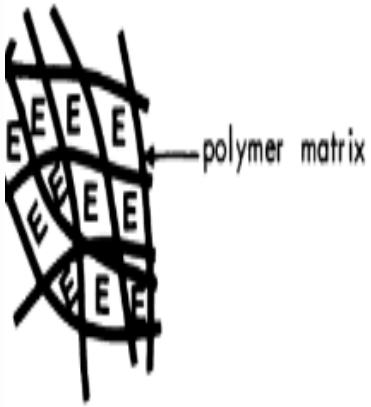
- *Among the factors that decide a technique, the enzyme catalytic activity, stability, regenerability and cost factor are important.*
- *The polymer material used for immobilization must be available in large quantities; it must be inert, non-toxic and cheap.*
- *It must be able to carry large quantities 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.*

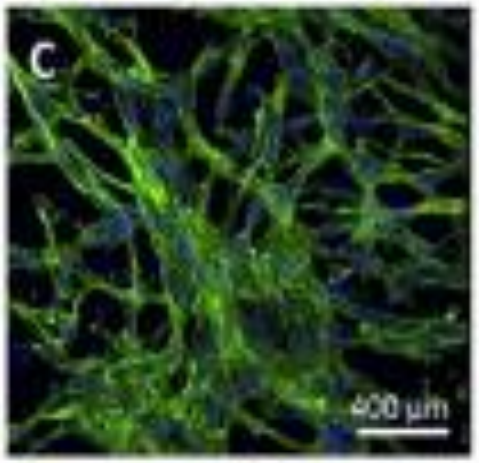
Gel entrapment

- *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.*
- *Immobilized Catharanthus roseus cells in calcium alginate, resulted in a threefold increase of extracellular ajmalicine.*
- *Some other gels are used for plant cell cultures too, but the reports on them are very few e.g. calcium pectate beads.*



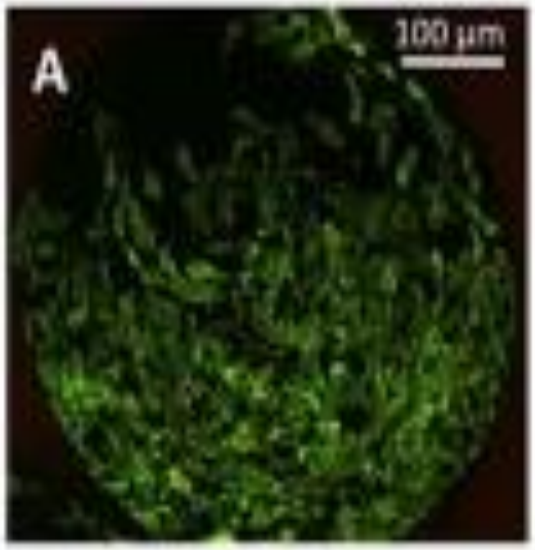
Immobilized *Catharanthus roseus* cells





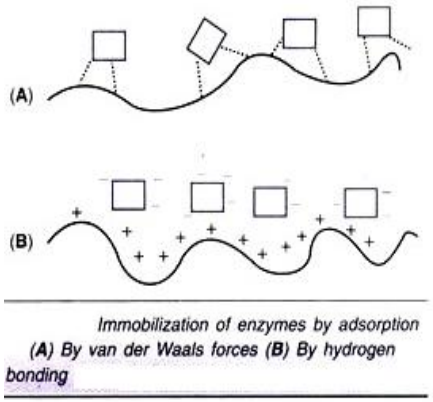
Surface immobilization

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.

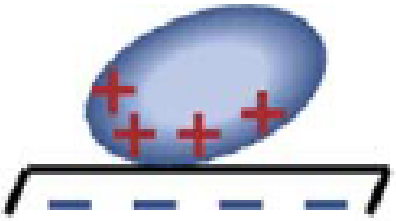


Adsorption

- *Net attractive forces pulling the specimen onto the solid substrate.*
- *Forces involved: van der Waals (vdW) forces, electrostatic double-layer (EDL) forces, hydration forces and hydrophobic effects.*
- *Depends on specimen concentration and purity, charge distribution on the surface of the specimen and the substrate, the ionic strength and pH of the buffer.*
- *Fibreglass, polystyrene, sulfonated polystyrene, fluorinated ethene/propene, metals, plastics and ceramics.*
- *Culture age is important to obtain good adsorption. When the cells had reached the age of 8-14 days, immobilization was best, probably due to the higher secretion of polysaccharides by the cells at that age.*



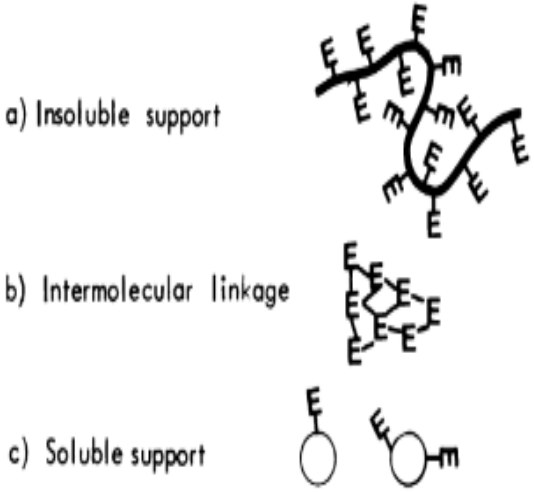
Immobilization of enzymes by adsorption (A) By van der Waals forces (B) By hydrogen bonding

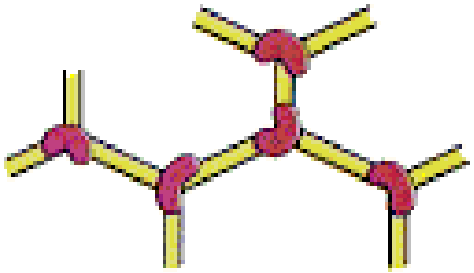


Whole-cell immobilization

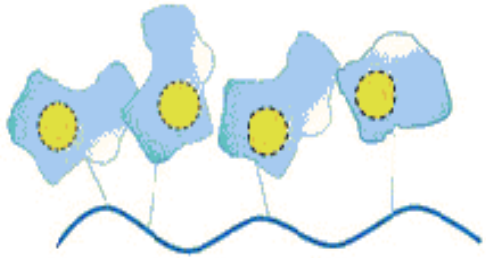
Covalent immobilization

- *Stable covalent bond is formed between chemical groups of the specimen and functionalities that are exposed at the substrate surface.*
- *An important strategy for applications where displacement or desorption is a critical issue, 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.*





Covalent Binding to a Solid Support

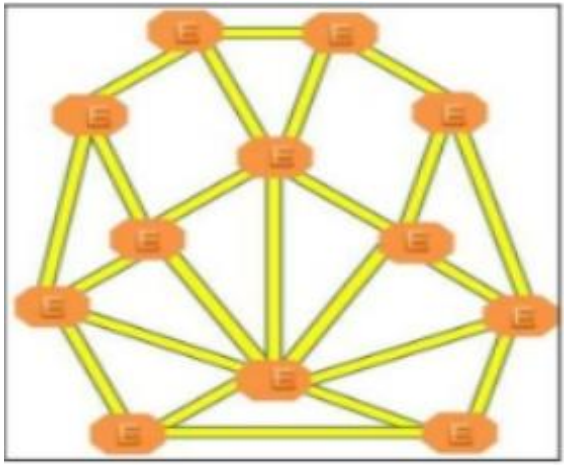


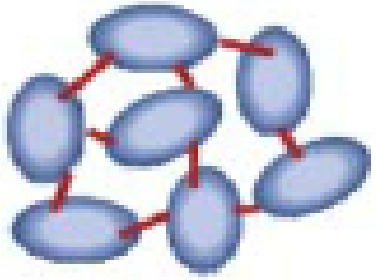
Covalent attachment

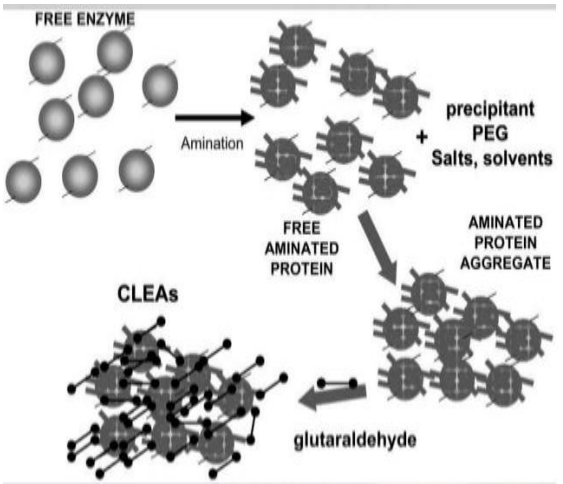
Whole-cell immobilization

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.

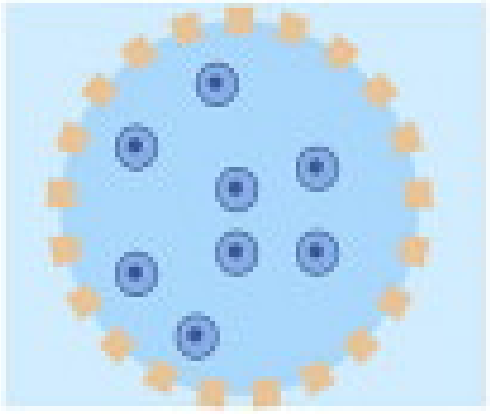


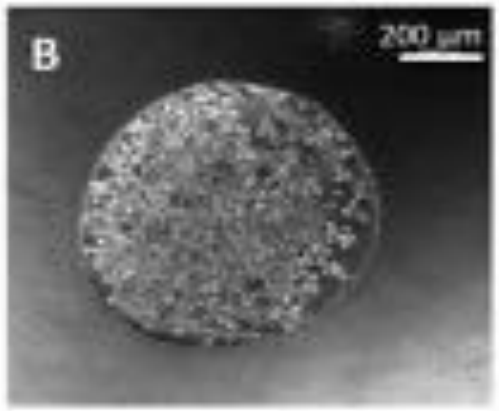


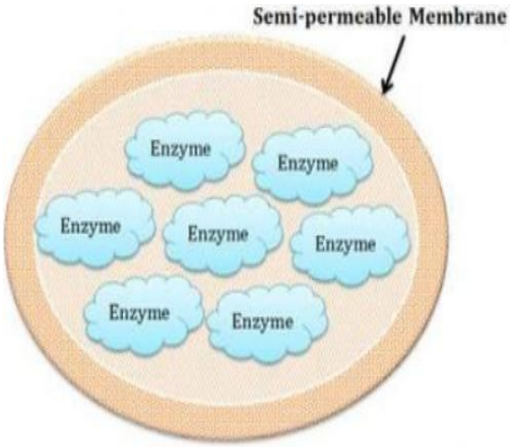


Microencapsulation

- 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







Advantages of Plant Cell Immobilization

- *Enables continuous **reutilization of biomass** as a production system e.g. Papaver somniferum have remained stable and active for up to six months.*
- *Allows the use of a **higher biomass level** compared to cell suspension culture, 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.*
- *It **separates cells from medium** which will simplify downstream processing compared to extract from tissue.*
- *It 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.*
- *It reduces susceptibility to mechanical damage (shear stress).*

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.
- 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.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