Tissue culture and Microbial Biotechnology

Module 7: Production of secondary metabolites



Princy

- Plant products can be classified into primary plant **metabolites** and **secondary metabolites**.

- The organic compounds such as carbohydrates, proteins, fats. Membrane lipids, nucleic acids, chlorophylls and hemes are found throughout the plant kingdom and are **central to the metabolism of plants**. These compounds are known as primary plant metabolites.

- But in many plants, particularly those of certain genera and families, synthesize a number of organic compounds in them which are not in the mainstream of metabolism and appear to have no direct function in growth and development of plants. These compounds are extremely numerous and chemically diverse in nature and are called as secondary plant metabolites.

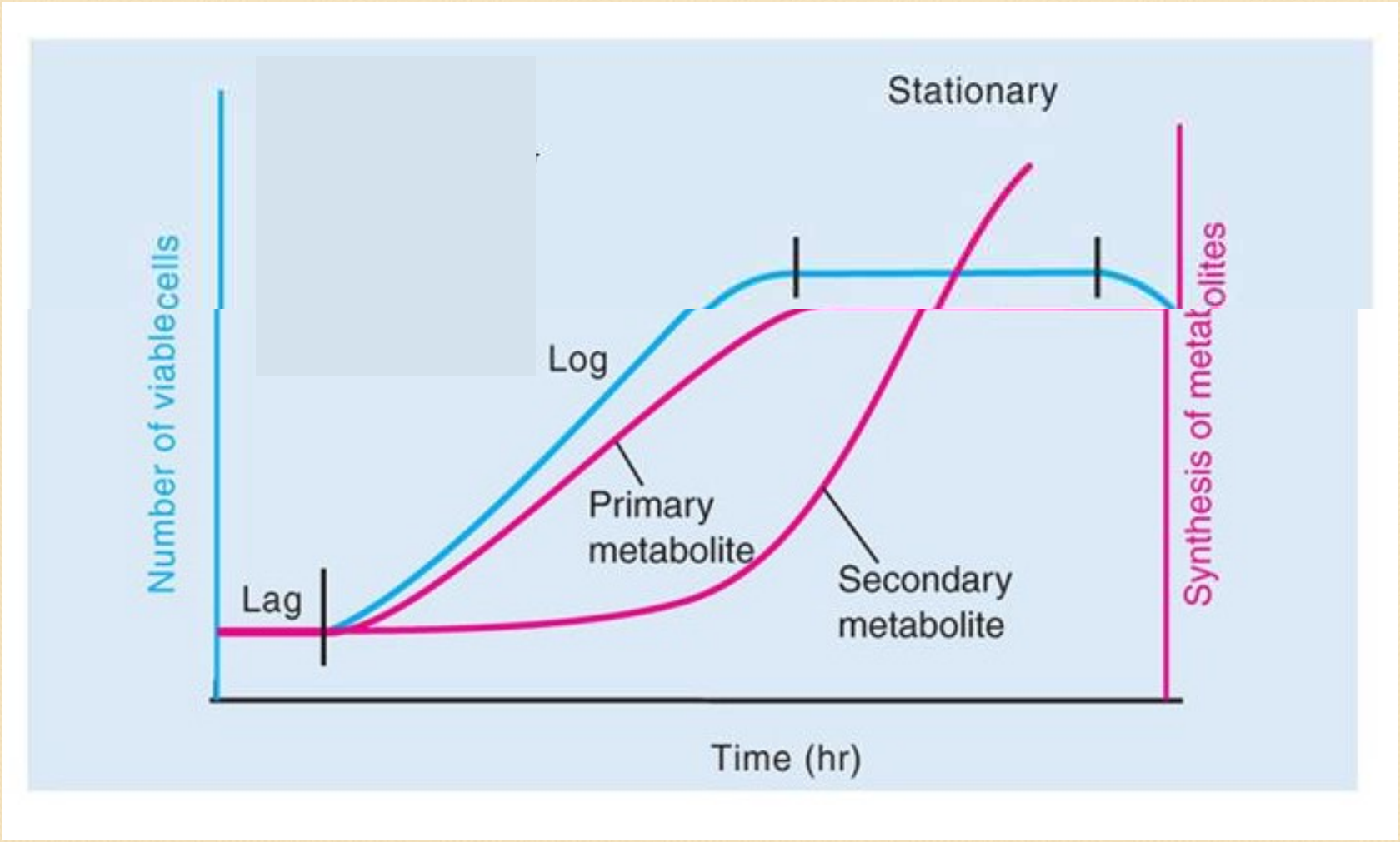
- **Primary plant metabolites** are essential for the growth survival of the plant.

- Therefore, plant tissue culture is being potentially used as an alternative for plant secondary metabolite production.

- **Primary metabolites are produced during active** cell growth (*lag, exponential, stationary).*

- **Secondary metabolites are produced near** the onset of stationary phase.

Primary and secondary metabolites



Secondary metabolites:

- Not essential for growth.
- Dependent on growth conditions (repression).
- Over-production often achievable (not growth related).
- Often produced as a series of closely related compounds.

Need & significance of plant cell culture for secondary metabolite production

- Higher plants produce a great variety of secondary products which play a minor role in the basic life processes of the plant but often have an ecological role, such as attractant of pollinators and chemical defence against microorganisms, insects and higher predators.

- Many of these natural products have been used as **sources of a large number of industrial products**, including agricultural chemicals, pharmaceuticals and food additives.

- Although, some of the natural products have been replaced by synthetic substitutes, because of cost considerations, a number of commercially important high value chemicals are still being extracted from plants.

- According to Lambie (1990), of the 30 medicinal alkaloids in use, 24 are obtained by extraction from plants.

- Our dependence on plants for natural products is expected to continue because some compounds are difficult to synthesize due to their structural complexity, and novel active compounds are still being detected in plant extracts as more and more until now unsurveyed plants are analyzed.

- Besides their direct application, the natural plant products serve as model compounds for the chemical synthesis of new, more potent analogues.

TABLE 17.1

Natural plant products of industrial importance

1. Pharmaceuticals	
a. Alkaloids	Ajmalicine, atropine, berberine, codeine, reserpine, vincristine, vinblastine
b. Steroids	Diosgenin
c. Cardenolides	Digitoxin, digoxin
2. Food and flavours	
a. Sweeteners	Stevioside, thaumatin
b. Bittering agent	Quinine
c. Pigment	Crocin
3. Pigments and perfumes	
a. Pigments	Shikonin, anthocyanins, betalins
b. Fragrances	Rose oil, jasmine oil, lavender oil
4. Agrochemicals and fine chemicals	
a. Agrochemicals	Pyrethrin, salannin, azadirachtin
b. Fine chemicals	Proteases, vitamins, lipids, latex, oil

- **Ajmalicine**, is an antihypertensive drug used in the treatment of high blood pressure.
- **Mainly found in plants** of the family Apocynaceae
- Direct culture of leaves in *Catharanthus roseus* intact **plant**.

- Digoxin, sold under the brand name Lanoxin among others, is a medication used to treat various heart conditions.
- Most frequently it is used for heart failure.
- Digoxin is taken by mouth or by injection into a vein.
- Digoxin was first isolated in 1930 from the foxglove plant, ***Digitalis lanata*.**
- It is on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system.

Digitalis lanata

- **Foxglove** is a native of Europe

- Codeine is an **opiate** used to **treat pain**, as a cough medicine, and for diarrhea. It is typically used to treat mild to moderate degrees of pain.

- Opium is an isoquinoline alkaloid **obtained** from poppy **plant** *Papaver somniferum* (Papaveraceae).

- **Codeine** is an alkaloid prepared from opium or morphine by methylation.

- Berberine is a quaternary ammonium salt alkaloid found in such plants as ***Berberis***.

- Berberine is usually found in the roots, rhizomes, stems, and bark.

- Due to berberine's strong yellow color, *Berberis* species were used to dye wool, leather, and wood.

- **Atropine** is a medication to treat certain types of nerve agent and pesticide poisonings as well as some types of slow heart rate and to decrease saliva production during surgery.

- Atropine is a poisonous **alkaloid** drug obtained from certain plants such as ***Atropa belladonna*** (deadly nightshade).

- **Reserpine** is an indole alkaloid, antipsychotic, and antihypertensive drug that has been used for the control of high blood pressure and for the relief of psychotic symptoms.

- The drug derived from the roots of certain species of the tropical plant ***Rauwolfia*** *(Rauwolfia serpentina)*

- **Diosgenin**, a phytosteroid sapogenin, is the product of hydrolysis by acids, strong bases, or enzymes of saponins.

- It can be **obtained** from several plants, namely, from ***Dioscorea***, *Trigonella*, *Costus* and *Smilax* species.

- **Stevioside** is a glycoside derived from the *Stevia* plant, which can be used as a sweetener.

- **Crocin** is a carotenoid chemical compound that is found in the flowers ***Crocus*** and ***Gardenia.*** Crocin is the chemical primarily responsible for the color of saffron.

Crocus sativus

Gardenia

- **Quinine** is an alkaloid derived from the bark of the *Cinchona* tree. It is used as an antimalarial drug, and is the active ingredient in extracts of the cinchona that have been used for that purpose since before 1633.

- The pyrethrins are a class of organic compounds normally derived from *Chrysanthemum cinerariifolium (Pyrethrum)* that have potent insecticidal activity by targeting the nervous systems of insects.

- **Thaumatin** is a low-calorie sweetener and flavour modifier. The protein is often used primarily for its flavour-modifying properties and not exclusively as a sweetener.

West African plant *Thaumatococcus daniellii*

- In addition, secondary metabolites can be applied as starting compounds for further chemical modification.

- For example, **podophyllotoxin** obtained from *Podophyllum sp. is used for the synthesis of the* clinically applied antitumour agents **etoposide** and **teniposide.**

- The increasing consumer preference for natural food colours and flavours over their synthetic counterparts further increases our dependence on plants.

- During the last 30 years there has been an increasing interest among scientists to produce high value natural plant products by cell culture which can **overcome many of the problems associated with industrial production of these phytochemicals by extraction from field grown plants (mass cultivated or natural populations).**

- In cultures, factory-type production of natural compounds can be carried out throughout the year, **unaffected by the season.**

- The risk of crop failure due to natural hazards and the danger of extinction of some species due to their mass extraction from natural populations are eliminated.

- Cell cultures not only provide means for de novo synthesis of natural products but also serve as 'factories' for bioconversion of low value compounds into high value products.

- Moreover, some novel compounds produced in cell cultures are not produced in intact plants.

- At least 85 novel compounds including 23 alkaloids, 19 terpenoids, 30 quinones and 11 phenyl compounds have been isolated from some 30 different plant culture systems.
- E.g. Paniculid A - from *Andrographis paniculata*

History

- Since the early 1950s, when the concept of tissue culture production of natural compounds was conceived, many technological advances have been made and in several cases **cell cultures have been shown to produce higher amounts of the products than the intact plants** from which they are derived.

- Of the various plant products produced by plant tissue culture, pharmaceuticals have received maximum attention. The two countries which have made substantial contributions to this field of research are Japan and Germany.

- Establishment of suspension cultures of plant cells in liquid medium, similar to microbes, in the mid-1950s prompted scientists to apply this system **for the production of natural plant products as an alternative to whole plant.**

- The first attempt for the industrial production of secondary metabolites in vitro was made during 1950-1960 by the **Pfizer** Company and the first patent was obtained in 1956 by Routien and Nickell.

- However, not much progress in this area was made for many years.

- Apparently, the industrial production of secondary metabolites required **large scale culture of cells**.

- In 1959, Tulecke and Nickell published the first report of plant cell culture in a bioreactor.

- Noguchi *et al.* (1977) **used bioreactor for the culture of tobacco cells.**

- Since plant cells are different from microbes in many respects the reactors traditionally used in microbiology had to be modified to suit plant cell culture.

- Several different kinds of bioreactors have been designed for large scale cultivation of plant cells.

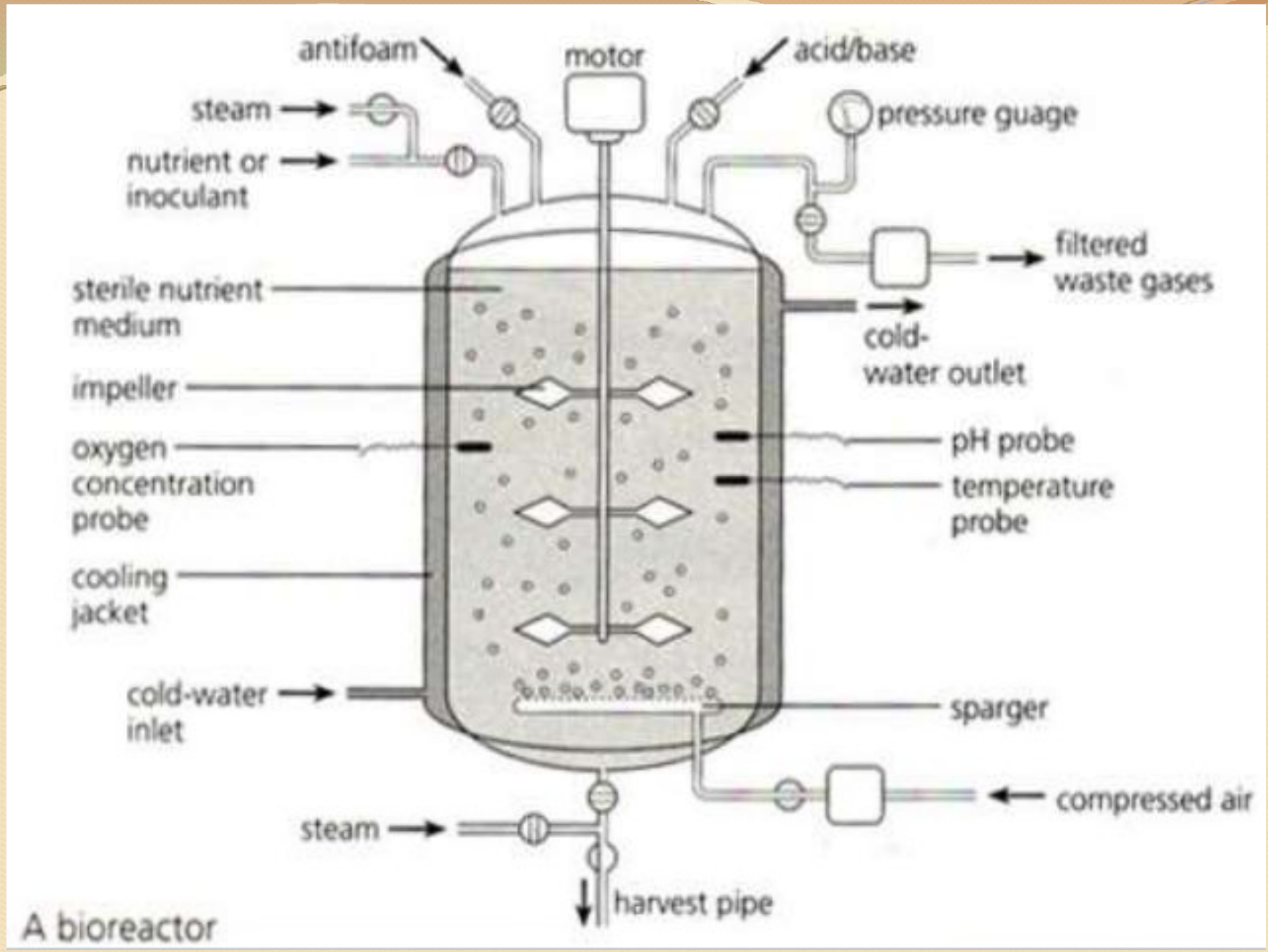
What is a Bioreactor?

- **Bioreactors are vessels in which raw materials are biologically converted into specific products**, using microorganisms, **plants,** animals, or human cells or individual enzymes.

- A bioreactor supports the natural process of cells by trying to maintain their environment to provide optimum growth conditions by providing appropriate temperature, pH, substrates, salts, vitamins, and oxygen.

- In most of the bioreaction processes the substrate of the biotransformation and the carbon source of the organisms will be the same.

A bioreactor



A bioreactor

- The technology for mass culture of plant cells is now available but there are some problems that makes tissue culture production of industrial compounds uneconomical.

Problems associated with mass culture of plant cells

1. Slow growth of plant cells.
2. Genetic instability of cultured cells.
3. Intracellular accumulation of secondary products.
4. Organ-specific synthesis of secondary products.

- Despite these problems in several cases cell cultures have been shown to produce certain metabolites in quantities equal to or many fold greater than the parent plant.

- In 1979, **Brodelius** *et al*. developed **the technique of immobilization of plant cells** so that the biomass could be utilized for longer periods, besides its other advantages.

- Culture of 'hairy roots', produced by transformation with ***Agrobacterium rhizogenes***, has been shown to be a more efficient system than cell cultures for the production of compounds which are normally synthesized in roots of intact plants.

- The first tissue culture product to be commercialized, by **Mitsui Petrochemical Co.** of Japan, is **Shikonin** from cell cultures of ***Lithospermum erythrorhizon***.

- *In 1988, another* Japanese company (Nitto Denko) started marketing **ginseng cell mass** produced in culture.

Lithospermum erythrorhizon

- The enantiomer of alkannin is known as **shikonin (which is a** naphthoquinone derivative).

- The colour of shikonin extracts in general is dependent on the pH of the solution, and varies from red, purple and blue in acidic, neutral and alkaline pH, respectively

- Alkannin is a **natural dye** that is obtained from the extracts of plants from the Boraginaceae family. The enantiomer of alkannin is known as **shikonin**, and the racemic mixture of the two is known as shikalkin.

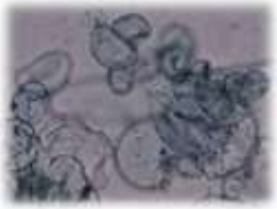
- The **chemical structure** of shikonin is as a naphthoquinone derivative.

- Shikonin is a major component in the dried root of *Lithospermum erythrorhizon*, a Chinese herbal medicine with various biological activities, including inhibition of human immunodeficiency virus type 1 (HIV-1).

- Shikonin also has anti-cancerous properties.

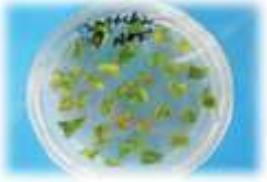
Plants (*Lithospermum erythrorhizon*)

Young leaves

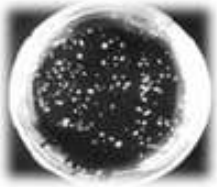


Plant cells

Cell suspension culture



Callus



Subculture

Cell culture in bioreactor

Dowmstream processing

Natural medicine

Ginseng (*Panax ginseng*)

Family Araliaceae

- The botanical genus name *Panax*, meaning "all-healing" in Greek, shares the same origin as "panacea“ (Goddess of universal remedy in Greek Mythology).

- Ginseng has wide use in Chinese medicine as a muscle relaxant.

- The English word "ginseng" derives from the Chinese term *rénshēn*. *Rén* means "person" and *shēn* means "plant root"; this refers to the root's characteristic forked shape, which resembles the legs of a person.

PLANT CELL REACTORS

- Mass culture of plant cells in vitro has been proposed as a **viable alternative for the production of vast arrays of high value, low volume phytochemicals.**
- Therefore, during the past two decades considerable work has been done to design bioreactors for plant cell culture.
- A bioreactor is a glass or steel vessel in which organisms/ cells are cultured.

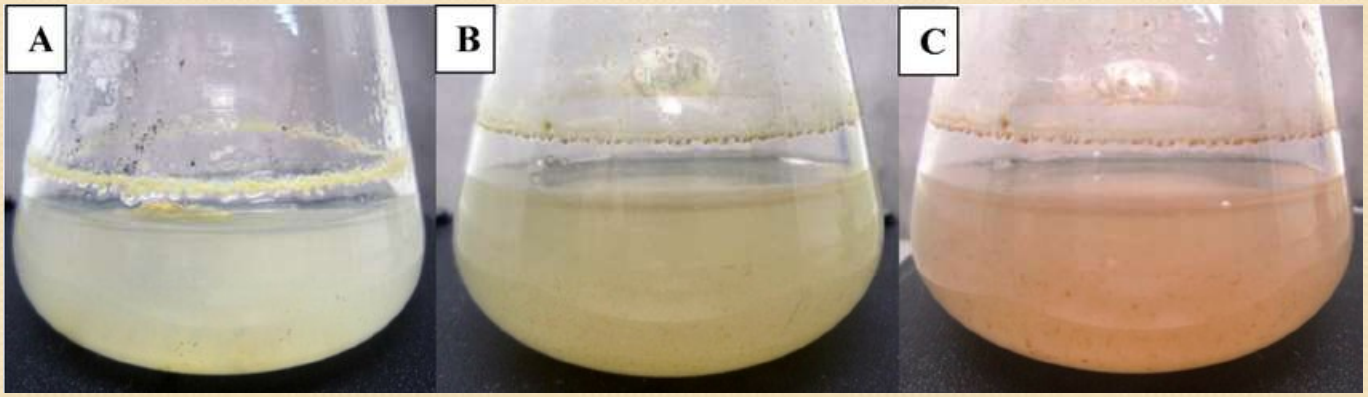
- Ideally, bioreactors are fitted with probes to monitor

1. pH.
2. Temperature and dissolved oxygen in the culture.
3. Provisions to sample the cultures and add fresh medium.
4. Adjust air supply and mixing of cultures without endangering the aseptic nature of the culture.

- It, thus, allows closer control and monitoring of culture conditions than is possible using shake cultures.

- Shake culture

- The basic requirements for suspension cultures of plant cells are similar to those of submerged microbial cultures.



- **Suspension culture** is a type of **culture** in which single cells or small aggregates of cells multiply while **suspended** in agitated liquid medium.
- It is also referred to as cell **culture** or cell **suspension culture**.

- The bioreactors/fermentors **used for microbial cell cultures are not suitable for plant cell cultures** because of striking differences in the nature and growth pattern of the two types of cells.

- Since plant cells are different from microbes in many respects the reactors traditionally used in microbiology had to be modified to suit plant cell culture.

Modifications for the construction plant cell bioreactors

1. Agitation
2. Optimum oxygen

1. Agitation

- Efficient mixing of plant cells cultured on large scale is extremely important.

Importance of mixing/agitation in bioreactors

1. To provide uniform physiological conditions inside the culture vessel.

2. To promote better growth by enhancing the transfer of nutrients from liquid and gaseous phases to the cells

3. Break-off and dispersion of air bubbles for effective oxygenation.

4. For uniform mixing of nutrient medium inside the vessel.

Plant cells require optimum agitation only!..

- Although, plant cells have higher tensile strength in comparison to microbial cells, their **large size**, **rigid cellulosic wall** and **extensive vacuole** make them sensitive to the shear stress restricting the use of high agitation for efficient mixing.

- Plant cells are, therefore, often grown in modified stirred-tank bioreactors at very low agitation speeds.

- **Air-lift reactors** may provide even better and uniform environmental conditions at low shear.

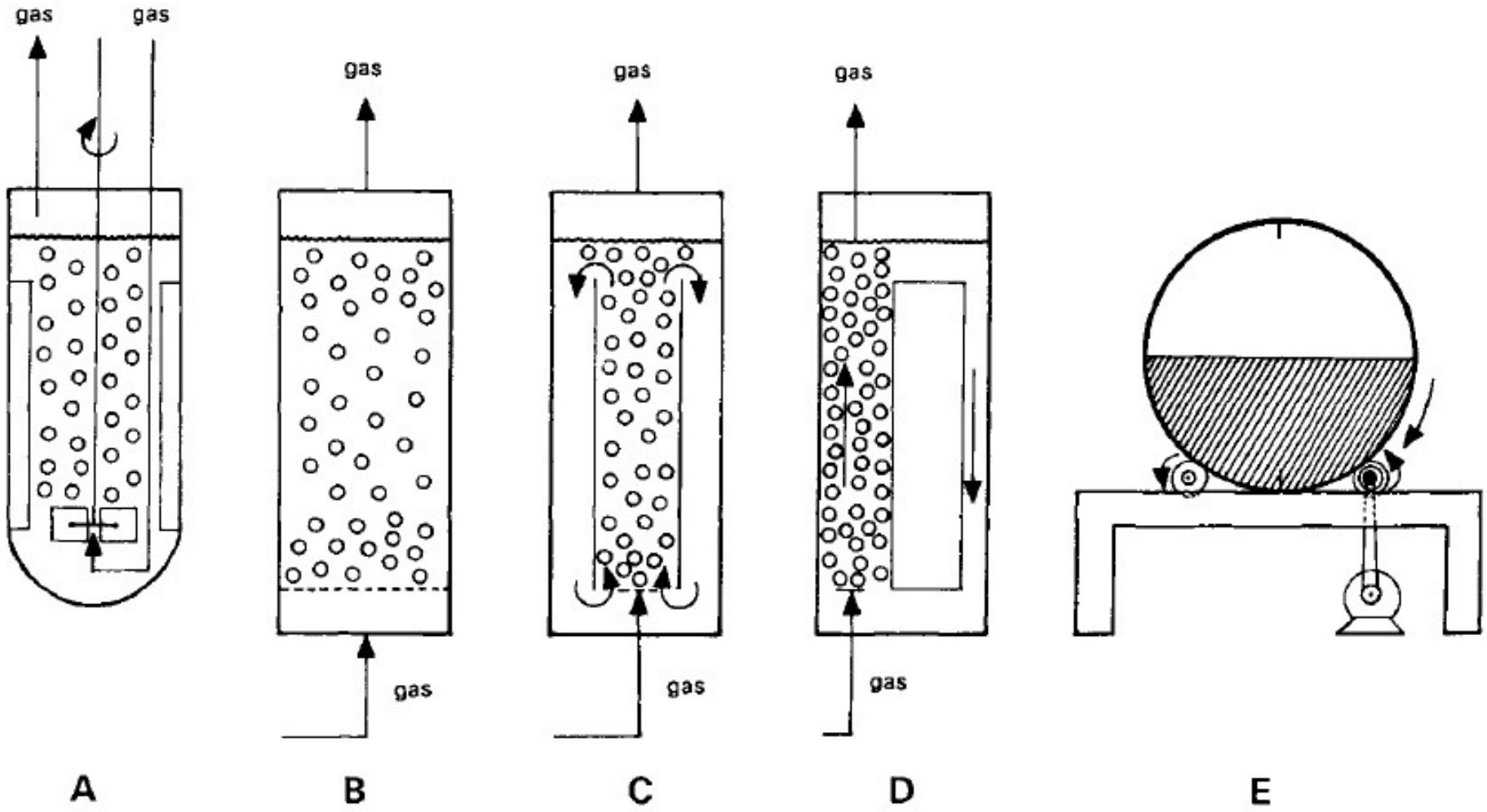


Fig. 4.7. Configuration of reactors used for plant cell cultivation. (A) Stirred-tank reactor; (B) bubble-column reactor; (C) air-lift reactor with draft-tube; (D) air-lift reactor with outer loop; (E) rotating-drum reactor.

Significance of agitation in a plant cell bioreactor

- Plant cells in suspension culture tend to form **aggregates of 2-200 cells.**
- During the **late exponential phase** of growth, cells become more sticky because of increased excretion of polysaccharides into the culture vessel.
- This leads to the adhesion of plant cells to the reactor wall, probes and stirring device and the formation of larger aggregates.

- Mixing is affected, as the aggregates tend to sediment or stick to the reactor surface, forming extensive **wall growth.**

- Large aggregates also create rheological problems by creating dead zones in the culture vessel and can **block the opening and pipe lines** of the reactor.

- Cell aggregation adversely affects the operation of the probes used to monitor culture conditions during growth and product formation.

- Diffusion-limited biochemical reactions may occur in large aggregates when nutrients can no longer penetrate to the aggregate's central core.

- In spite of these effects, certain degrees of cell aggregation (cell-cell contact) and cell differentiation seem to be essential for secondary metabolite production.

- Hence, **controlled aggregation** of plant cells is of interest from the process engineering point of view.

2. Optimum oxygen

- All plant cells are aerobic and require continuous supply of oxygen.
- However, plant cells **require less oxygen than micro-organisms** because of their slow metabolism.
- In some cases, **high oxygen concentration is even toxic to the metabolic activities of cells**.
- Air is normally sparged or blown in at the base of the bioreactor.

Selection of a bioreactor

- The suitability of a particular bioreactor for plant cell cultivation could be evaluated by considering the following factors:

1. Capacity of oxygen supply and intensity of air bubble dispersion in broth.

2. Intensity of hydrodynamic stresses generated inside the reactor and their effect on the plant cell system.

3. Adequacy of mixing of culture broth at high cell concentration.

4. Ability to control temperature, pH, and nutrient concentration inside the reactor.

5. Ability to control aggregate size (which may be helpful for increasing product formation).

6. **Ease of scale-up**.

7. **Simplicity of aseptic operation** for long durations.

- The major types of bioreactors currently in use for suspension culture of plant cells are

1. Stirred-tank reactor
2. Bubble column reactor
3. Air-lift reactor
4. Rotating-drum reactor

Basic parts of bioreactor

- A culture vessel
- Associate supply and environmental systems
- Measurement and control systems

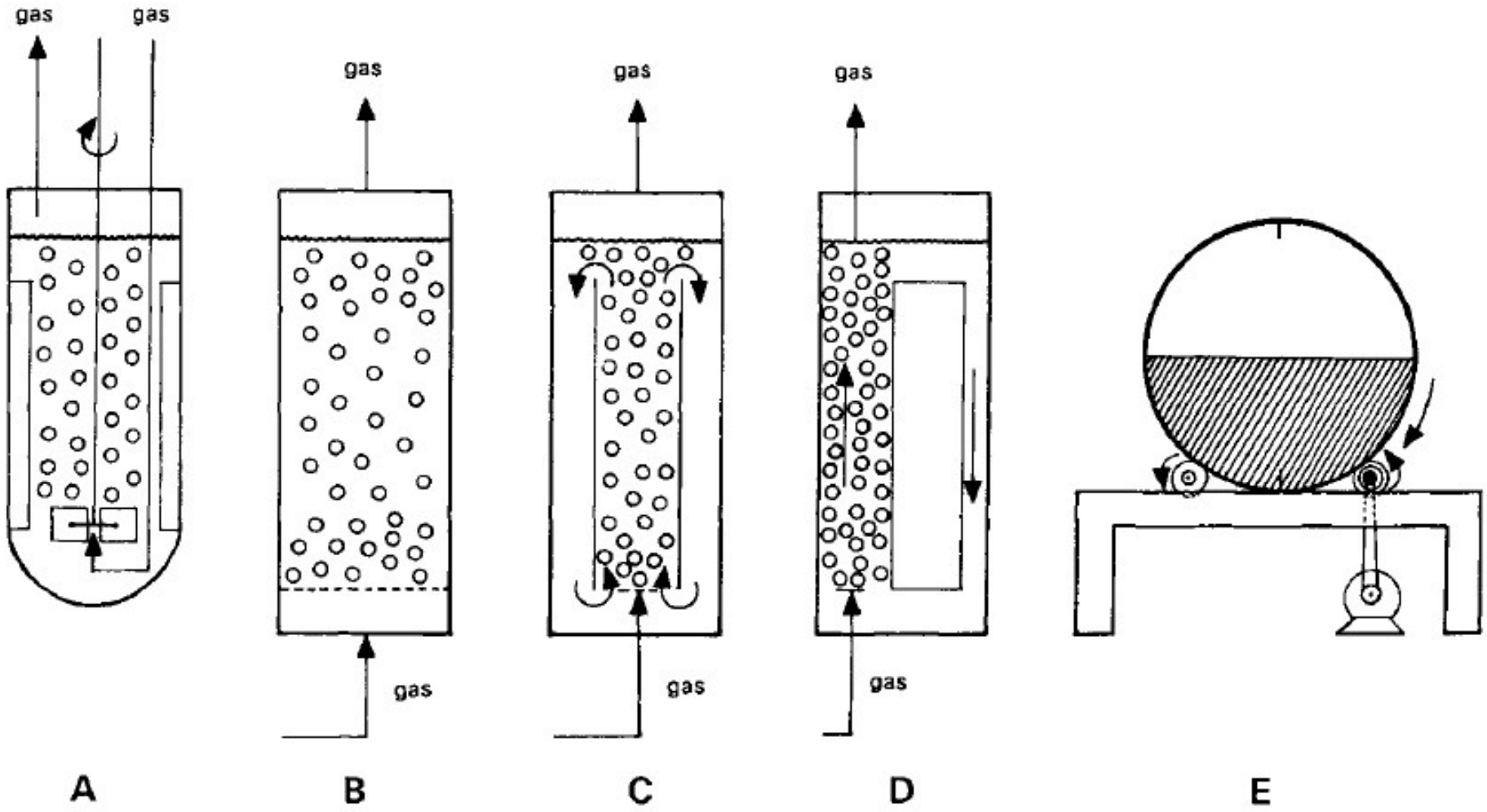


Fig. 4.7. Configuration of reactors used for plant cell cultivation. (A) Stirred-tank reactor; (B) bubble-column reactor; (C) air-lift reactor with draft-tube; (D) air-lift reactor with outer loop; (E) rotating-drum reactor.

(i) *Stirred - tank reactor*

- *The stirred-tank reactor , in which* air is dispersed by mechanical agitation, represents the classical bioreactor for aerobic fermentations.

- Its behaviour has been well studied in a number of biological systems.

- **Temperature**, **pH**, **amount of dissolved oxygen**, and **nutrient concentration** can be controlled better within this reactor than any other reactor.

- It is an upright cylindrical vessel with both the ends are closed with hemi-spherical basins.

- **A stirrer** is fitted inside the vessel in a horizontal position. The stirrer properly mixes the air and the nutrients during fermentation.

- At lateral side of the fermenter there is an opening through which culture medium is being pumped into the fermenter. It has another opening (outlet) at the base of the fermenter for harvesting the products.

Drawbacks

- A major drawback of the stirred-tank reactor is the **shearing stress** generated by its stirring device to which plant cells may be sensitive.

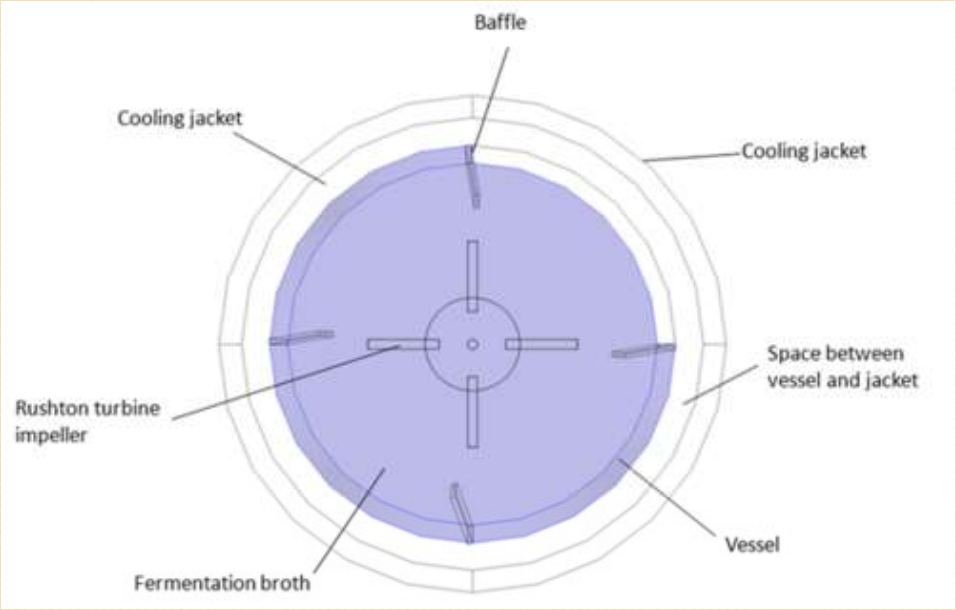
- Some other disadvantages of stirred-tank reactors are their high energy requirements and complexity of construction and the fact that they are difficult to scale up.

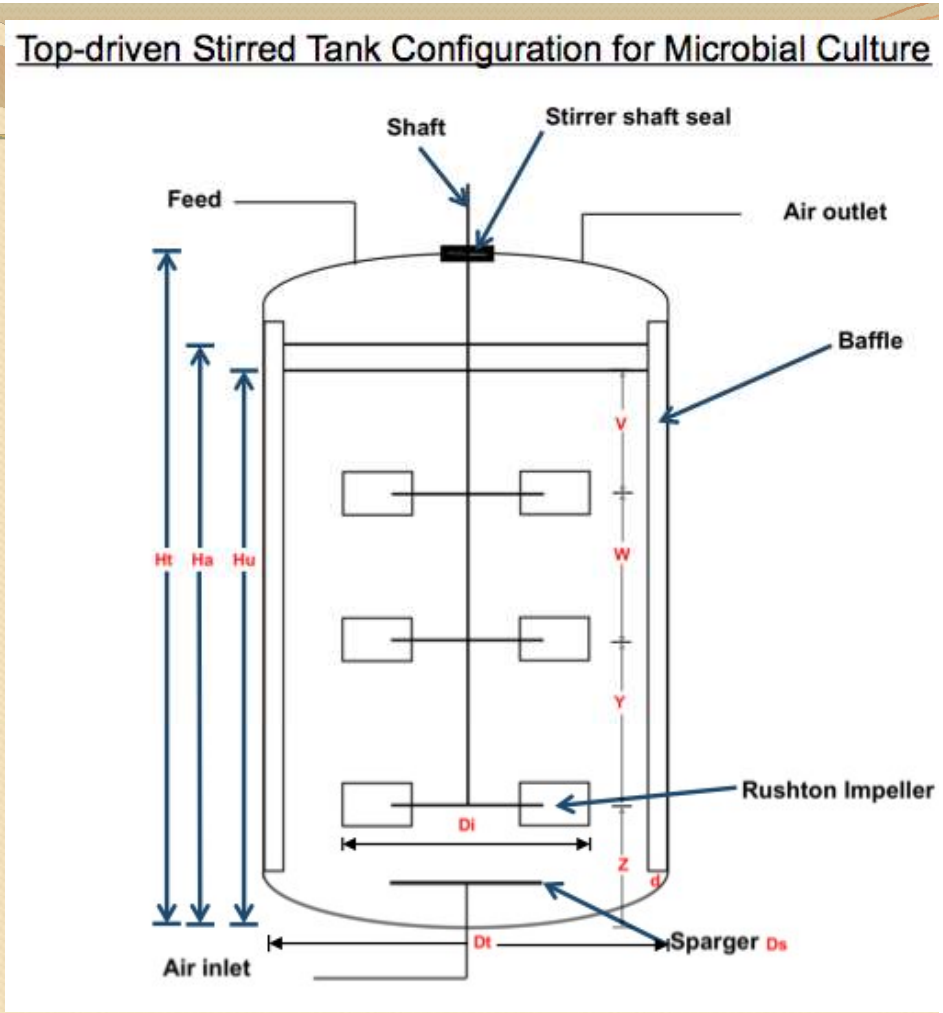
- Despite this, most existing laboratory and commercial bioreactors are of the stirred-tank design which have been suitably modified for plant cell culture, such as:

(1) The impeller speed is reduced to 50-150 rev./min and in some cases the turbine impeller is replaced by a marine screen or paddle.

(2) Removal of baffles, pH probe and other probes not required.

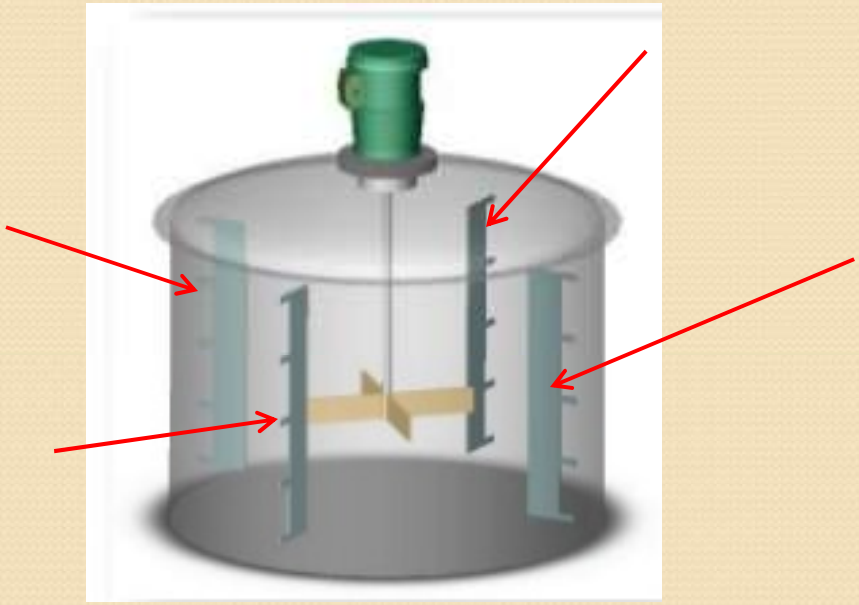
(3) The **sample ports are enlarged to about 1 cm** to reduce blockage caused by cell aggregates.





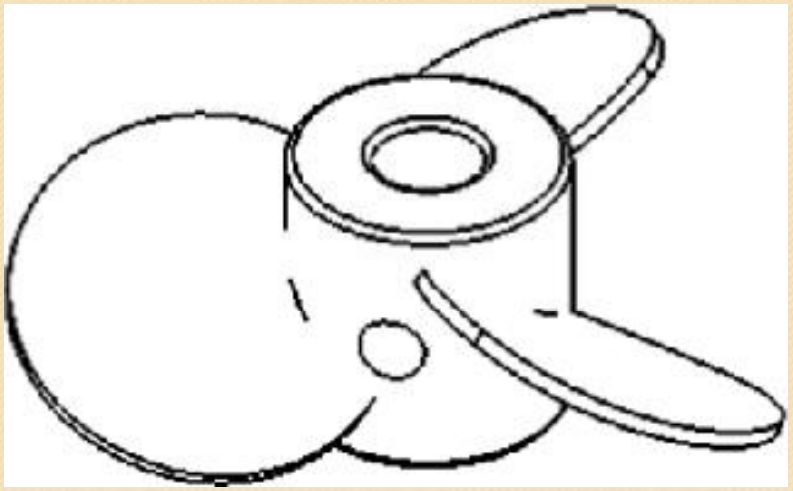
- Cells of ***Catharanthus roseus*** *have been grown in stirred-tank reactors.*

Baffles – to prevent vortex



- For the first industrial plant cell culture process, ***stirred-tank reactors* were used** for the production of shikonin from ***Lithospermum erythrorhizon*.**

Turbine impeller



marine screen or paddle or paddle impeller

(ii) *Bubble-column reactor*

- The bubble-column reactor is one of the simplest types of gas-liquid bioreactors used for aerobic fermentation.
- It consists of vertically arranged cylindrical vessel aerated at the bottom.
- In such a system the gas is dispersed pneumatically through a deep pool of liquid by means of nozzles or perforated plates.
- Bubble columns are devices in which gas, in the form of bubbles, comes in contact with liquid.

- A bubble-column reactor has been used for the cultivation of ***N. tabacum***, *but insufficient* mixing at such a scale reduced the specific growth rate of the cells.

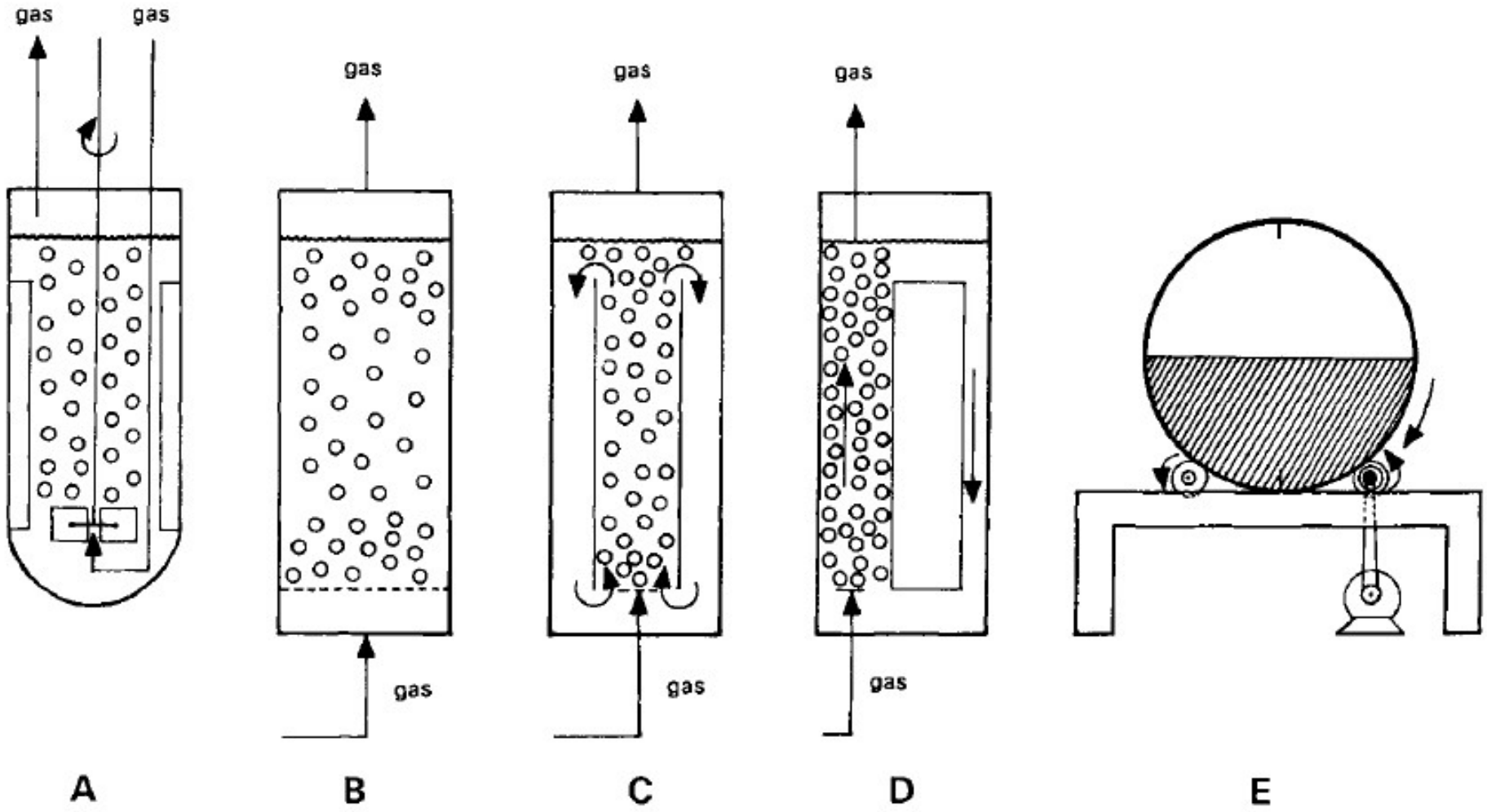


Fig. 4.7. Configuration of reactors used for plant cell cultivation. (A) Stirred-tank reactor; (B) bubble-column reactor; (C) air-lift reactor with draft-tube; (D) air-lift reactor with outer loop; (E) rotating-drum reactor.

Merits of bubble-column reactors

- (i) It facilitates sterile operation because of the absence of moving parts.
- (ii) It provides high mass and heat transfer areas without the input of mechanical energy and may, thus, be suitable for shear-sensitive systems such as plant and animal cell culture.
- (iii) The **scale-up is relatively easy**, and the reactor requires minimum maintenance.
- (iv) Less internal parts and easy cleaning.

Disadvantages

- 1. the **undefined fluid flow pattern inside the reactor** and its non-uniform mixing.

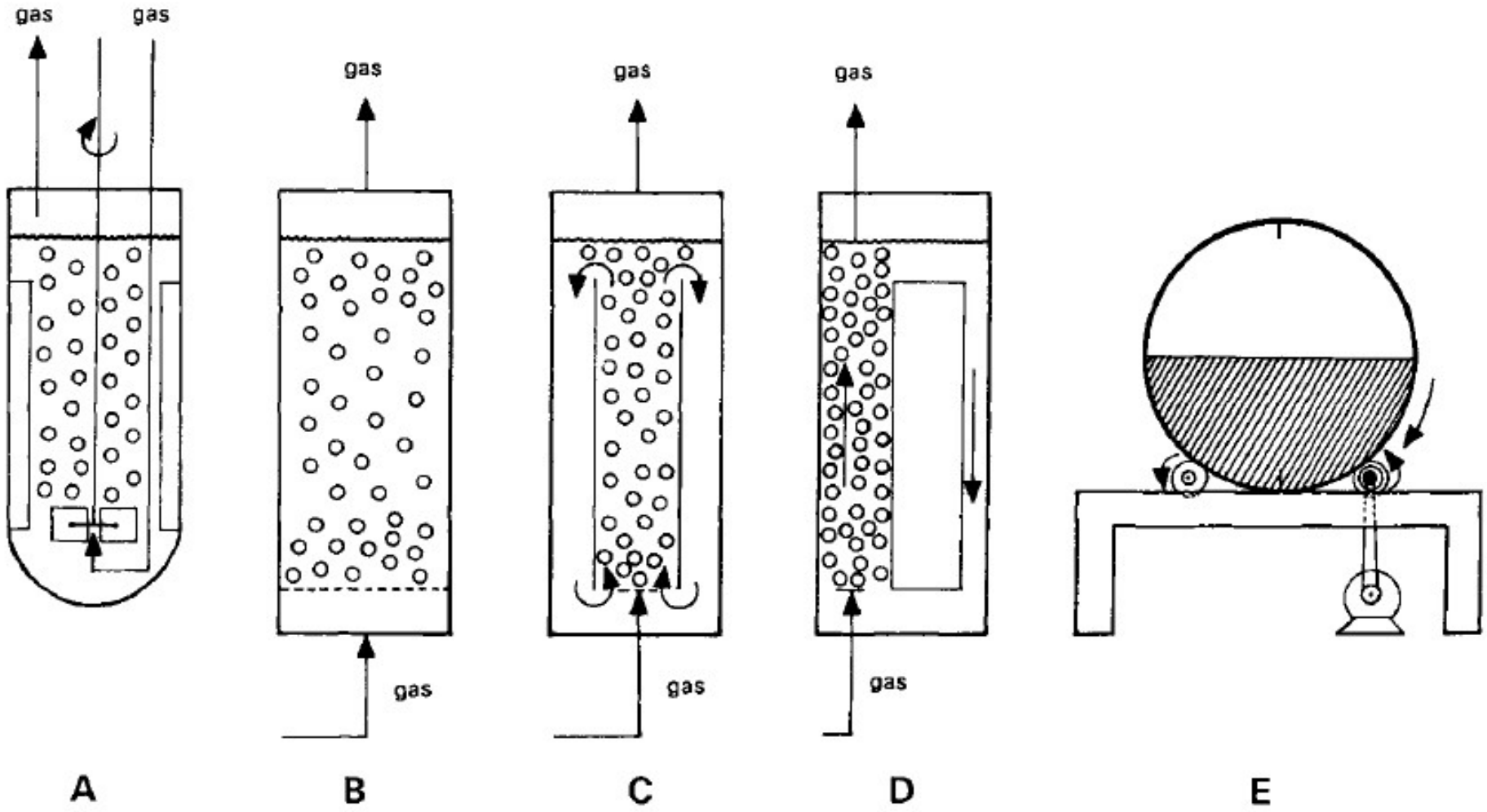
(iii) *Air-lift bioreactor*

- *In air-lift reactor, as its name implies, compressed* air is used for aeration and mixing of the contents of the reactor vessel.
- Its operation is based on the draught tube principle.
- Air sparged into the base of the reactor lowers the density of the medium which rises up the draft tube pulling fresh medium in at the base and, therefore, a flow is achieved.
- Schematic diagrams of the draught tube (inner loop and outer loop) air-lift vessels are given.

- Non- mechanically agitated bioreactors.

- Sterile air is pumped into the base of the up flow tube (small) and it lifts the medium inside to the top of the tube. At the top, air moves up leaving the medium into the down flow tube and gives a pressure to force the medium downwards.

- As a result, culture is in a continuous circulation without mechanical agitations.



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- A more uniform flow pattern is achieved in the air-lift reactor compared with the bubble column reactor, where a random flow pattern exists.

- Air-lift reactors have been used extensively for cultivation of

C. roseus cells,

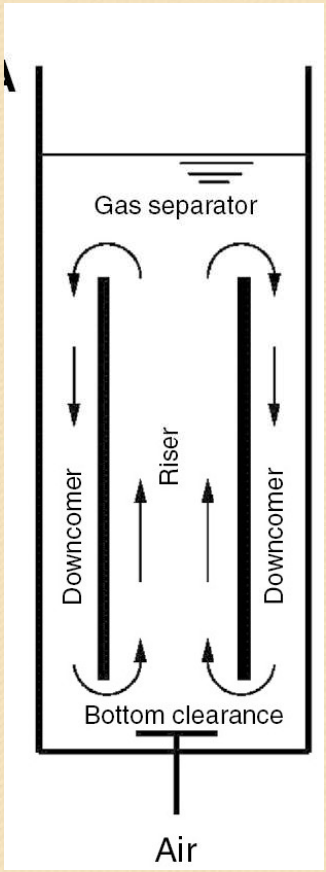
Cinchona ledgeriana,

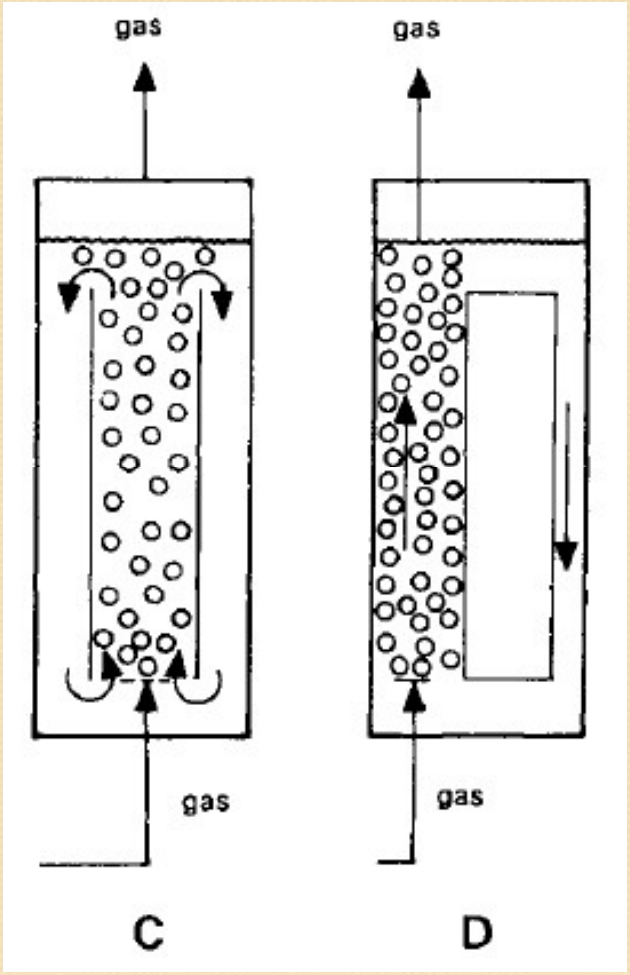
Digitalis lanata,

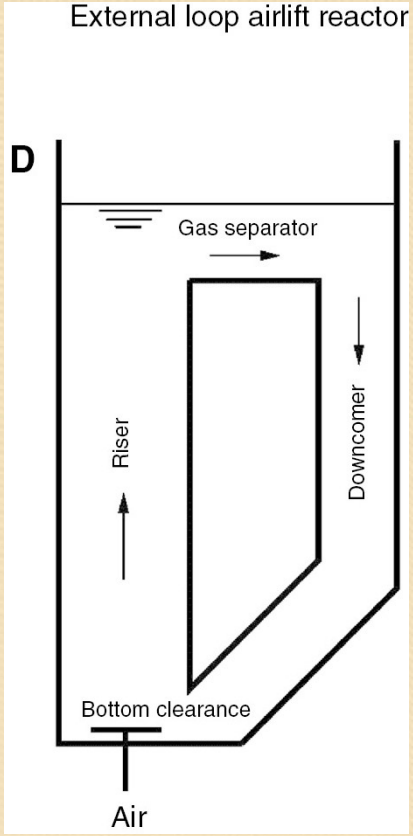
Morinda citrifolia *etc.*

- Recently, ***Lithospermum erythrorhizon*** *have also been cultured in air-lift* reactors.

Airlift reactor (ALR) is generally classified as pneumatic reactors without any mechanical stirring arrangements for mixing.







C – Air-lift reactor with draft tube / internal loop

D - Air-lift reactor with outer/external loop

- The air-lift reactor is one of the **most suitable bioreactor types for cultivation of plant cells on a large scale.**

- It provides **reasonable mixing and oxygen transfer at low shear**, and **less contamination** occurs because there are no moving parts and no intrusion of impeller shaft.

- The **operating cost**, compared to the stirred-tank reactor, is low because of its **simple design** and it does not require power input for the stirrer.

- Despite these advantages air-lift reactors have not been used as extensively as stirred tank reactors.

Disadvantages

- The development of dead zones inside the reactor and insufficient mixing at high cell densities.
- Moreover, little information is available on the engineering analysis of the reactor behaviour in complex systems such as the plant cell cultures.
- Greater **air input and higher pressures** needed.

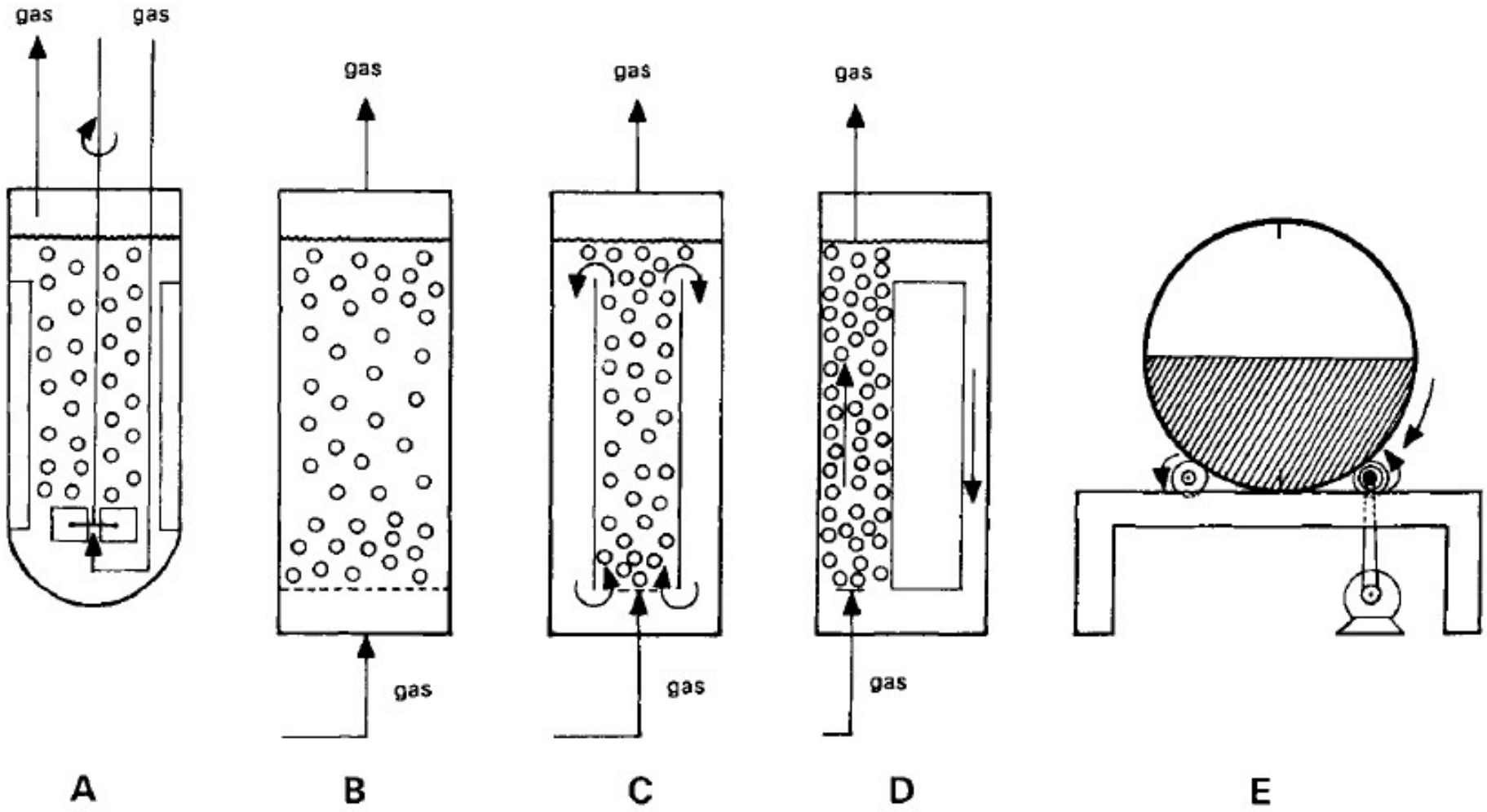
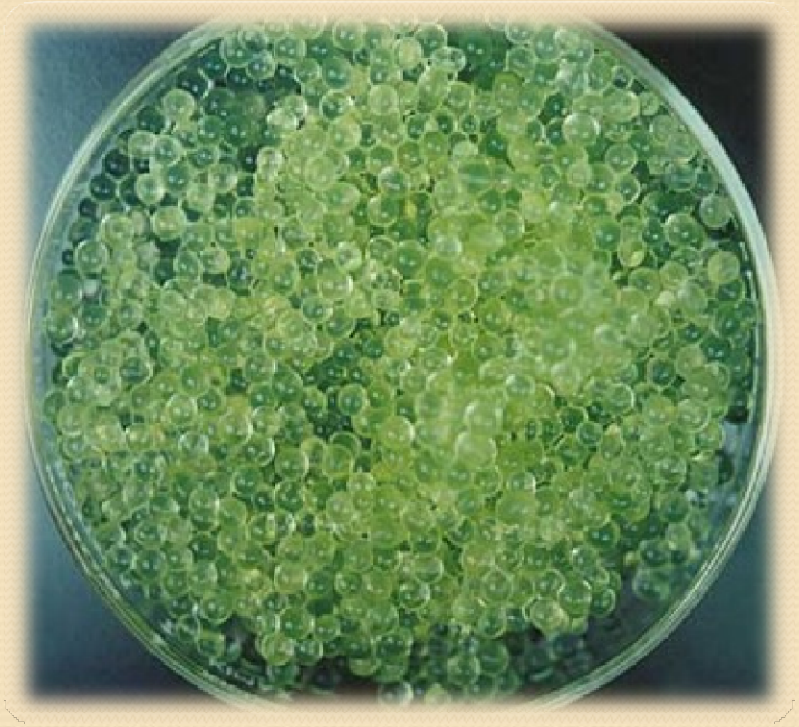


Fig. 4.7. Configuration of reactors used for plant cell cultivation. (A) Stirred-tank reactor; (B) bubble-column reactor; (C) air-lift reactor with draft-tube; (D) air-lift reactor with outer loop; (E) rotating-drum reactor.

(iv) *Rotating-drum reactor*

- The rotating-drum reactor consists of a horizontally rotating-drum on rollers connected to a motor.
- The rotating motion of the drum **facilitates good mixing** and **aeration** without imposing a high shear stress on the cultured cells.
- Baffles in the inner wall of the drum help to increase oxygen supply.
- This type of reactor has the capacity to promote high oxygen transfer to cells at high density.

- **It has been used to grow cultures of *C. roseus and Lithospermum erythrorhizon*.**
- The rotating-drum reactor facilitates better growth and imparts less hydrodynamic stress.
- In the stirred-tank reactor growth rate was low at low agitation speed because of insufficient oxygen supply, while at high agitation speed the cells died.
- Hence, **for cultivation of cells at high densities,** the rotating-drum reactor was preferred.
- The major disadvantage of this reactor type is the **restriction in scale up.**



Immobilization of plant cells

- Immobilization is the newest culture technology of plant cell, and considered as to be the most “natural”.

- It is defined as a technique, which confines the cells to a defined region in a space while retaining their catalytic activity and prevents its entry into the mobile phase, which carries the substrate and product.

- Immobilization of plant cells, protoplast or embryos is achieved by binding these materials onto or within a solid support.

Immobilized plant cell reactors

- *Immobilization of plant cells into a* suitable carrier and cultivation of the immobilized cells in different types of reactors has been **developed as an alternative to free cell culture systems** for the production of industrial phytochemicals.

Immobilized plant cell reactors

- General methods employed to immobilize plant cells

1. Entrapment in natural (alginate, agar, agarose, carrageenan) or synthetic (polyacrylamide)polymers
2. Adhesion to reticulate polyurethane foam, and
3. Confinement behind semi-permeable membranes.

- **Alginate** has been the most popular polymer used to immobilize plant cells.

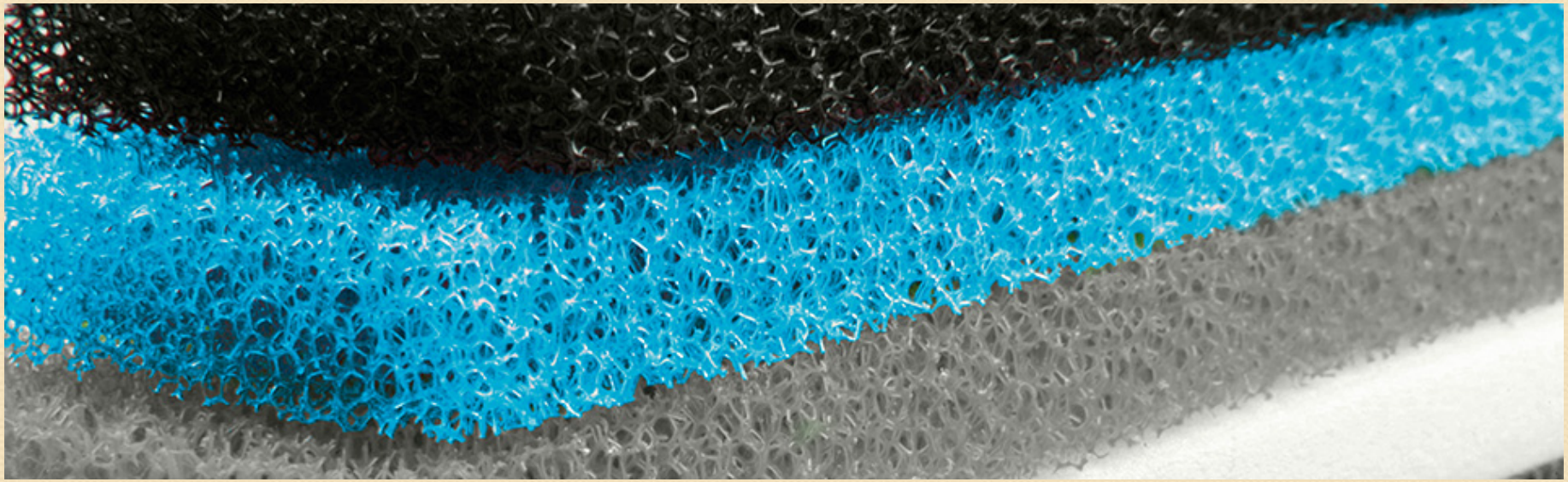
- Cell suspension in 4% sodium alginate solution is allowed to fall as 2 mm drops in a beaker containing 0.2 M solution of $CaCl_2$.

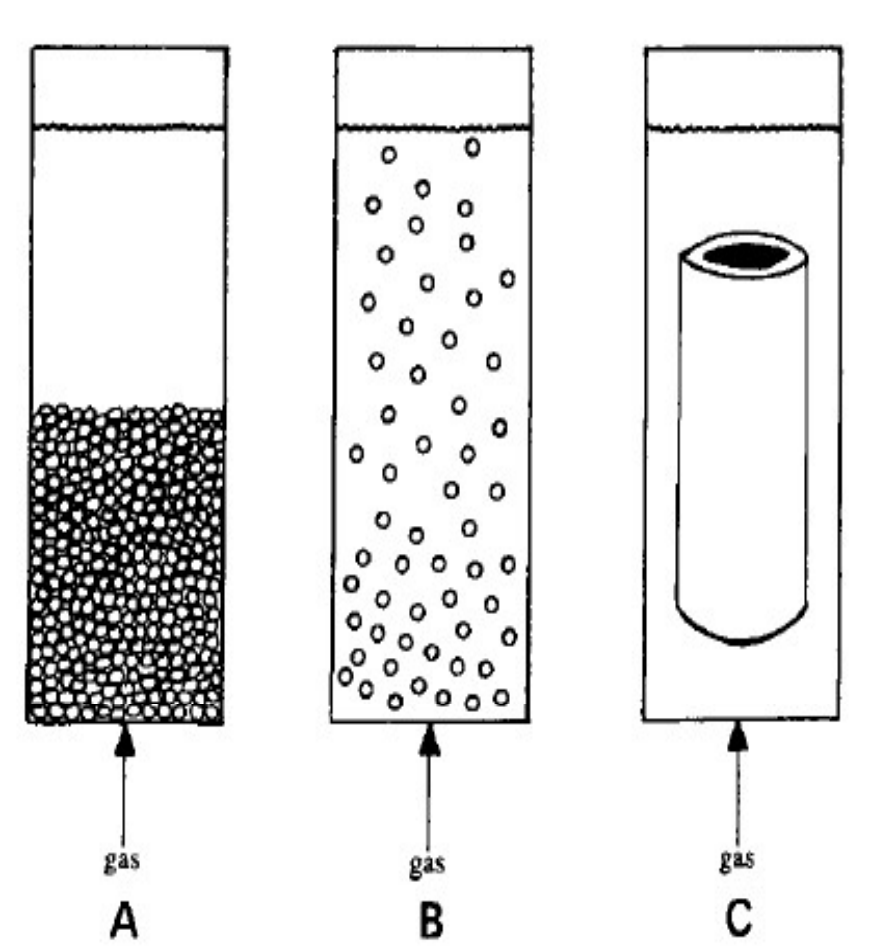
- **Calcium-alginate** is formed by ion exchange reaction and the drops harden as beads within 20-30 min.

Alginate entrapped cells can be cultured in 3 ways.

- 1. Packed-bed
- 2. Fluidized-bed
- 3. Air-lift bioreactors.

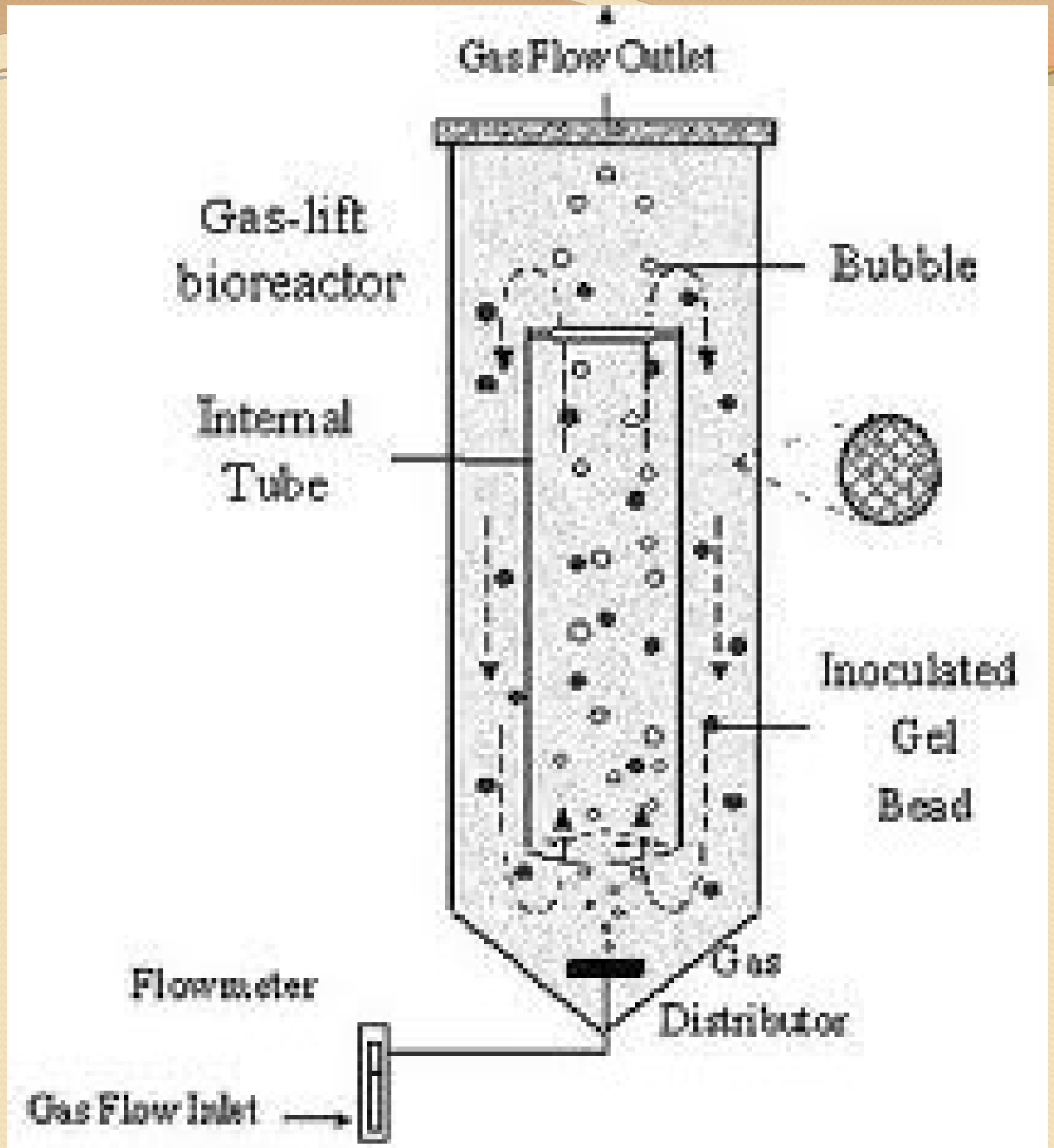
Polyurethane foam (a polymer composed of organic units joined by carbamate links**)**





Configuration of some reactors used to culture immobilized plant cells.

- (A) **Packed-bed reactor**
- (B) **Fluidized – bed reactor**
- (C) **Polyurethane draft-tube reactor**


Gas Flow Outlet
Gas-lift bioreactor
Bubble
Internal Tube
Inoculated Gel Bead
Flowmeter
Gas Distributor
Gas Flow Inlet

Polyurethane foam has been used to immobilize a range of cell lines

- **The cells are immobilized in these matrices by flowing cells and medium through the foam or by adding sterile foam to growing cultures**.

- The foam can be cut into various shapes.

Polyurethane entrapped cells have been cultured in both packed and fluidized beds as

1. **Cubes**
2. Shaped into **draft tube** or
3. Threaded **as strips on stainless steel rods**.

Membrane reactors - examples

- Hollow-fibre units
- Flat membrane reactors

Confinement behind semi-permeable membranes

- Membrane reactors (e.g. hollow-fibre units and flat membrane reactors), in which **cells are separated from the growth medium by membrane,** are particularly suitable for fragile cells which can be entrapped more readily on membrane and allow better control over cell density.

- The environment in a membrane reactor is more homogeneous; pressure drop and fluid dynamics are more easily controlled and are relatively independent of the scale of operation.

Hollow-fibre reactor

- Bioreactor consists of a cartridge containing bundles of synthetic, semi permeable hollow fibers.

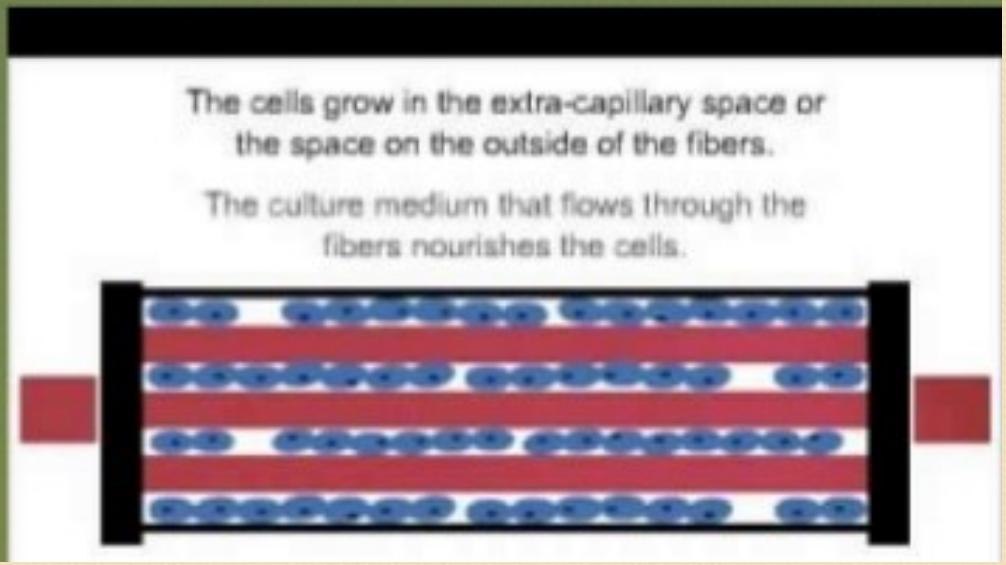
- In a **hollow-fibre reactor** cells are introduced into the shell side/ lumen side of the hollow-fibre cartridge and the medium is circulated through the fibre lumen/ shell side and aerated using a separate reservoir.

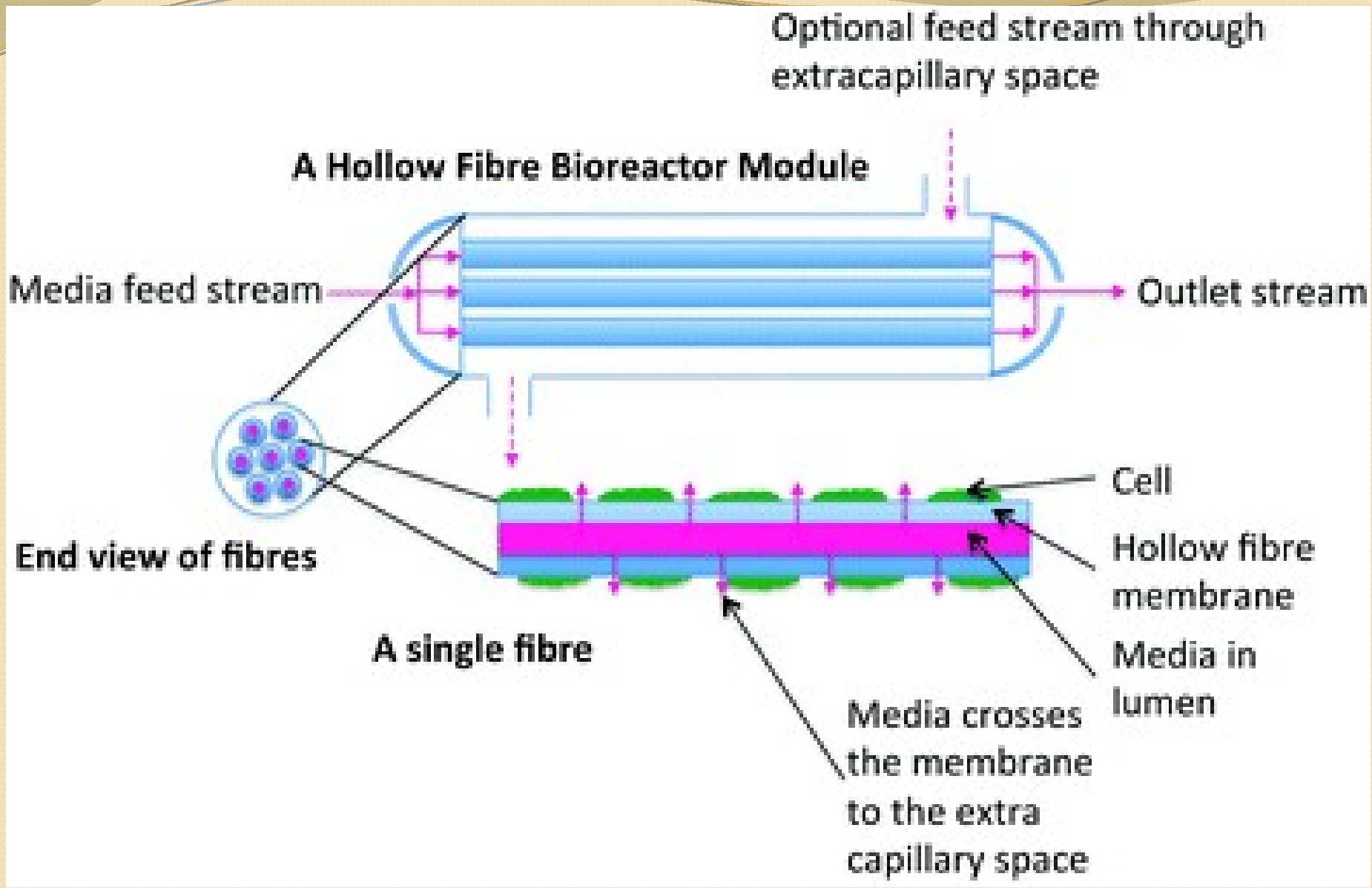
- It is a non-agitated bioreactor.

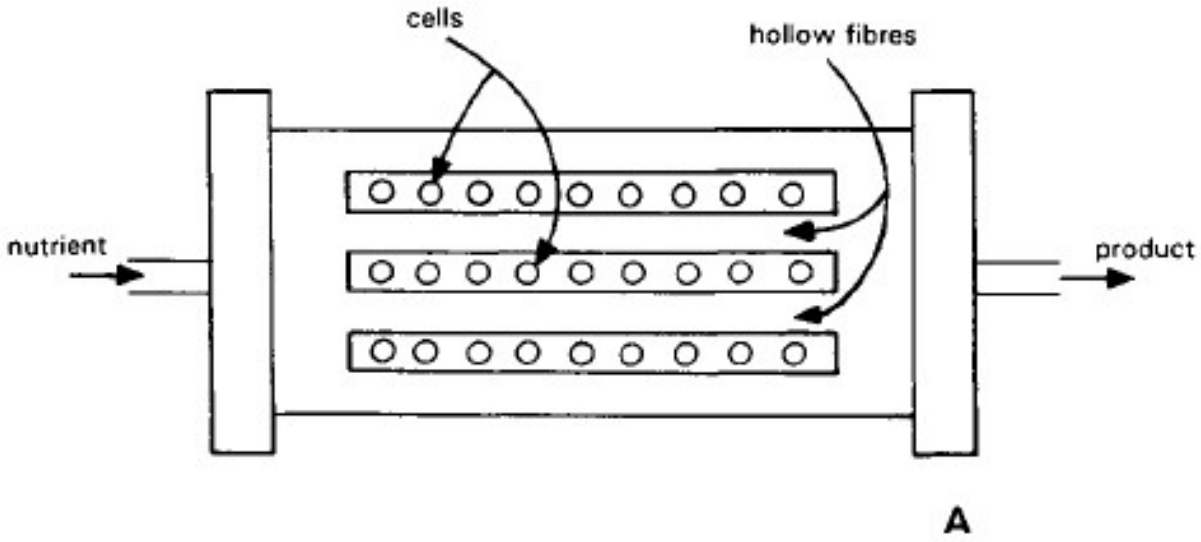
- Since the cells do not stick to the fibre membrane (unlike in the case of alginate beads) the reactors may retain their mechanical integrity for a longer period of time and may be reusable.

Hollow Fiber Bioreactor

- Fibers are made of a porous material
- Intraluminal (Cells inside fibers)
- Extra luminal (Cells outside fibers)
- Permits movement of small molecules (O_2,glucose) , but not cells
- High cell densities
- Good oxygenation
- Difficult to remove cells




Optional feed stream through extracapillary space
A Hollow Fibre Bioreactor Module
Media feed stream
Outlet stream
Cell
Hollow fibre membrane
Media in lumen
End view of fibres
A single fibre
Media crosses the membrane to the extra capillary space



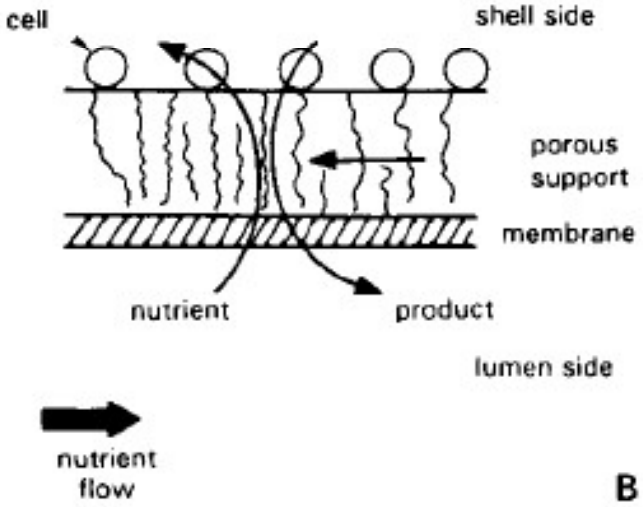
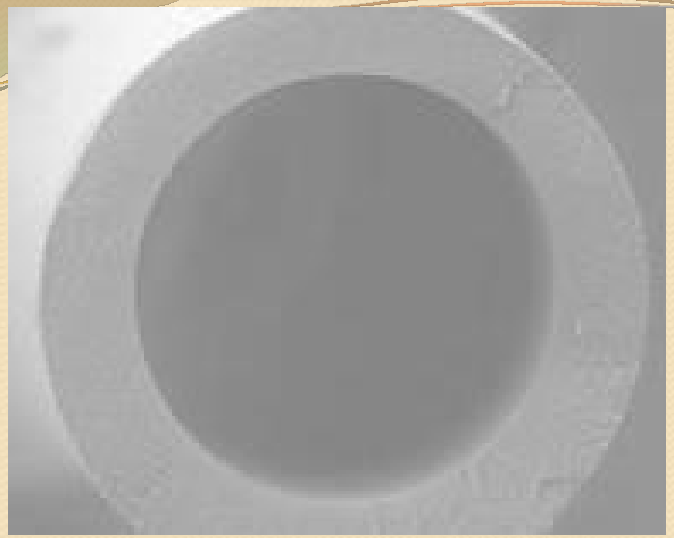
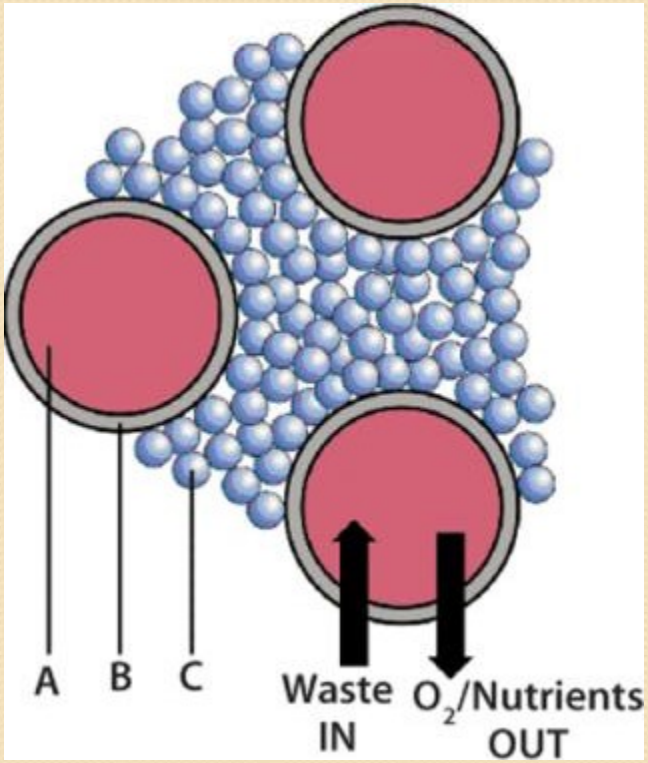
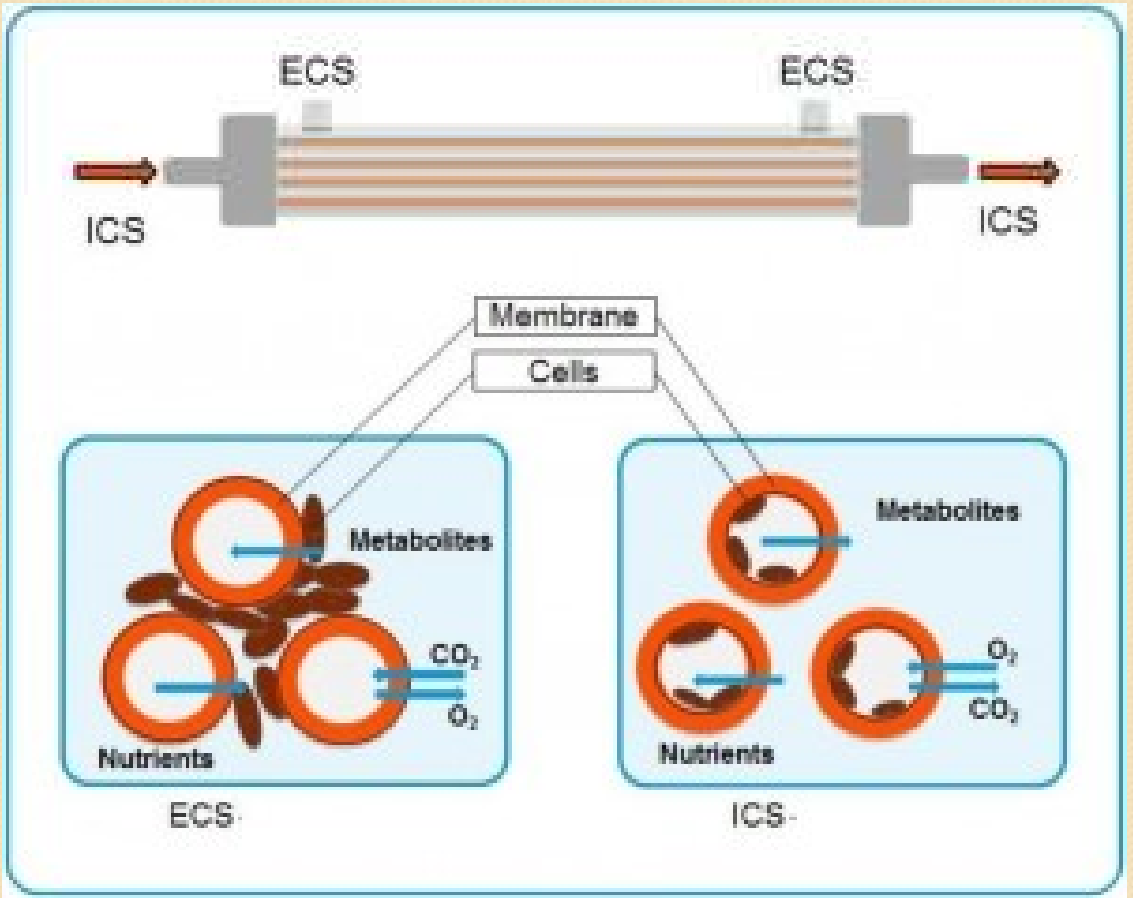
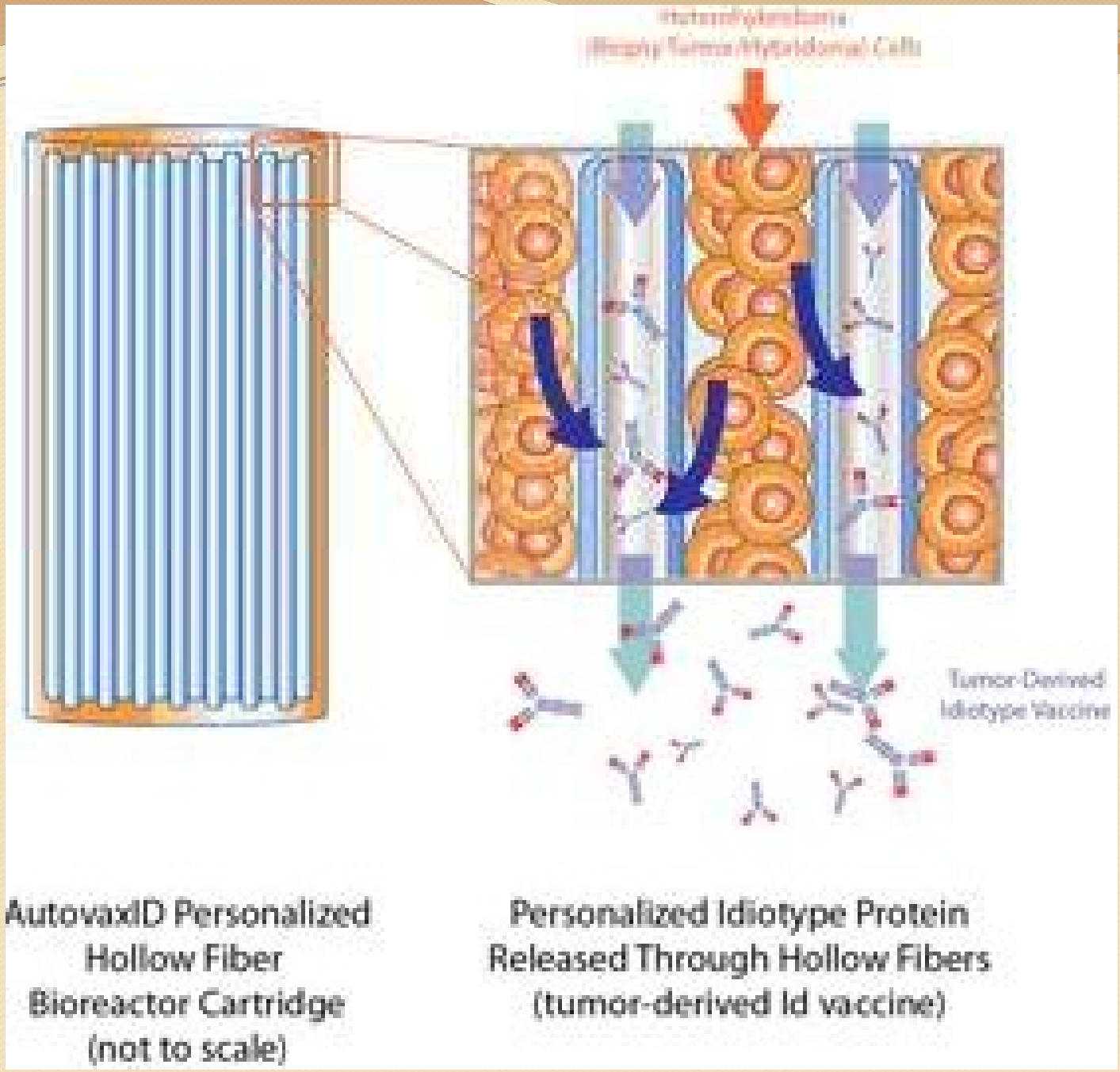


Fig. 4.9. (A) Hollow-fibre reactor for immobilized cell culture; (B) the portion marked in (A) enlarged to show the details of the reactor and the flow of nutrients and products across the membrane and the porous support of the hollow-fibre cartridge (adapted from Prenosil and Pederson,



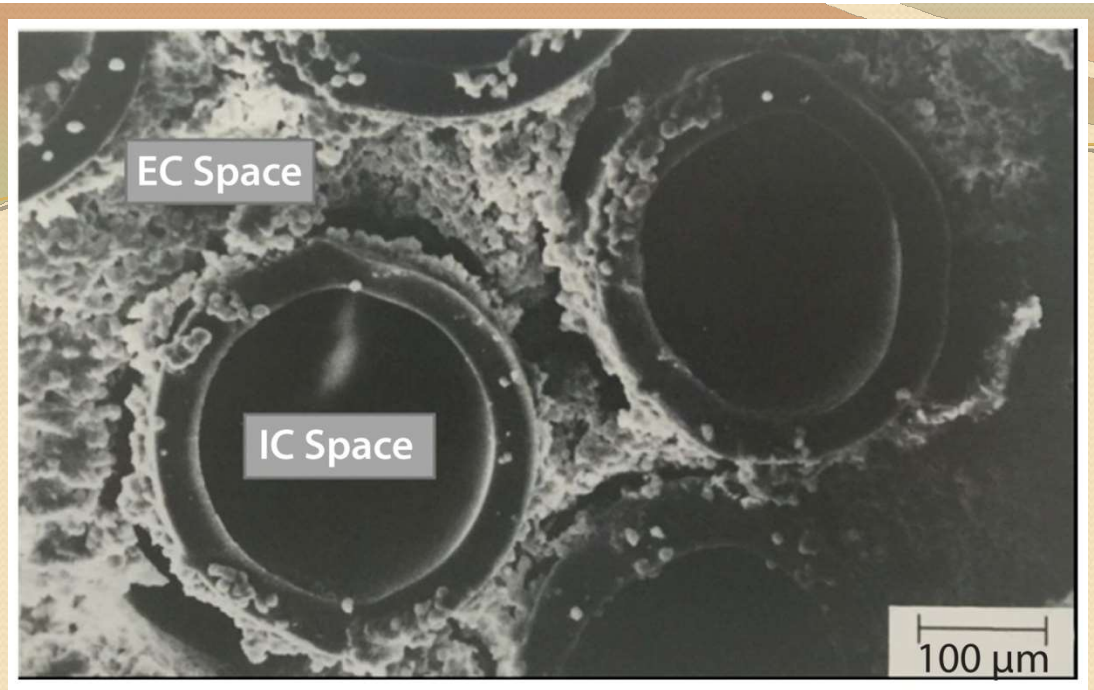

A
B
C
Waste IN
O_2/Nutrients OUT

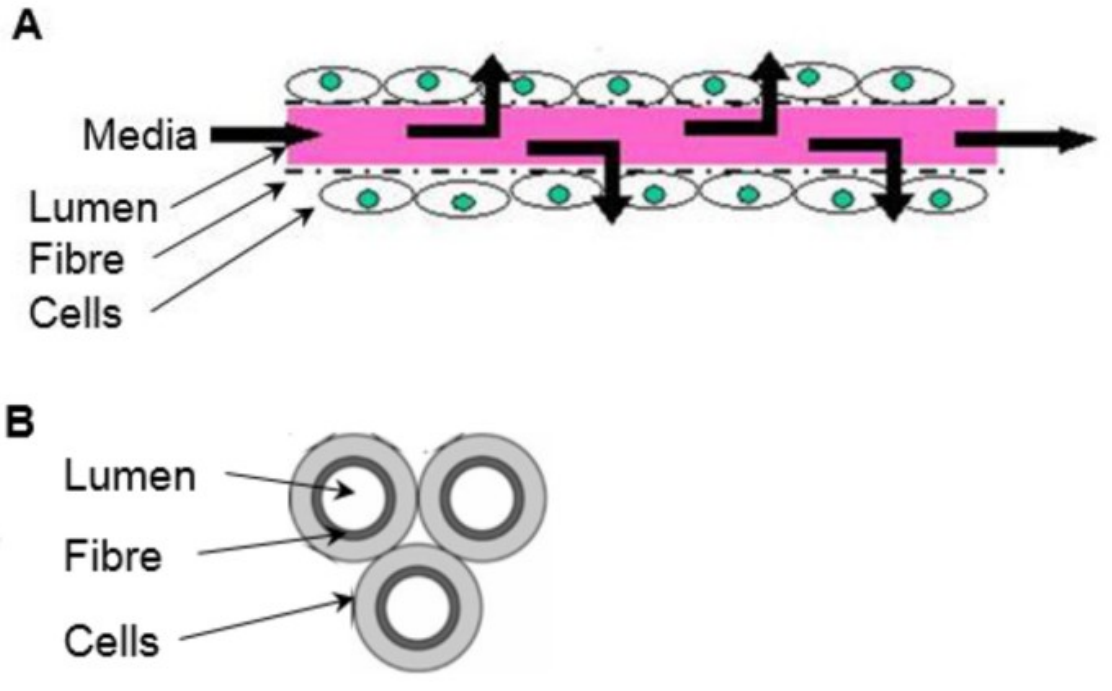

ECS
ECS
ICS
ICS
Membrane
Cells
Metabolites
CO_2
O_2
Nutrients
ECS
Metabolites
O_2
CO_2
Nutrients
ICS

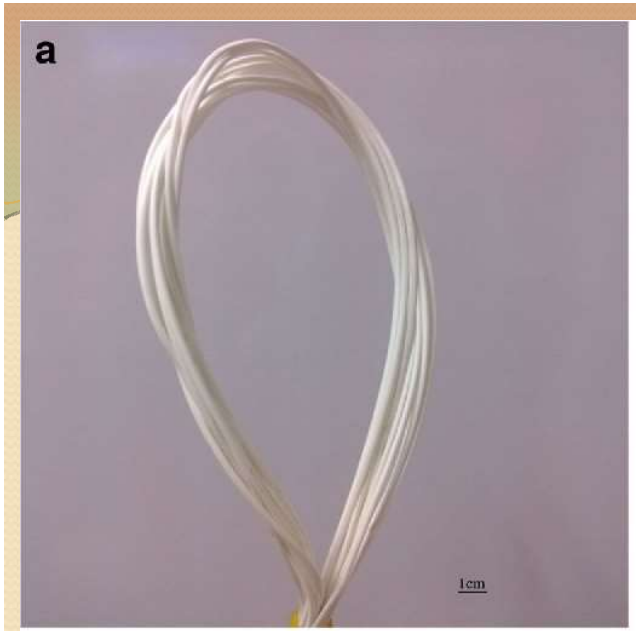


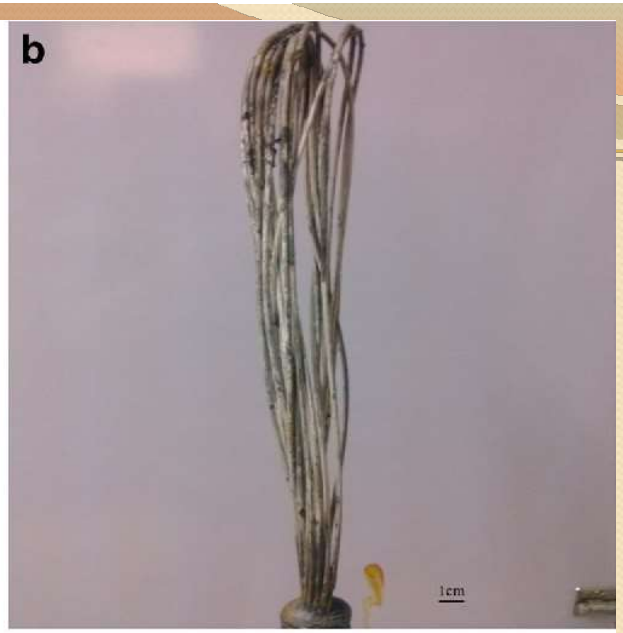
AutovaxID Personalized
Hollow Fiber
Bioreactor Cartridge
(not to scale)

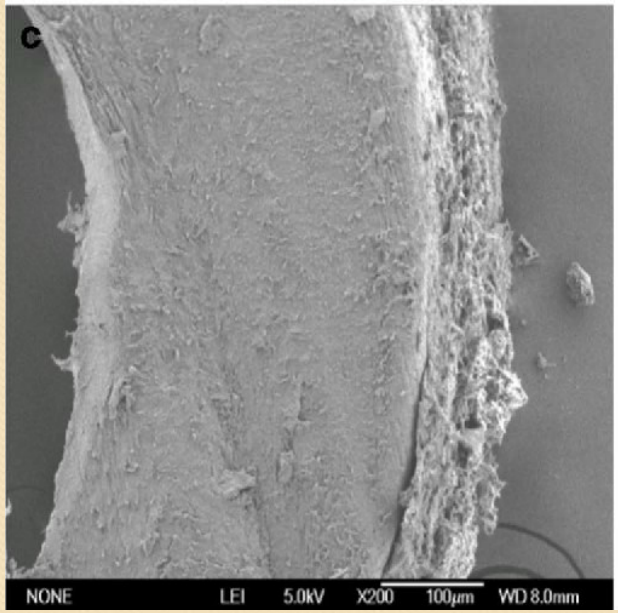
Personalized Idiotype Protein
Released Through Hollow Fibers
(tumor-derived Id vaccine)

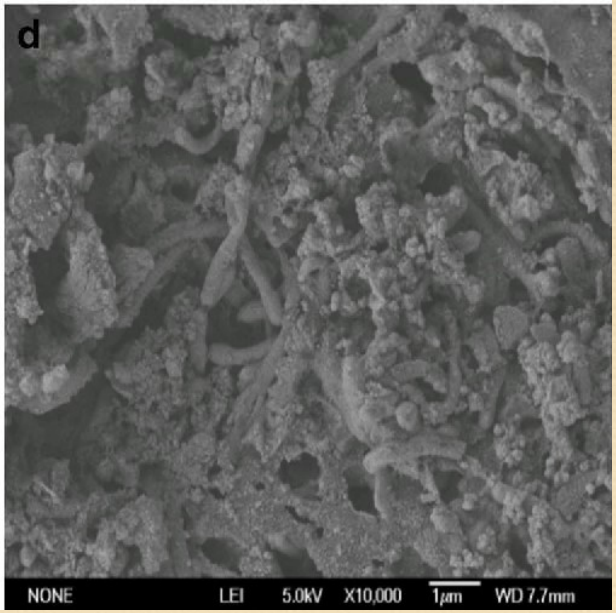

EC Space
IC Space
100 µm

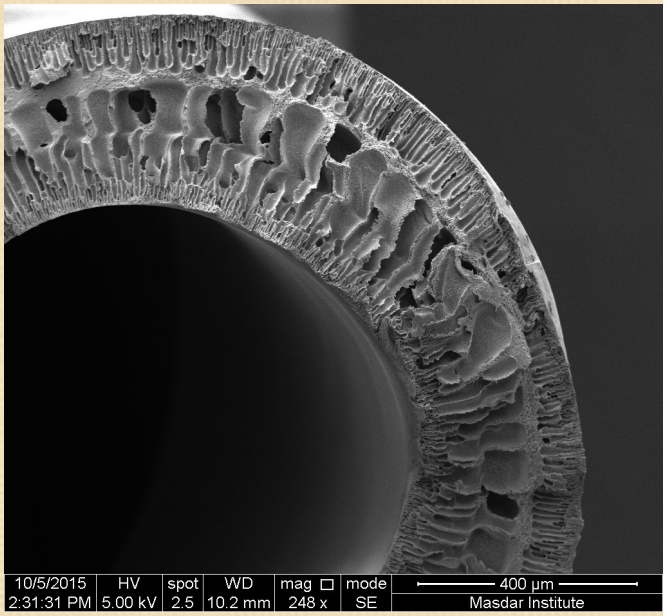

A
Media
Lumen
Fibre
Cells
B
Lumen
Fibre
Cells


a
1cm


b
1cm


c
NONE LEI 5.0kV X200 100µm WD 8.0mm

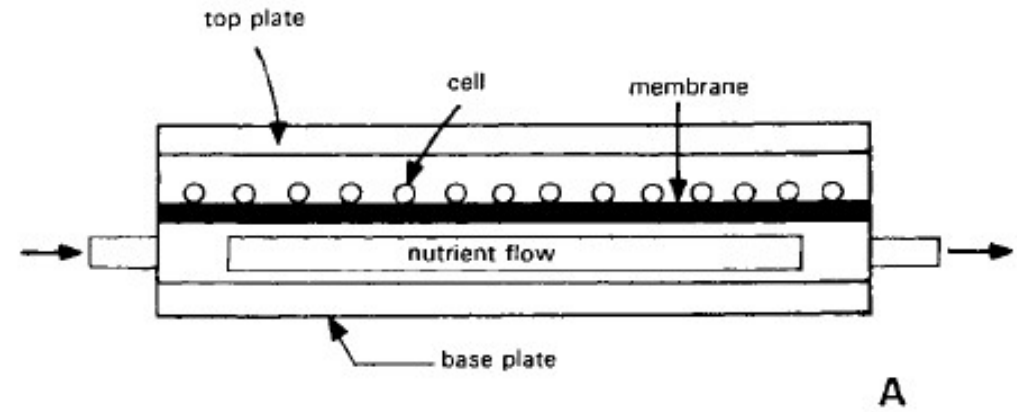

d
NONE LEI 5.0kV X10,000 1µm WD 7.7mm

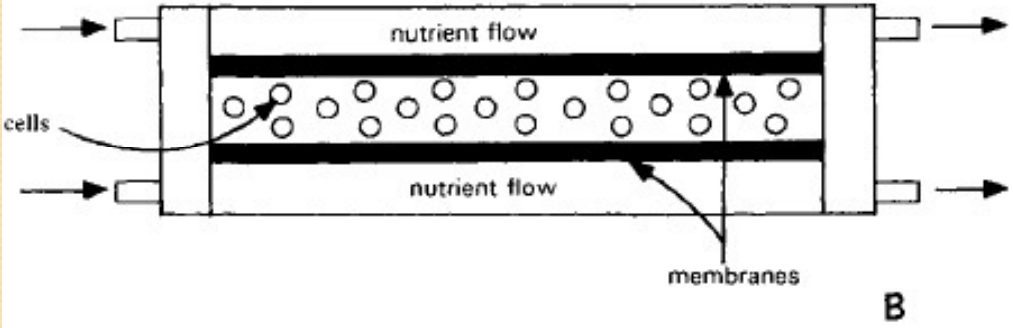

10/5/2015 2:31:31 PM
HV 5.00 kV
spot 2.5
WD 10.2 mm
mag 248 x
mode SE
400 µm
Masdar Institute

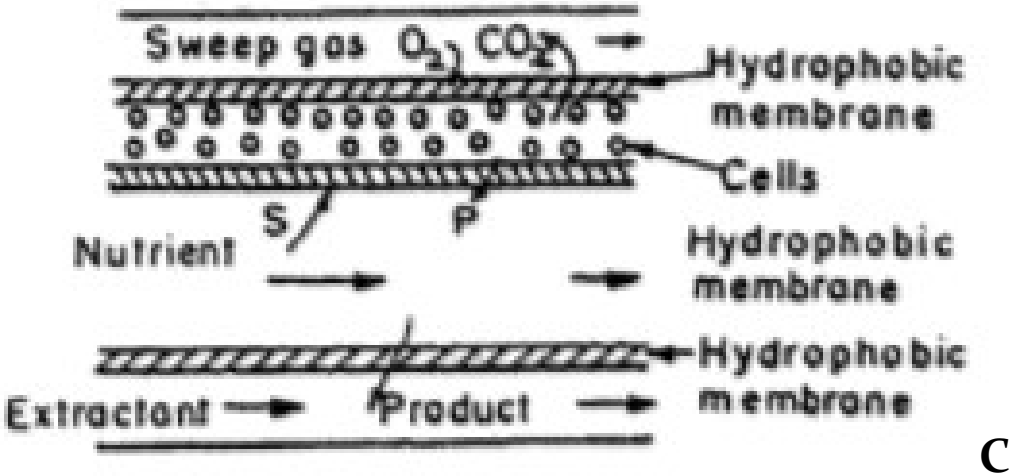
- When **cells are no longer productive**, when an **experiment is over**, or when **a new cell-product combination is desired**, it is potentially possible to flush out the old cells and refill the device with the new cells.

- In **FLAT-PLATE MEMBRANE REACTOR** systems, with one side flow and two side flow, the cells are loaded manually into the membrane cell layer and direct sampling can be achieved through a removal cap plate.

- Substrate enters the cell layer by diffusion or pressure driven flow and is converted into product which diffuses into the cell-free compartment.







- Membrane reactor systems for immobilized plant cell culture.

- (A) Flat plate membrane reactor with **one side flow** of nutrients
- (B) Flat plate membrane reactor with **two side flow** of nutrients
- (C) **Multimembrane** reactor system.

- Multi-membrane reactors have also been proposed for immobilized plant cell cultures. The main advantage of this reactor is that the desired metabolites are produced and selectively separated from the reactant simultaneously.

The potential advantages of immobilized cell culture

(1) It may enable prolonged use of biomass.

(2) By immobilization of cells the cell density in a bioreactor can be increased 2-4 times that in suspension cultures and this enables the use of small reactors, reducing the cost of medium, equipment installation and downstream processing.

(3) The entrapped cells are protected against shear forces and, consequently, a simple bioreactor design may be used.

(4) It separates the cells from the medium and, therefore, if the product is extracellular it can simplify downstream processing.

(5) It uncouples growth and product formation which allows product optimization without affecting growth.

(6) The non-dividing immobilized cells are less prone to genetic changes and, therefore, provide a stable production rate.

(7) It minimizes fluid viscosity, which in cell suspensions cause mixing and aeration problems.

(8) It promotes secondary metabolite secretion in some cases.

- An immobilized system which could maintain viable cells over an extended period of time and release the bulk of the product into the extracellular medium in a stable form could dramatically reduce the cost of phytochemical production.

- **STRATEGIES USED TO OPTIMIZE PRODUCT YIELD IN PLANT CELL CULTURE**

1. Culture conditions

a. Medium type

b. Plant growth regulators

c. pH of the medium

d. Light

e. Gaseous environment

2. Selection of high yielding lines

3. Elicitation

1. Culture conditions for producing optimum secondary metabolites

A. Medium

- The productivity of cell lines is greatly influenced by the culture conditions, of which culture medium is the most important.

- In general, growth and production of secondary metabolites are inversely related, both, in whole plant and in cell cultures.

- Consequently, in cell cultures on media defined for optimum growth, production of secondary metabolites generally occurs in the late stationary phase when the medium gets depleted of some of its important constituents.

- Growth inhibition is often associated with cytodifferentiation and the induction of enzymes for secondary metabolism. In such cases, a **'dual culture system'** is preferred.

- Growth medium
- Production medium

- It involves biomass production in a medium optimum for cell proliferation ('growth medium') followed by transfer of healthy cells to a different medium ('production medium') which does not support good growth of the cells but is favourable for product yield.

- E.g. usually used media is LS (Linsmaier and Skoog, 1965) medium.

- Zenk *et al*. (1977) were the first to use such a two stage culture system for the production of indole alkaloids by *Catharanthus roseus cells*.

- The same strategy was used for commercial production of shikonin by cell cultures of ***Lithospermum erythrorhizon.***

- The most useful modification made in the growth medium for use in secondary metabolite production are:

(a) reduction or elimination of 2,4-D or other phytohormones.

(b) reduction of phosphate level and

(c) increase in sucrose level or alternation of carbohydrate (C)/nitrogen (N) ratio.

- The effect of nitrogen on alkaloid production is dependent on the carbon available to the cells which makes the C/N ratio an important factor to be taken into account.

- The optimum media for the production of different metabolites by a cell line are likely to be different.

- In **some cases it has been possible to combine growth and production steps in the same medium.**

- Increased sucrose concentration (6%) in the medium improved both growth and production in batch cultures of C. *roseus developed a modified* MS medium that promoted growth and alkaloid production by *C. roseus* cell cultures in a single stage.

b. Plant growth regulators

- Plant growth regulators affect growth and differentiation and, thus, affect secondary metabolite production by cultured cells.

- In general an **increase of auxin level,** such as **2,4-D**, which stimulates dedifferentiation and proliferation of cells, reduces the level of secondary metabolites.

- Therefore, generally auxins are added to the growth medium but omitted or used at a lower level in the production medium.

- **Berberine** production by cell cultures of *Thalictrum minus* *was greatly influenced by the hormonal composition* of the medium.

- In the presence of 2,4-D the cells grew rapidly, producing little berberine. The alkaloid yield was remarkably increased by the combined presence of an auxin and a cytokinin.

- **GA3 generally inhibits the secondary metabolite production.**

c. The pH of the medium

- The pH of the medium is shown to **enhance permeability of the cell membrane** and, thus, helps in the release of intracellular alkaloids.

d. Light

- Light is an important regulatory factor in the production of alkaloids in plant cell cultures.

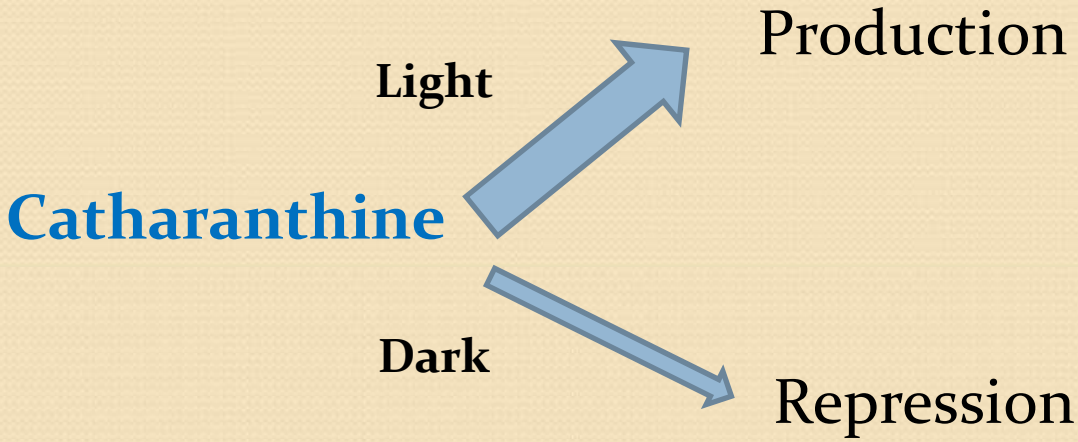
- The importance of light for the optimal expression of some pathways in cultured cells has been demonstrated, including for flavonoids, cardenolides and betacyanins.

- In ***C. roseus***, *light influences the ajmalicine/serpentine accumulation* ratio.

- Here, observed higher accumulation of **serpentine** in the cultures under a 15 h photoperiod instead of 24 h illumination.

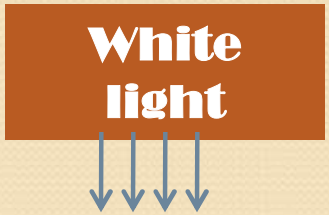
- **15 h light**  **serpentine**  **24 h light**

- **Catharanthine** production was completely repressed in the absence of light. Moreover, light affected not only production but also the site of product accumulation.



- In **dark grown** cultures of *C. roseus* 79% of the serpentine and 78% of ajmalicine were excreted into the medium but in light it dropped to 14% and 18%, respectively.

- In the calli grown under white light, ajmalicine accumulation preceded that of serpentine.



Calli ⟶ ajmalicine ⟶ serpentine

- However, the calli exposed to red or blue light had a constant ajmalicine content and their serpentine content was always lower than that observed under white light.

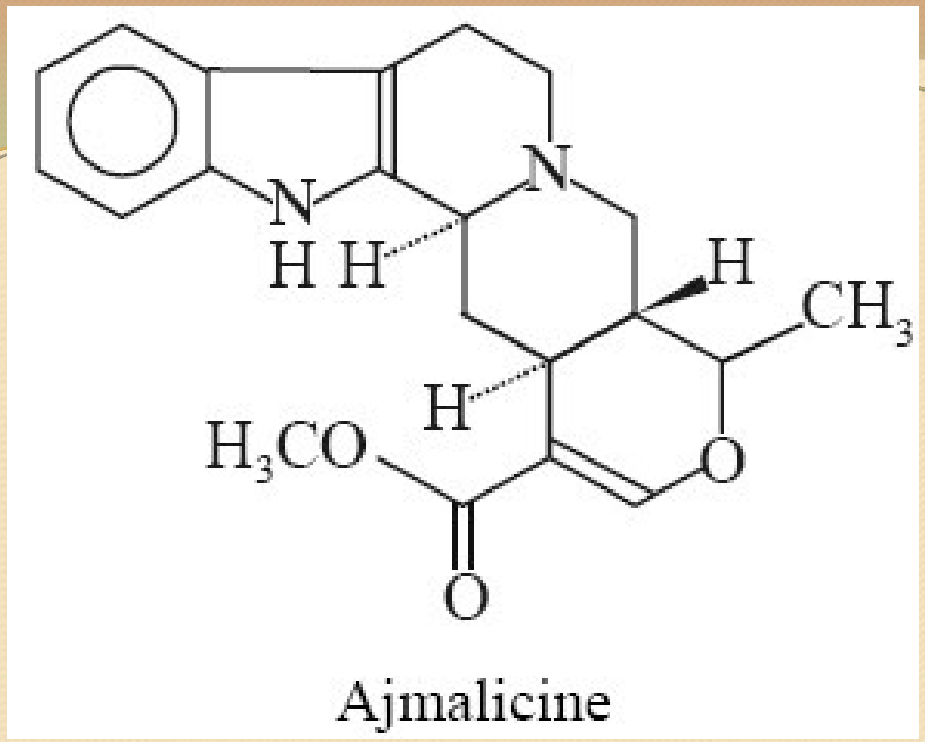
e. Gaseous environment

- The gaseous environment, mainly the availability of oxygen and carbon dioxide, also plays an important role in the production of secondary metabolites by cell cultures.

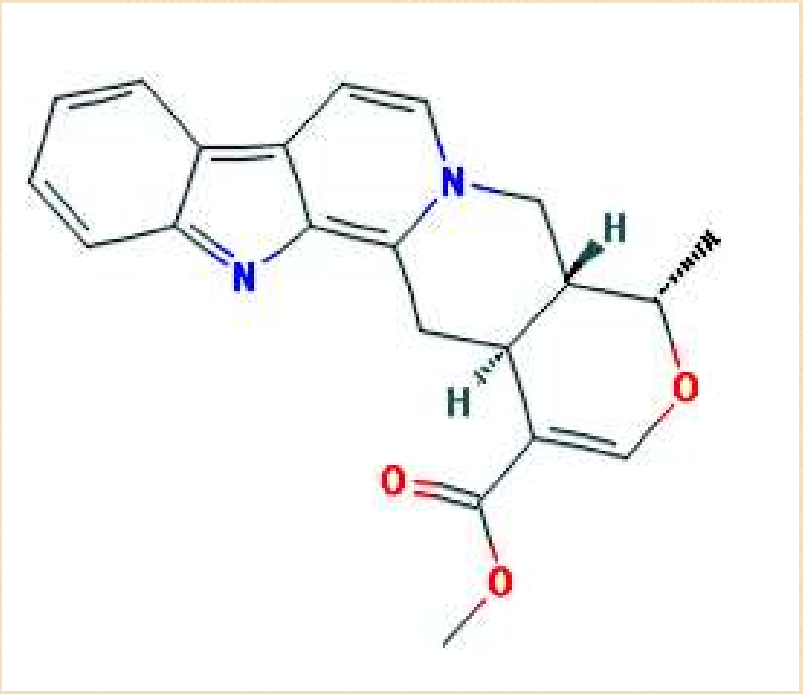
- It was observed that by increasing the initial oxygen mass transfer coefficient in batch cultures of *C. roseus*, it was possible to shorten the time for which cells **accumulate serpentine** without altering the final yield.

- Apparently, the increased availability of dissolved oxygen (DO) stimulated the oxidative metabolism responsible for the conversion of ajmalicine into serpentine.

- The cultures of ***C. roseus with limited gas exchange*** **accumulated ethylene and carbon dioxide** and showed inhibition of ajmalicine production.



Ajmalicine



Serpentine

Selection of high yielding lines

- The explants used to initiate tissue cultures are highly heterogeneous with regard to the metabolic productivity of its constituent cells.
- The heterogeneity is expected to increase under culture conditions which are known to induce genetic and epigenetic changes.
- Consequently, cells and cell clusters from a culture exhibit considerable variation for the accumulation of secondary metabolites.

- **The productivity of a heterogeneous culture would be an average of the productivity of its high and low yielding cells.**

- Selection and cloning of high yielding cells from such cultures is, therefore, regarded as an effective method to improve in vitro production of secondary metabolites.

- It requires, firstly, initiation of cultures from selected high yielding genotypes.

- One should then screen a large number of individual cultures for the best producing variant.

- From established cultures of these lines, selection can be made for better yielding subclones.

- Selection of cell lines of *Papaver* helped improve the yields of sanguinarins considerably.

- Since cultured cells are prone to spontaneous genetic changes it would be necessary to make periodic selections to maintain high productivity of the cultures.

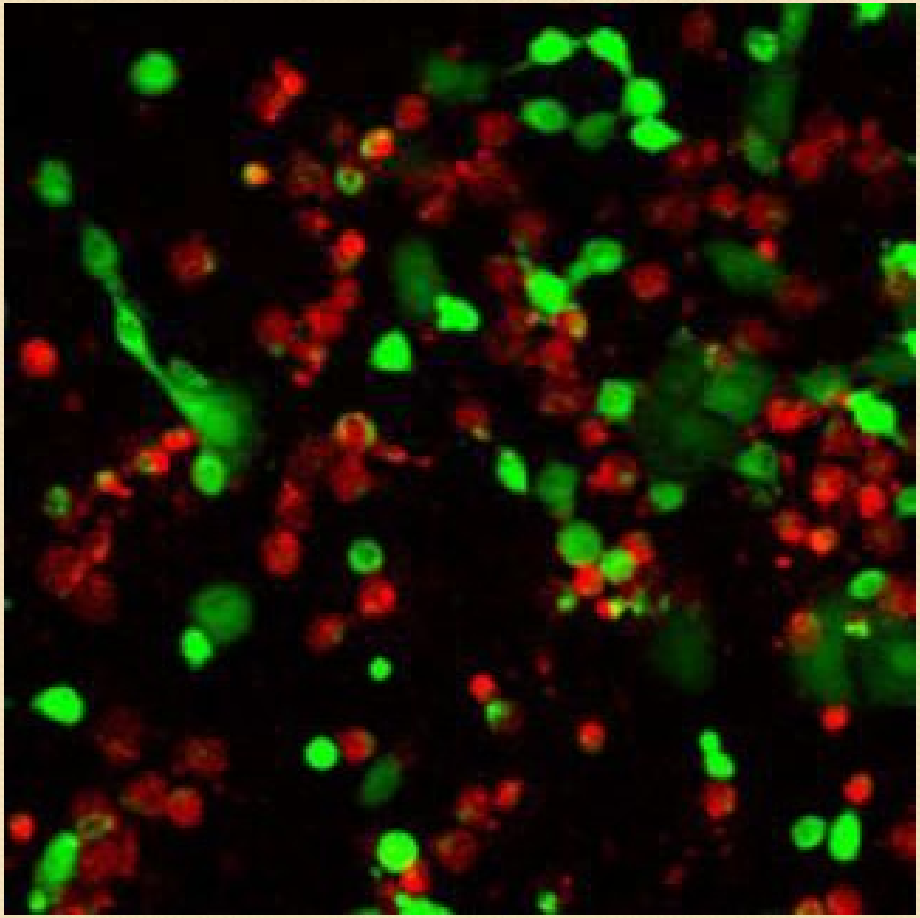
- Selections should be made under conditions which are suitable for product formation.

- Screening for high yielding lines for coloured compounds, such as **shikonin (red), berberine (yellow)** and **betanin (red)** is very simple.

- The most coloured areas of cell clumps can be easily isolated and cultured separately.

- Cells with highly fluorescent compounds may be detected under UV light by naked eye or fluorescence microscope.

- Flow cytometry enables rapid analysis and subsequent sorting of a large amount of cells on the basis of their fluorescence. However, it requires single cells.



- For colourless compounds, specific reactions of squashed cells or immunological tests of extracts may help to find the best clones.
- It is important that the selected lines are reasonably stable.
- Since metabolite synthesis by plant cells is highly influenced by the physiological state of the cells, the high yield shown by the selected lines may be due to altered gene expression.

- Screening of *C. roseus* cells for high ajmalicine and serpentine production yielded lines with up to 10-fold increased productivity).

- However, the lines were highly unstable and often reverted back to the level of unselected lines

- **For the isolation of stable lines, repeated selections should be made**, and only if the product of a cell line remains stable over several selection cycles, will **a true variant** be identified.

- In the case of *Euphorbia millii and sweet-potato*, about 30 consecutive clonings of cell aggregates with the highest anthocyanin content was made and established stable cell lines that maintained increased anthocyanin content without further selection.

Elicitation

- *When an insect or microbe attacks a plant it triggers a chain of reactions leading to the production of some compounds that can act against the invading organisms and chemical. This process is called **elicitation** and the compounds produced in responds to the chemical or insect/microbial attack are called **phytoalexins.***

- In nature, these chemicals or microbial/insect attacks act as stress conditions to the plants.

- Increased production of secondary metabolites in media supporting poor growth of cells is also regarded as a stress response.

- Consequently, a number of biotic (fungal extracts) and abiotic (inorganic and organic chemicals, UV irradiation) factors have been tested as elicitor and shown to improve the production of secondary metabolites in plant cell and organ cultures.

- **Elicitor-induced products are frequently released into the medium.**

- The addition of **conidia of *Verticillium dahliae*** to cell cultures of *Gossypium arboreum* increased the yield of **gossypol** within 5 days.

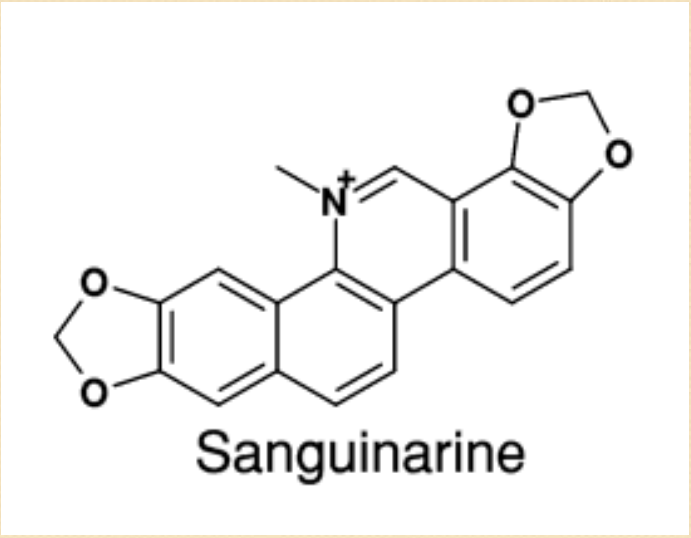
- Fungal elicitors may inhibit the growth of plant cells. The amount of *Verticillium dahliae* required to stimulate metabolite synthesis by suspension cultures of ***G. hirsutum*** could be reduced by adding **oxalate to the medium.**

- Similarly, a 26-fold increase of **sanguinarine** (2.9% of dry mass) was noted after addition of the homogenate of an isolate of ***Botrytis*** *species*.

- Kurz et al. (1990) used **solubilized chitin** to elicit sanguinarine production by cell cultures of *Papaver somniferum*.

- Several cell lines of *C. roseus* respond to elicitation by biotic and abiotic agents.

Sanguinaria canadensis (bloodroot plant)



- A combination of the fungal elicitor and oxalate did not reduce the cell mass and, therefore, secondary metabolite synthesis was increased up to 10-fold.

- It was reported that a combination of phosphate limitation with elicitation by *Rhizoctonia solani* synergistically increased the production of sesquiterpene solativone by *Agrobacterium rhizogenes-transformed* hairy-root cultures of ***Hyoscyamus muticus.***

- *The increase was substantially* higher than that with phosphate limitation or fungal elicitation alone.

Hyoscyamus muticus

- *Hyoscyamus muticus*, is a shrub in the family of Solanaceae that is native to desert areas of North Africa. It contains alkaloids that are useful in pharmaceuticals. It is used locally as a painkiller and a recreational drug.

- Simple organic and inorganic molecules can also induce product accumulation in cultured cells. (Smith *et al.)*
- Enhanced accumulation of catharanthine in the cells of *C. roseus* in response to **NaCl, KCl** and **sorbitol** individually.
- Addition of **vanadyl sulphate** to cell suspension cultures of *C. roseus* resulted in the production of catharanthine, serpentine and tryptamine.
- The effect was concentration dependent. At a lower concentration catharanthine and ajmalicine accumulated while at higher concentrations tryptamine accumulation occurred.

- The production of dimeric alkaloids by shoot cultures of *C. roseus* *can* be induced by irradiation with near-ultraviolet light.

- **The time of application of elicitor is critical for the yield of secondary metabolites by cultured cells**.

- Most of the cultures respond to an elicitor only during the growth phase.

- For the production of sanguinarine by *P. somniferum* cell cultures, elicitation with the homogenate of ***Botrytis*** *or* **solubilized chitin** was optimum when applied 5.8 days after culture initiation.

- Elicitation at days 7 and 8.5 was 3 and 8 times less effective, respectively.

Elicitation

P. somniferum ⇧ *5.8 (6) days* 7 and 8.5 days ⇩

- Maximum increase in the production of benzophenanthridine alkaloids in suspension cultures of *Eschscholzia californica* *occurred when treated with an elicitor from* ***yeast extract*** *on* the 6th day after culture which corresponds to the exponential phase of growth.

- The time of elicitor application may affect not only the quantity of the product but also the production pattern.

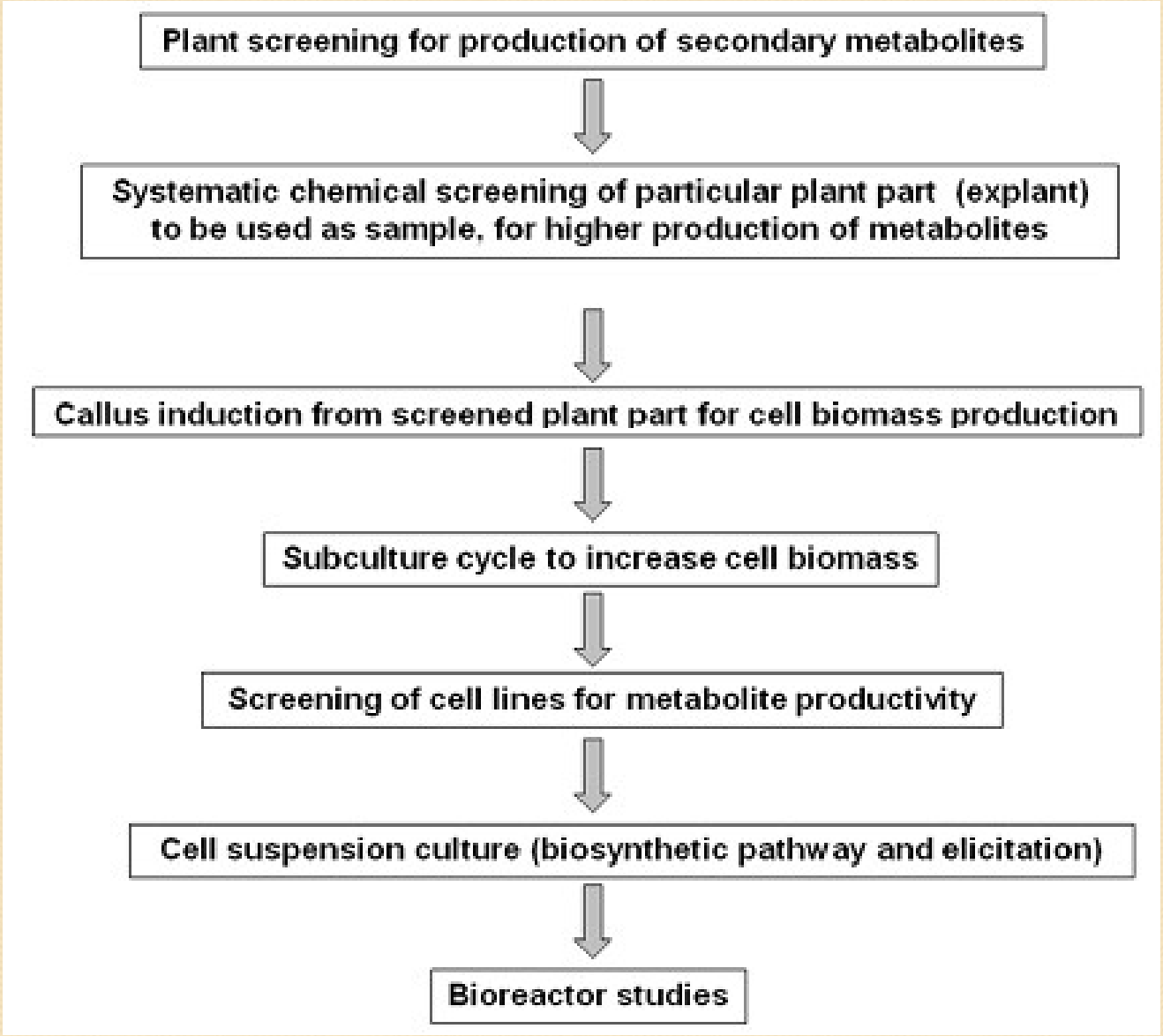
California poppy

- *Eschscholzia californica* is a species of flowering plant in the Papaveraceae family, native to the United States and Mexico.

- In 5-day-old cultures of *C. roseus* *the homogenate of* ***Pythium cultures*** stimulated N-acetyl tryptamine formation.
- In 10-day-old cultures, it induced the accumulation of a whole spectrum of monoterpene indole alkaloids.

- Elicitor treatment after a culture has already started to accumulate the inducible compound does not enhance or accelerate its production.

Stages in mass culture of plant cells in bioreactor



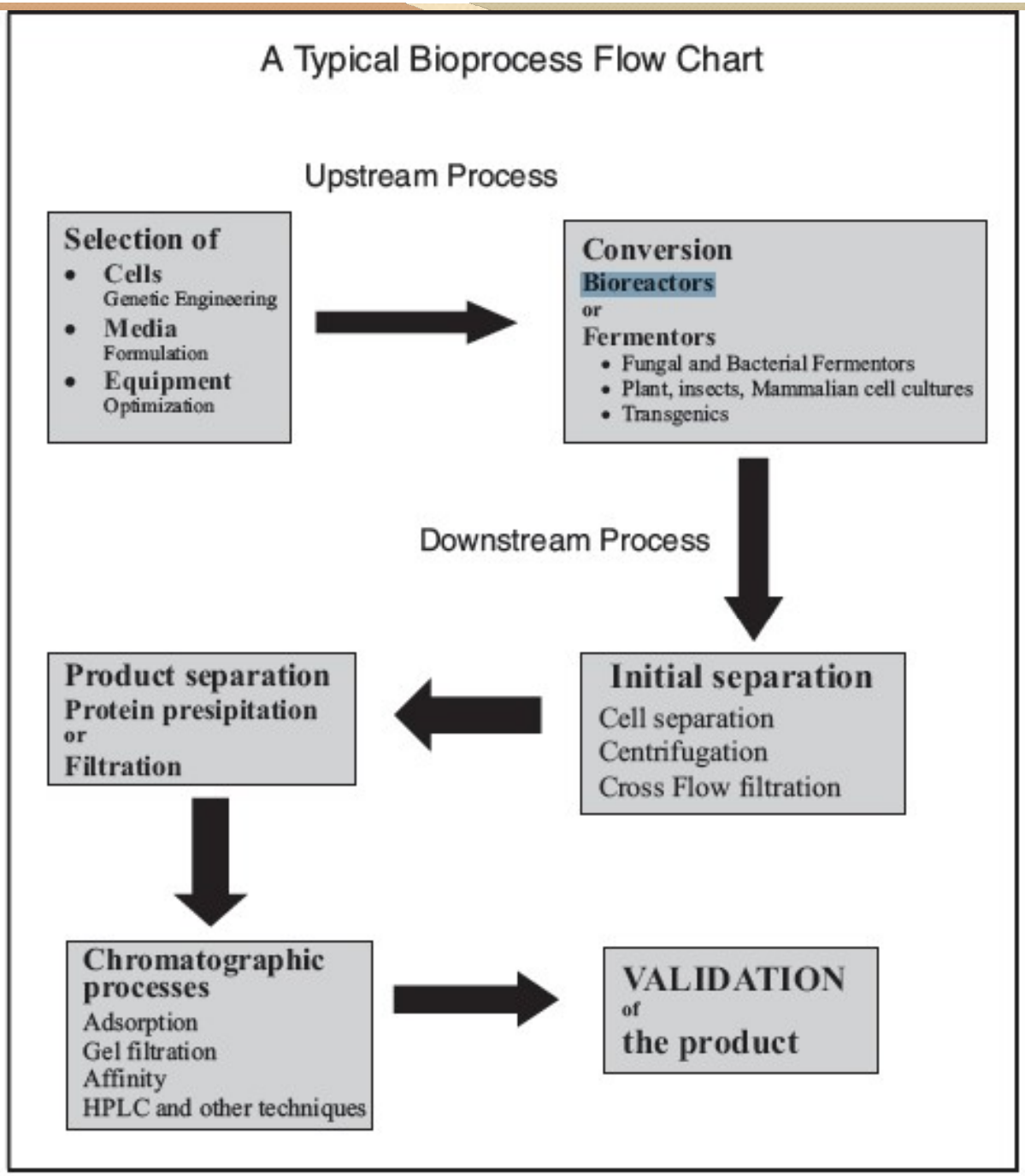


FIGURE 1.1 **An overview of modern bioprocessing.**


Thank you!