In Vitro Production of Secondary Metabolites

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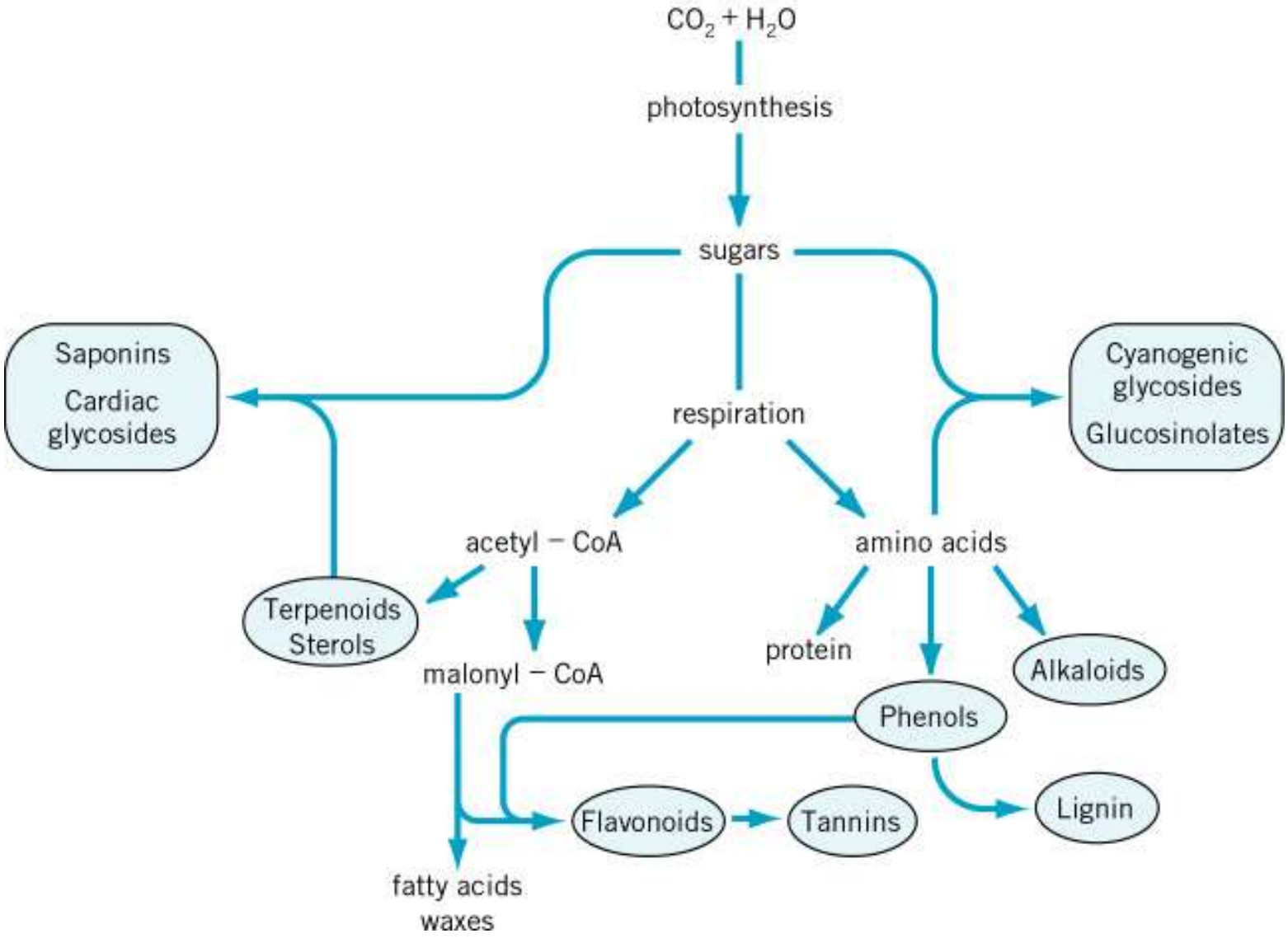
Metabolites

- Primary metabolites: Molecules that are essential for growth and development of an organism.

- Secondary metabolites: molecules that are not essential for growth and development of an organism.

Secondary metabolites are derived from primary metabolites



Why secondary metabolites?

- Chemical warfare to protect plants from the attacks by predators, pathogens, or competitors
- Attract pollinators or seed dispersal agents
- Important for abiotic stresses
- Medicine
- Industrial additives

Secondary metabolites

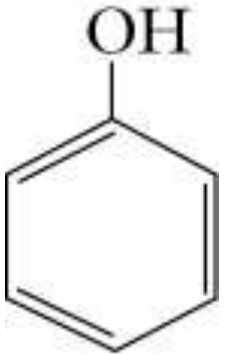
- Possibly over 250,000 secondary metabolites in plants
- Classified based on common biosynthetic pathways where a chemical is derived.
- Four major classes:

  Alkaloids, glycosides, phenolics, terpenoids

Alkaloids

- Most are derived from a few common amino acids (i.e., tyrosine, tryptophan, ornithine or argenine, and lysine)
- Compounds have a ring structure and a nitrogen residue.
- Indole alkaloids is the largest group in this family, derived from tryptophan
- Widely used as medicine

Phenolics



Phenol

- Derived from aromatic amino acids, such as phenylalanine, tyrosin, and trytophan.
- All contain structures derived from phenol
- Some examples:
  Coumarins: antimicrobial agents, feeding deterrents, and germination inhibitors.

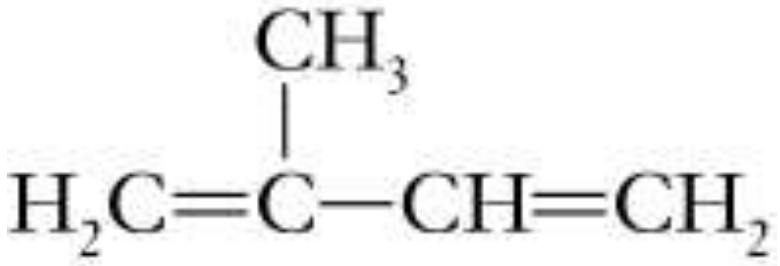
  Lignin: abundant in secondary cell wall, rigid and resistant to extraction or many degradation reagents.

Glycosides

- Compounds that contain a carbonhydrate and a noncarbohydrate
- Glucosinolates: found primarily in the mustard family to give the pungent taste.

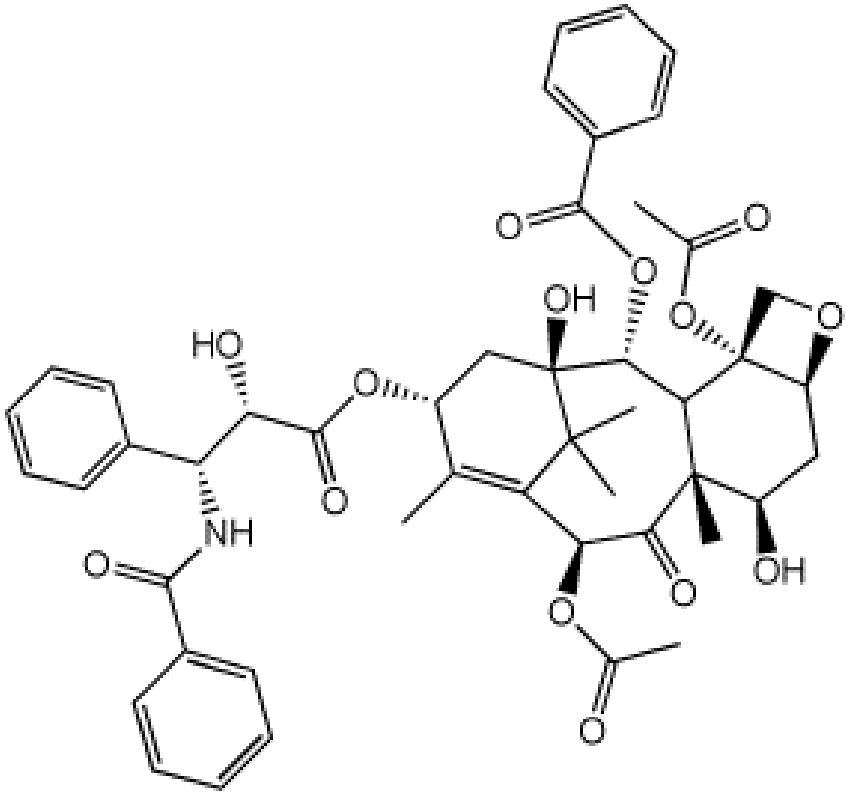
Terpenoids

- Terpenes are generally polymers of 5-carbon unit called isoprene
- Give scent, flavors, colors, medicine…
- Three plant hormones are derived from the terpenoid pathway.



Isoprene

Taxol



- Taxol is a terpenoid
- "the best anti-cancer agent" by National Cancer Institute
- Has remarkable activity against advanced ovarian and breast cancer, and has been approved for clinical use.

How can we make more usual plant secondary metabolites?

- Plant more trees, extract directly from plants
- Genetic engineering
  - Increase the yield
  - Introduce the biosynthetic pathway to other plants that normally does not produce certain compound
- **Tissue culture to produce a compound**
- Chemical synthesis
- Semi-chemical synthesis

Culture Conditions

- Environment – Culture Medium : Simple nutrient medium – MS, B5, - Mineral Salts Macro and Micro.
- Carbon Source – Sucrose, glucose, fructose, maltose.
- GRs- Auxins, Cytokinins, GA_3
- Amino acids- glycine, glutamine, proline, phenyl, alanine, arginine
- Vitamins – nicotinic acid, Pyriodoxine Hcl, thiamine Hcl, biotin, folic acid, cyanocobalamine
- Organic supplements: Yeast extract, malt extract, casein hydrolysase, coconut water.
- Gelling agents : Agar – Agar

- Temp- of incubation 22 -28 ° C
- Illumination – 0- 5000 lux, photoperiod – 8-16 h
- Subculture of tissues : 2-8 weeks for Static cultures & 1-2 weeks for cell suspensions.

Culture initiation

- ✓ Explant – callus induction
- ✓ Growth & Secondary Metabolite Production
- ✓ Growth curve – sigmoid

- Lag –no growth cells adjust to the new media after subculture

- Stationary - primary metabolism & cell proliferation come to a halt as nutrients in the medium are exhausted. Dry weight may decline as cells utilize stored reserve material.

- Exponential – primary metabolism increases & tissue proliferates rapidly with the consumption of medium nutrients

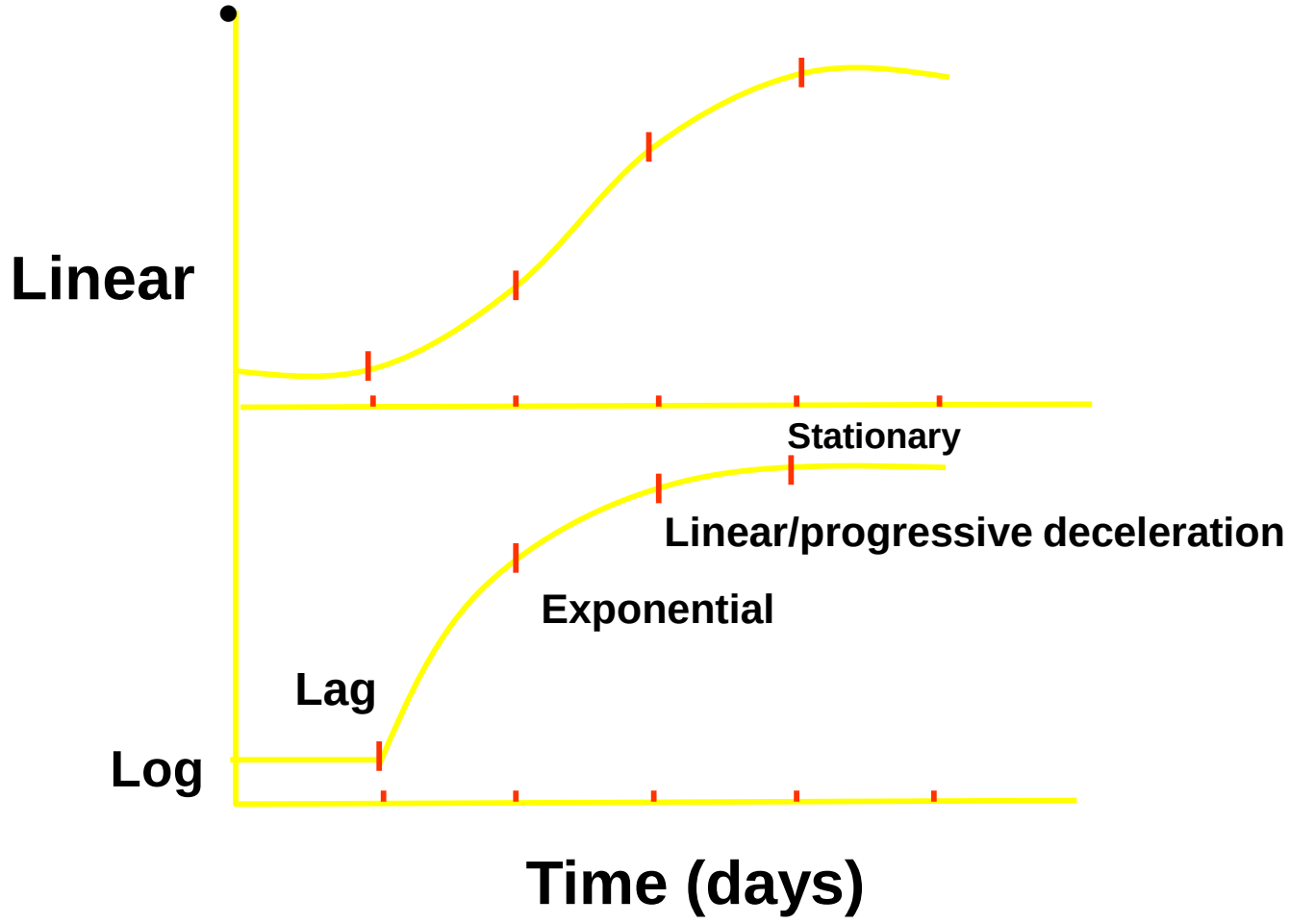
Growth and Metabolism of Plant in *In Vitro*

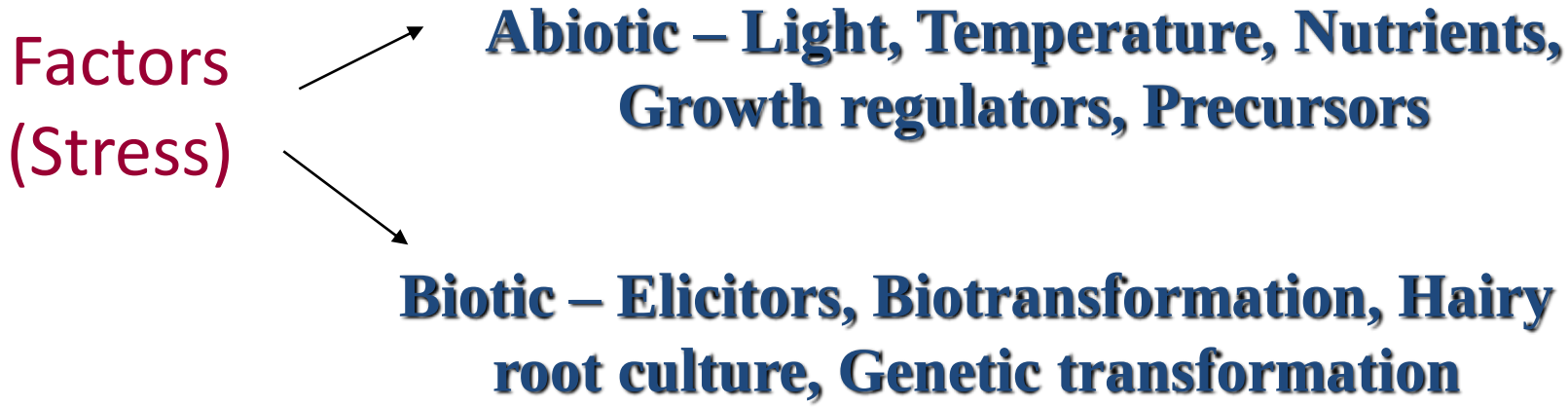
(i) Batch / Solid Culture

1. Lag phase
2. Log / Exponential Phase
3. Stationary Phase
4. Declining Phase

(ii) Continuous cultures

Batch Culture Growth Cycle (Passage)




Factors
(Stress)
Abiotic – Light, Temperature, Nutrients,
Growth regulators, Precursors
Biotic – Elicitors, Biotransformation, Hairy
root culture, Genetic transformation

Temperature

- **Effect on enzymes 25 - 28° C**

- **pH – 5-6 effect on anthocyanin, anthraquinones, alkaloids, anthocynanin degrades at higher pH.**

- **Aeration – in liquid shake flasks or in bioreactors – secondary metabolite production**

- **Anaerobic – direct effect on primary metabolism & consequently production of secondary metabolites or direct effect on secondary metabolites. Anaerobic - ethanol production**

Nutrients

- **Carbon – 2-3 %**

- **Nature & Concentration of ‘C’**

- **Sucrose is better than all other carbohydrate sources for growth and concentrations >3% often enhance the biosynthesis of phytochemicals by heterotrophic plant cells.**

- **Interactions of carbohydrates & nitrogen in important in cell dry weight, fresh weight, protein content and cell proliferation.**

- Maximum amount of mineral nitrogen assimilated by cultured cells is used for the biosynthesis of a – acids, proteins (enzymes also) & nucleic acids.

- High NH_4 + - inhibitors to secondary metabolites

- Low or nil – increase to secondary metabolites.

- Phosphate

- Increased phosphate – alkaloid, anthraquinone

- Inorganic phosphate - photosynthesis, respiration, phospholipids synthesis.

Light

- **Blue Light – anthocyanin in *Haplopappus gracilis***
- **White Light – anthocyanin in *Catharanthus roseus,* Populus sps.,**
- **Napthoquinone inhibited – *Lithospermum erythrorhizon* in blue or white light**
- **Anthocyanin – *Daucus carota* – in white light**

Helianthus tuberosum

Linum usitatissimu

Vitis vinifera

- **UV (280-320 nm) – stimulate flavone glycoside synthesis in *Petroselinum hortense* cell suspension cultures.**

- **UV glyceollin in *Glycine max***

  White light

- **Coumarins, alkaloids – *Ruta graveolens***

- **Indole alkaloids – *Catharanthus roseus***

- **Light – direct on development of choloplasts enzymes of photosynthesis, lipid metabolism, phytochrome**

Precursors

Precursors molecules which are directly incorporated into synthesis of secondary metabolites

Ruta graveolens	**4 – OH 2 – Quinoline**		**Dictamine – 0.6% DW**
Chinchona ledgerianer	**Tryptophan**		**Quinoleines – 0.9% DW**
Lithospermum erythrorhizon	**Phenylalanine**	**Shikonin**	**37- 126 µg -1 FW**
Ephedra gerardiana	**Phenylanine**	**Ephedrine**	**0.17 – 0.5 % DW**

Need to optimize the growth and production conditions for each species and strain, and also for each metabolite

Advantage of callus and cell suspension culture

- The production of secondary metabolites from plant cell and tissue culture, which are immediate relevance to the industry.
- Independence from environmental factors.
- The production system is not limited by seasonal consideration.
- The more consistent product quality and yield.
- The product is free from microbes.
- The synthesis of novel natural products, which are not normally produced in normal plants.
- A means of synthesizing novel natural product where the source plant is difficult to grow.

Immobilization

Type of immobilization

Gel entrapment
Biofilms
Adsorption
Foam immobilization
Membrane

Advantages of immobilization

1. Facilitate the use of continuous flow process.
2. Facilitate sequential chemical treatment of the cells or organelles and harvest of metabolites from the medium.
3. The biocatalyst can be reused and can be easily separated from the reaction medium.
4. Control aggregation of cells.
5. The cell/ cell contact induced by immobilization can be beneficial for differentiation process and for secondary metabolites production. Since differentiation process is a prerequisite for the production of secondary plant products.
6. Immobilization protects the sensitive plant cells against shear forces.
7. Immobilized cells can be used for much longer periods than free cells.

Disadvantages

Some of the problems with immobilized cells are the introduction of gradients in the gel beads, the necessity of product excretion to the use of immobilized cells and the fact that some immobilization materials can affect cell viability in a negative way. Besides immobilization introduces an extra cost factor.

Plant species with immobilized cells employed for the production secondary Metabolite (s)

Plant culture species	Immobilization method	Substrate	Product
Catharanthus roseus	Entrapment in agarose	Cathenamine	Ajmalicine
Digitalis lanata	Entrapment in alginate	Digitoxin	Digoxin
Capsicum frutescens	Entrapment in polyurethane foam	Sucrose	Capsaicin
Catharanthus roseus	Entrapment in alginate, agarose, carrageenin	Sucrose	Ajmalicine
Petunia hybrida	Entrapment in hollow fibres	Sucrose	Phenolics
Morinda citrifolia	Entrapment in alginate	Sucrose	Anthraquinone
Solanum aviculare	Attachment polyphenylene beads	Sucrose	Steroid gylcosides
Glycine max	Entrapment in hollow fibres	Sucrose	Phenolics

Elicitors

- **Keen – Coworkers (1972) – elicitation response.**

- **Fungal cultures – fresh cultures homogenized, autoclaved at 121°C for 20 min, and suitably diluted fungal preparations or chemicals are used to evaluate the elicitation effect.**

- **Eg. *Pythium, Fusarium, Phytopthora, Alternaria, Penicillium* etc.**

Elicitor-induced secondary metabolites production in plant cell culture

Elicitor microorganism	Plant cell culture (s)	Secondary metabolite (s)
Aspergillus niger	*Cinchona ledgeriana, Rubia tinctoria*	Anthraquinones
Pythium aphanidermatum	*Catharanthus roseus*	Ajmalicine, Striclosidine, Catharanthine
Botrytis sp	*Papaver somniferum*	Sanguinarine
Phytophthora megasperma	*Glycine max*	Isoflavonoids, Gluceollin
Dendryphion sp	*Papaver sominiferum*	Sanguinarine
Alternaria sp	*Phaseolus vulgaris*	Phaseollin
Fusarium sp	*Apium graveolens*	Furanocomarins
Phythium aphanidermatum	*Daucus carota*	Anthocynins
Penicillium expansum	*Sanguinaria canadensis*	Benzophenanthridine Alkaloids

Biotransformations

- Biotransformations is of two types

(i) Transformation of low cost precursors into valuable product or conservation of racemic /inactive compounds into active forms

Eg. Conversion of D- menthol to L – menthol

(ii) Transformation with the help of Agrobacterium

Biotransformation by plant cell culture

Plant cell culture	Substrate	Product
Digitalis lanata	Digitoxin	Digoxin
Papaver somniferum	Codeinone	Codeine
Nicotiana tobacum	Carvoxine	Cavaxone
Daucus carota	Digitoxigenin	Periplogenin
Mucuna pruriens	L – Tyrosine	L- Dihydroxy phenylalanine (L-DOPA)
Mentha sp	(-)- Methone	(+) – Neomenthol
Coffea arabica	Vanillin	Vanillin –D – glucoside
Solanum tuberosum	Solavetivone	Hydroxylated derivatives
Galium mollugo	2-Succinyl benzoate	Anthra quinones
Datura sp	Hydroquinone	Arbutin
Citrus sp	Valencene	Nootkatone
Chiisya ternata	Ellipticine	5-Formyl ellipticine
Digitalis purpurea / Stvia rebandiana	Steviol	Steviocide, Steviobiocide

Hairy root – *Agrobacterium rhizogenes*

Eg. *Atropa belladonna* – Atropine

- ***Datura stramonium* – Hyoscamine**
- ***Hyscyamus multicus* – Hyoscamine**
- ***Cathyranthus roseus* – Ajmaline, Serpentine, Catharnthine**
- ***Lithospermum, Erythrorhizon* – Shikonin**
- ***Chinchona ledgeriana***

❑**The formation of transformed roots following infection of plants with *A. rhizogenes* is limited to dicotyledonous species only.**

❑**The use of transformed root cultures for the production of secondary metabolites may also be restricted to species in which the products are synthesized in roots of intact plants.**

Plant species used in hairy root cultures for the production of secondary metabolite (s)

Plant species	Secondary metabolite (s)
Nicotiana tabacum	Nicotine, anatabine
Atropa belladonna	Atropine
Datura stramonium	Hyoscyamine
Lithospermum erythrorhizon	Shikonin
Catharanthus roseus	Ajmalicine, serpentine
Cinchona ledgeriana	Quinine alkaloids
Mentha vulgaris	Monoterpenes
Solanum laciniatum	Steroid alkaloids

Production

Bioreactors

- **Optimization of secondary metabolite production in plant cells.**
- **1984- *Lithospermum erythrorhizon* cells in Japan – 750 bioreactor for shikonin (a dye & chemical compound).**
- **Sanguinarine – *Papaver somniferum* cells. USA**
- **Vanilla flavour USA**
- **Germany Co., Taxol, Taxus cell cultures 75 m^3.**

Growth period – bacteria - < 1hr doubling time
plant cells – 24 – 72 h

Batch cultivation

Phase I - lag period – adaptations to the new environment of the bioreactor

II - exponential growth – growth required nutrients are present in excess (O_2 also)

III - nutrient- limited rate – (C,N,P)

IV Stationary (Sec. metabolites) – growth ceases & the cell density reaches maximum, cells remain metabolically active.

V Cell lysis – generally not in bioreactors

Plant cell process systems is divided into three areas

- Upstream processing

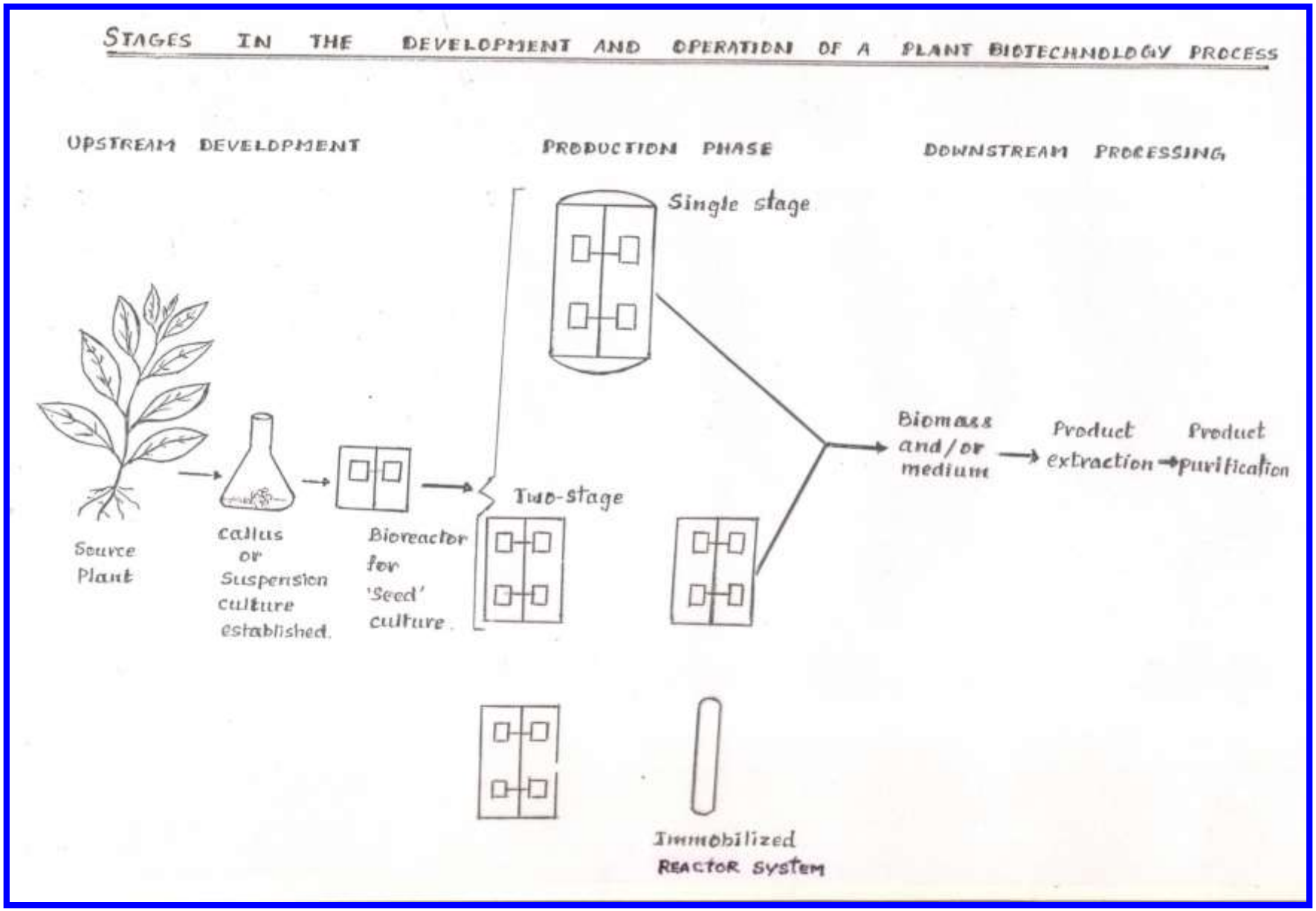
- the production or reactor phase

- downstream processing

First phase – batch/ continuous system

Second phase – batch cultures / immobilized cell technology

Steps of large scale secondary metabolites production



List of some culture secondary products obtained through in vitro culture

Species	Products/Compounds	Culture type
Anethum graveolens	Scopoletin	Root
Atropa belladonna	Hyoscyamine, Scopolamine	Root
Calystegia sepium	Hyoscyamine, Scopolamine	Root
Catharanthus roseus	Ajmalicine, Catharanthine Cephaeline	Root
Cephaelis ipecacuanha	Valepotriates	Root
Centranthus macrosiphon	Valepotriates	Root
Centranthus ruber	Quinine, Quinidine	Root
Cinchona ledgeriana	Hyoscyamine, Scopolamixie	Root

Species	Products/Compounds	Culture type
Citrus paradisi	Naringin	Shoot
Cinchona ledgeriana	Quinine, Quinidine	Shoot
Cinchona succirubra	Cinchonidine	Shoot
Chrysanthemum cinerariaefolium	Pyrethrine, Cinerin	Shoot
Chrysanthemum americanum	O-methylated flavanol glucosides	Shoot
Datura innoxia	Hyoscyamine,Scopolamine	Shoot
Digitalis purpurea	Digitoxin	Shoot
Digitalis lanata	Digitoxin	Shoot
Digitalis lutea	Digitoxin	Shoot
Digitalis mertonensis	Digitoxin	Shoot
Digitalis ferruginea	Digitoxin	Shoot
Digitalis ambigua	Digitoxin	Shoot
Dioscorea composita	Diosgenin	Shoot
Foeniculum vulgare	Anethole	Shoot
Heimia salicifolia	Vertine, Lyfoline	Shoot
Lavadula angustifolia	Vertine, Lyfoline	Shoot
Origanum vulgare	Monoterpenes	Shoot
Papaver bracteatum	Thebaine	Shoot
Papaver somniferum	Thebaine	Shoot
Pelargonium fragrans	Pinene	Shoot
Pelargonium graveolens	Geraniol, citronellol	Shoot
Pelargonium tomentosum	Methone, isomenthone	Shoot

Species	Products/Compounds	Culture type
Atropa belladdona	Artopine	Hairy root
BetaBeta	Betanin	Hairy root
Bidens	Thipenes	Hairy root
Catharantthus roseus	Ajrnalicine,Catharanthine	Hairy root
Cinchona ledgeriana	Quinine, Quinidine	Hairy root
Coreopsis	Thiophenes	Hairy root
Datura stramonium	Hyoscyamin, Scopolamine	Hairy root
Dubosia hopwoodii	Hyoscyamin, Scopolamine	Hairy root
Digitalis lanata	Digoxin	Liquid culture
Digitalis purpurea	Digitoxin	Liquid culture
Dioscorea deltoidea	Diosgenin	Suspension
Papaver somniferum	Codeine	Suspension
Papaver somniferum	Morphine	Suspension
Rauwolfia serpentina	Reserpine	Suspension
Matricaria chamomilla	Sesquiterpenes	Suspension
Pimpinella anisum	Anethole	Suspension
Coleus blumei	Rosemarinic acid	Suspension
Catharanthus roseus	Serpentine	Suspension
Catharanthus roseus	Ajmalicine	Suspension
Nicotiana tabacum	Glutathione	Suspension

Species	Products/Compounds	Culture type
Papaver bracteatum	Thebaine	Callus
P. somniferum	Thebaine	Callus
Mentha piperita	Geraniol,linolol	Callus
Mentha piperita	Menthone, menthol	Callus
Matricaria chamomilla	Sesquiterpenes, a-bisabolol	Callus
Pelargonium spp.	Monoterpenes	Callus
Cassia tora	Anthraquinones	Callus
Panax ginseng	Ginsenosides	Callus
Lithospennum erythrorhizon	Shikonin	Callus

Sources: Charlwood et al. (1990), Petersen (1993) Starford (1991), Rhodes et al 1990)