Module 2. PRODUCTION OF SECONDARY METABOLITES & ANTIBIOTIC TECHNOLOGY:

Syllabus

A. Secondary metabolite production- strategies for optimizing product yield,

1. culture conditions,
2. selection of high yielding lines,
3. elicitation,
4. immobilization of cultures,
5. hairy root culture and biotransformation.

B . Factors affecting secondary metabolites,

C. Industrial application of secondary metabolites.

D. Hybridoma technology for monoclonal antibody production. Applications of custom made monoclonal antibodies.

E. Bioreactors considerations for animal cell cultures – Production of Monoclonal antibodies and therapeutic proteins.

C**ourse outcome 1**: Describe the factors affecting secondary metabolite production and its industrial importance.

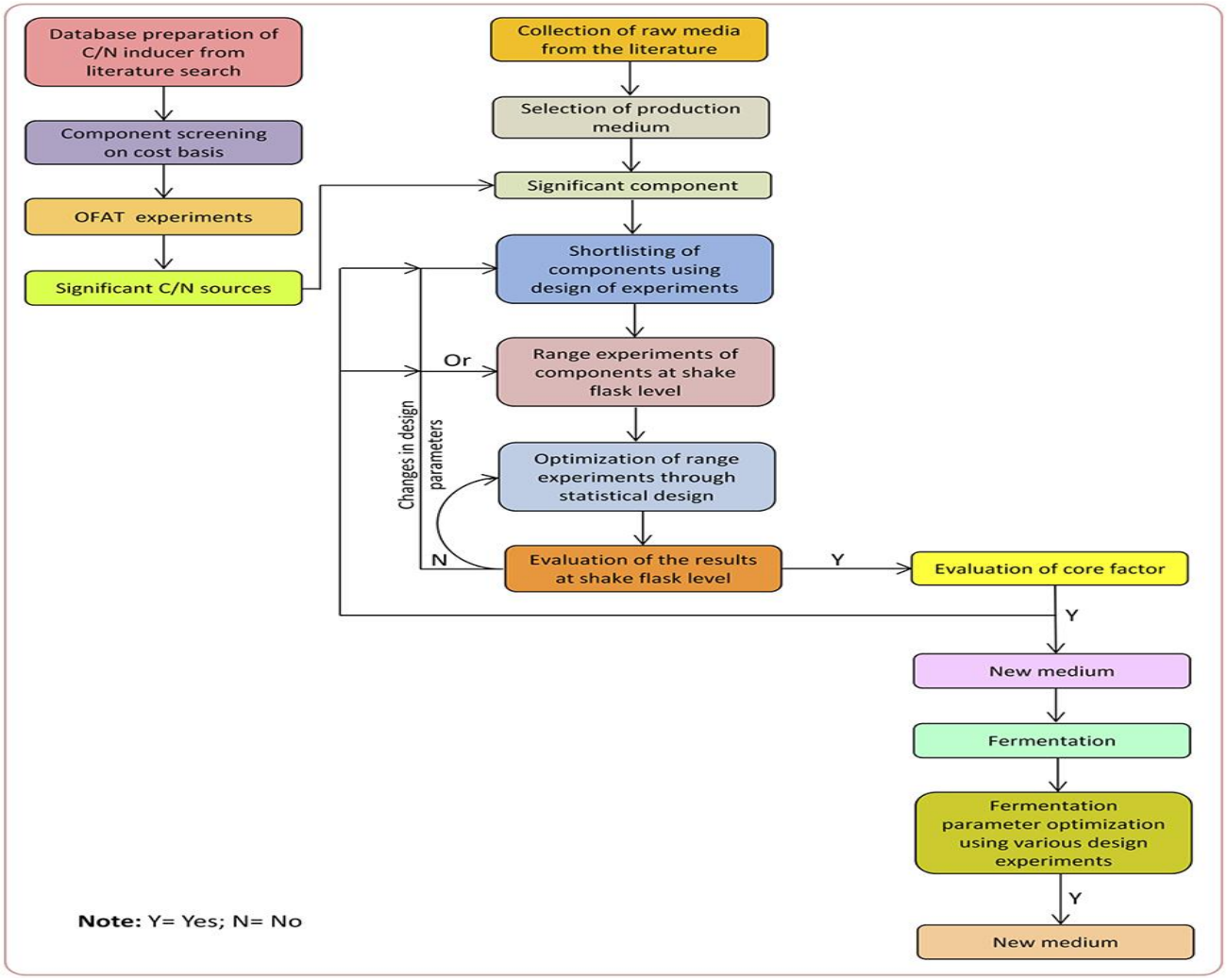
Course outcome 5: Analyzing both analytical and process validation issues that are critical to successful manufacturing

Chapter 1. Secondary metabolite production-strategies for optimizing product yield, culture conditions, selection of high yielding lines, elicitation, immobilization of cultures, hairy root culture and biotransformation.

Secondary metabolite production

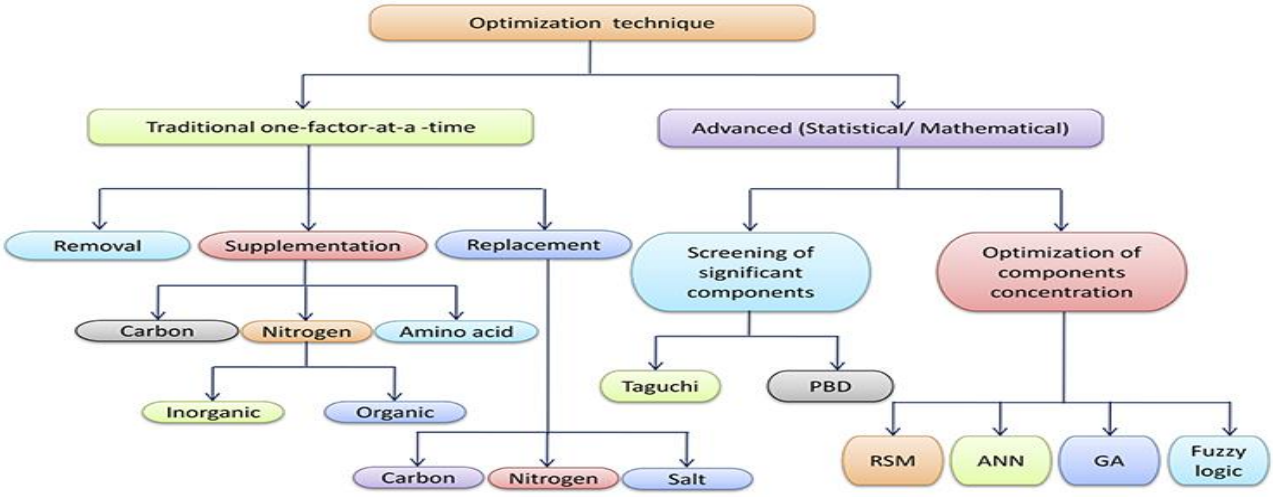
1. **Strategies for optimizing product yield,**
2. **Culture conditions,**
3. **Selection of high yielding lines,**
4. **Elicitation,**
5. **Immobilization of cultures,**
6. **Hairy root culture and biotransformation.**

1. Strategies for optimizing product yield,



2. Culture conditions

During the medium designing and optimization, there are various strategies available which are frequently used to improve the efficiency of the production medium.



Classical Medium Optimization Methods

One-Factor-at-a-Time (OFAT): In the classical medium optimization technique, one-factor-at-a-time (OFAT) experiments, only one factor or variable is varied at a time while keeping other variables constant.

Removal experiments : In this type of experiment, all the medium components are removed from the production medium one-by-one, and after proper incubation period, their effects on the production of secondary metabolite or the product of interest is observed in terms of suitable parameters.

Supplementation experiments: Supplementation experiments are generally performed to evaluate the effects of various carbon and nitrogen supplements on metabolite production.

Replacement experiments: For medium formulation, carbon/nitrogen sources showing enhancement effect on the desired metabolite production in supplementation experiments are generally tried to be used as a whole carbon/nitrogen source.

Physical parameters: In addition to chemical and biological variables, several researchers used OFAT experiments to standardize the physical parameters such as pH, temperature, agitation and aeration requirements of the fermentation process

Nutritional Control of Metabolite Production

3. SELECTION OF CELL LINES FOR HIGH YIELD:

- Separation **of producer cells from the non- producer ones**.

- The physiological characteristics of individual plant cells are not always uniform
- Therefore a rapid assay method is crucial in the selection of a high yielding cell line
- The specific cell line is obtained from the selection a number of strains producing high level of desirable product
- The strains then were subjected to further cell cloning to increase the level of secondary metabolites

3) Strain improvement with programs analogous to those used for microbial systems

- The physiological characteristics of individual plant cells are not always uniform
- In tissue culture, selection of cell lines for specific compound production will increase in yield.
- Selection using radioimmunoassay. Similar method to monocolony isolation of bacteria
- radioimmunoassay (RIA), highly sensitive laboratory technique used to measure minute amounts of substances including antigens, hormones, and chemicals present in plant cells

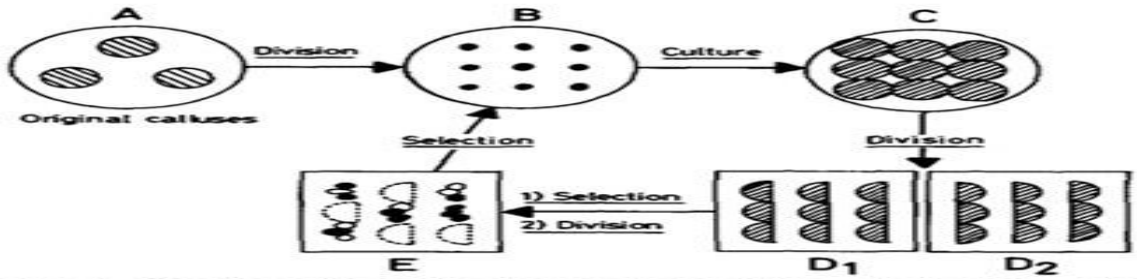


Fig. 1. Outline of the selection method. *A* The original *E. millii* calluses were divided into 128 segments, and a segment was placed on agar-medium in one section of a 9-section Petri dish and coded. *B* Segments were cultured at 28 °C under light (6000 lux) for 10 days. *C* Each grown segment was then divided into two cell-aggregates; one (D_1) for subculture and the other (D_2) for quantitative analysis of the pigment. D_1 The reddest of the nine aggregates were removed and placed in an empty Petri dish (*E*). *E* Each of these red aggregates was divided into several segments, of which the reddest pieces were removed, then coded and placed on agar-medium (*B*). ○ = unselected segment; ● = selected segment; ▫ = unselected aggregate; ⊘ = grown aggregate

VTU: Write Short notes on Elicitation

4. ELICITATION

- ENDOGENOUS EXOGENOUS ABIOTICBIOTIC
- Elicitors are the compounds of biological origin which stimulate the production of secondary metabolites, and the phenomenon is called ELICITATION.
- All elicitors of biological origin
- Physical agent: heat, cold, UV, osmotic pressure
- Chemical agent: antibiotics, fungicide, etc..
- Produced by microbes. Eg: chitin, chitosan, glucans.
- Within plant cell: pectin, pectic acid, cellulose, etc METHODOLOGY FOR ELICITATION
- a) Selection of microorganisms
- b) Co-culture
- Effect of light
- Effect of incubation temp
- Effect of pH Aeration of culture

Elicitors that have been used in plant cell cultures are yeast extract, chitosan, inorganic and organic molecules and many more

♣ Plants grow under stress condition also show elicitation effects. Phosphate limitation in hairy root cultures of Hyoscyamus muticus had increased production of the sesquiterpene solavetivone

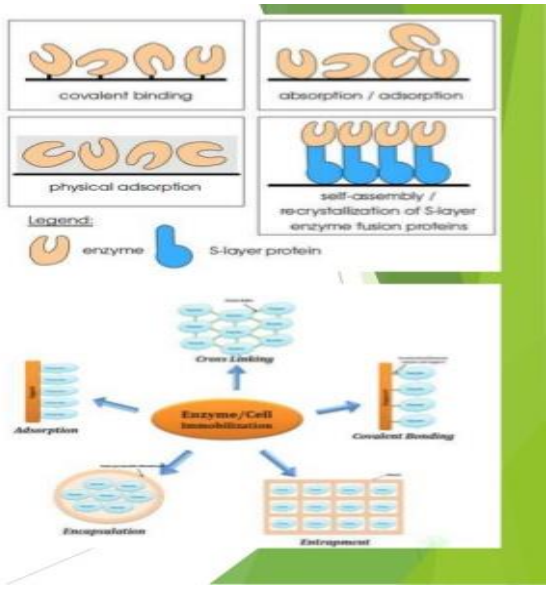
♣ Examples of inorganic compounds used are sodium chloride, potassium chloride, sorbitol and abscisic acid

♣ For economical use of the elicitors, they should be cheap and easy to obtain.

VTU: Write Short notes on immobilization of culture

5. Immobilization of cultures,

- Similarly Cells can be immobilized in cell culture.
- The cells can be immobilised by
  - covalently attached to the base
  - Adsorbed or absorbed
  - Cross-linked
  - Encapsulate
  - Assembled to a protein
  - Entrapped



The two basic cell immobilization techniques include

- Immurement Culture/Encapsulation
- Entrapment
- Immurement Culture/Encapsulation
  - Cells are encapsulated in a polymeric matrix by adsorption.
  - Materials used in matrix: gelatin, polylysine, alginate(most common), Agarose.
  - Medium diffuses freely into the matrix and into the cells, while product moves out into the medium.
  - For larger molecule production like MAB, Agarose in suspension of paraffin oil is preferable to alginate since alginate does not allow diffusion of products out of alginate beads.
  - Uses
    - Encapsulation protects cells from mechanical damage in large fermenters.
    - Production of hormones, antibodies, immunochemical and enzymes over much longer periods than possible in suspension culture.
    - Helps in MAB production.

VTU: 1. Write a note on Hair root culture and biotransformation

6. Hairy root culture and biotransformation.

× Definition:

- It is the culture produced after the infection of explants or cultures by the gram negative soil bacterium *Agrobacterium rhizogenes*.
- This processes take advantage of the naturally occurring hairy root disease in Dicotyledons.

PROPERTIES OF HAIRY ROOTS

- Genotype and phenotype stability
- Autotrophy in plant hormones
- Fast growth
- High levels of secondary metabolite production

PRODUCTION OF HAIRY ROOTS *IN VIVO*:

a) *Agrobacterium* recognizes some signal molecules exuded by wounded plant cells and becomes attached to it.

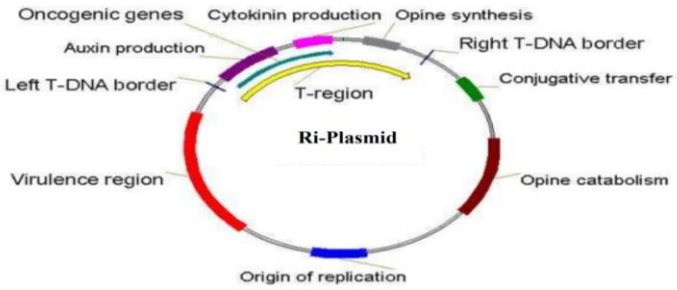
b) The bacteria contain the Root inducing plasmid (Ri-plasmid)

c) The bacteria genetically transfer part of the Ri-plasmid called the transfer DNA (T-DNA) to the plant genome, where it gets expressed and make the plant cell to:

PRODUCTION OF HAIRY ROOTS *IN VIVO*:

1. Proliferate by increasing the rate of cell division (cytokine expression) and cell elongation (auxin expression) to produce the hairy roots.
2. Produce the opines which is a type of unusual amino acids (octopine, agropine,nopaline, mannopine, and cucumopine) which is used by the bacterium as a carbon, nitrogen and energy source.

- More than 450 species of many different genera and families of higher plants are known to be susceptible to the infection by *Agrobacterium rhizogenes*.
- *A. rhizogenes* is a gram negative, non-sporing, motile, rod shaped bacterium, closely related to rhizobium which, produce nitrogen fixing nodules on leguminous plants.
- The ability of root induction by *A. rhizogenes* lies in its typical genome structure, the molecular biology of which has not been properly understood till date.
- Virulent strains of *A. rhizogenes*, contains a **large mega plasmid (more than 200 kb) which plays a key role in hairy root formation**.
- Most of the **genes involved in hairy root formation** are not borne on the chromosome, but on this root inducing (Ri) Plasmid.



- This plasmid carries three genetic components,

  -first is a **mobile DNA element**, **t-DNA** which is **integrated into the nucleus of infected cells** where it is subsequently **stable and integrated** into the host genome and **transcribed** causing the formation of **proliferative multi-branched adventitious roots at the site of infection**; called as hairy root diseases in dicotyledonous plants. The **transferred DNA (t-DNA)** located on the Ri plasmids are approximately 10 to 30 kbp in size, generally represent less than 10% of the Ri plasmid.

  -**The second on**e is the **virulence area (vir),** which contains several **vir genes** which do not enter the plant cell but, **together with the chromosomal DNA, cause the transfer of t-DNA.**

  - **The third one** is, so-called border sequences (25 bp), resides in the *Agrobacterium chromosome*.

  Formation of Hairy Root Culture:

  The t-DNA of the **agropine-type Ri-plasmid** consists of **two separate t-DNA region tl-DNA and tr- DNA**.

  -The **genes encoding auxin synthesis (tmsl and tms2)** and

  The **genes encoding** agr**opine synthesis (ags)** have been localized on the **tr-DNA** of the agropine type of Ri-plasmid.

  ti-DNA regions of t-DNA consists of **4 genetic loci, rolA, rolB, rolC, and rolD**, which affect hairy root induction.

  Gene Transfer Mechanism from Agro-bacterium Rhizogenes to Plant Genome:

  steps of transfer of DNA from Agrobacterium to plant cell.

1. The vir gene expression,
2. generation of t-DNA copy,
3. formation of T strand protein complex,
4. movement of the T-complex through the bacterial membranes,
5. targeting of the T complex into and within the plant cell,
6. targeting of the T complex into the cell nucleus, it's stabilization, and
7. finally integration of T strand into cell DNA are seven successive

   Step 1:

   Bacterial colonization on the wounding site of plant tissue is prerequisite for transformation. The production of phenolic compounds at the wounding site is sensed by one of the **Vir A gene product** which initiates induction of expression of remaining **Vir loci.**

   Step 2:

The product **of Vir C and Vir D** play pivotal role in this step. Two Vir D specific product Vir D_1 and Vir D_2 are essentially required for synthesis of t-DNA strand. The Vir C locus decodes for two polypeptides Vir Q and Vir C_2 that are shown to enhance t-DNA border nicking.

Step 3:

The t-DNA strand is likely to exist as a DNA protein complex. The Vir E, specially Vir E_2 protein is the most abundant protein synthesized in Vir induced Agrobacterium cells. The Vir D_2 bounds to the leading end of the T- complex. Thus T-complex is compressed of the t-DNA strand, Vir D_2 and Vir E_2.

Step 4:

The product of Vir B locus **produces trans membrane channel outside** the bacterial cell wall because of its 11 open reading frame known as Vir B_1 to Vir B_{11} the last one helps to pump the T complex out of the bacterial cell.

Step 5:

The uptake of T-complex into the plant cell though yet not understood clearly but assume this mechanism somewhat analogues to bacterial conjugation.

Step 6:

The T-complex (T- DNA strand, Vir D_2 and Vir E_2) in this step enters within plant cell – nucleus. The N terminal of Vir D_2 has role to nick the T-DNA border while C terminus helps in the nuclear uptake of the T strand. The Vir E_2 help to Vir D_2 to target the T complex to the nuclear pore in a polar direction which facilitates it's linear uptake.

Step 7:

Generally t-DNA insertions can occur in any chromosome of the plant genome or it may occur randomly.

.**Transformation Protocol:**

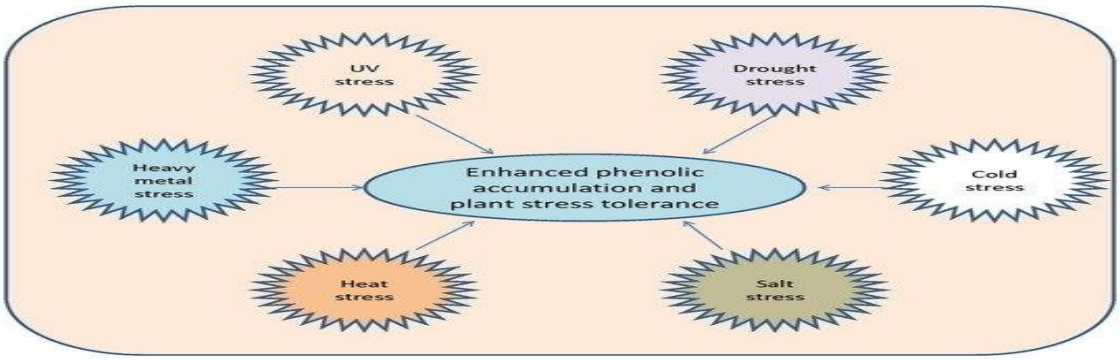
Different scientists in different time proposed many protocols for successful transformation. Surface sterilization, overnight co culture and direct infection at the wound site of plant tissue with A. rhizogenes are commonly used procedure for transformation. However successful infection procedure depends on particular plant species and specific plant tissue.

VTU 2: What are the factors affecting secondary metabolite production

Chapter 2: Factors affecting secondary metabolites

Factors affecting secondary metabolites, industrial application of secondary metabolites

- Medicinal plants constitute **main resource base of almost all the traditional healthcare systems**.
- Most of the herbal drugs produced currently in majority of the developing countries **lack proper quality specification and standards**.
- Herbal drugs used in traditional medicine may contain a **single herb or combinations of several different herbs** believed to have **complementary** and/or **synergistic effects**.
- Both the **raw drugs** and the **finished herbal products manufactured** contain **complex mixtures of organic compounds**, such as **fatty acids, sterols, alkaloids, flavonoids, polyphenols, glycosides, saponins, tannins, terpenes** etc.
- The quality of the finished product is based on the **quality of the raw materials**, which is again **depends on mineral composition of soil, geographical area** etc.
- As many as 35% of the medicinal plants used in Indian systems of medicine are highly cross pollinated which indicate the **existence of a wide range of genetic variability in the populations of these medicinal plant species which in turn reflected in the variations in the composition of secondary metabolites**.
- **Ecological and edaphic** as well as **seasonal variations** also cause changes in the chemical composition of medicinal plants.
- These **facts have to be considered while developing quality parameters** standards of medicinal plants and their finished products.
- SMs that are useful in medicine are mostly **polyphenols, alkaloids, glycosides, terpenes, flavonoids, coumarins, tannins** etc.
- The production of **secondary metabolites although controlled by genes** but their specific expression is greatly influenced by various factors including **biotic and abiotic environments** such as **climate** and **edaphic** factors or **other associated living organisms**.
- During the course of evolution plants have evolved various physical and chemical mechanisms to **protect themselves from the vagaries of nature** (drought, heat, rain, flood, etc.) and also to **defend or offend the predators or to protect from predators and pathogens**.



- The most successful adaptation of plants while developing various physiological mechanisms is through the **production of a variety of phytochemicals** by which they were able to **face both biotic and abiotic stresses and threats**.

- In this process of **defence/ offence from abiotic stress** or the invading diseases causing organisms or the predators (animals, birds, insects and herbivorous animals), the **plant synthesize a variety of chemical compounds**.
- Apparently, plants produce many **antioxidants for protecting themselves from the oxidative stress**.
- These compounds are in general stored in the leaves or other parts such as, bark, hardwood, fruits, etc., so that the predators or the disease-causing organisms can be either **knocked down or paralyzed or even get killed**.

Physiological variations

1. **Organ development** The stage of development of the plant organ (leaf, flower and fruit ontogeny) can be a determinant for the **composition of the volatiles**.
2. **Pollinator activity cycle** Except for ornithophilous flowers, the great majority of zoophilic **flowers are scented**. Apart from the **visual attraction** (colour and shape), the odours are the most important cause for the attraction and orientation of pollinators, particularly for night insects.
3. **Type of plant material** Although quite a few species yield a similar essential oil composition for their different organs, the **composition can also be largely dependent on the plant part used**: flowers, green parts (leaves and stems), bark, wood, whole fruits, pericarp or seed only, or roots
4. **Type of secretory structure** The differences found in the composition of essential oils obtained from diverse plant parts can be partly explained by the **existence of distinct secretory structures** that **are heterogeneously distributed over** the plant body.
5. **Seasonal variation** In some species, the **composition of the essential oil also changes with the time of year**, and thus the right time of harvest may be of major importance from an agronomic and economic point of view
6. **Mechanical or chemical injuries** The **emission of volatiles** not only has a **stimulant and/or attractive role** but also works in a **direct or indirect defensive way**. Nevertheless, the effect of mechanical or chemical injuries, e.g. wounds, infestation by predators or treatment with herbicides, on the yield and composition of essential oils has been little studied

Environmental conditions

7. **Climate** despite all kinds of technological advances, there is one element that remains far from human control, namely the climate. **Essential oil production and that of secondary metabolites** in general is extremely dependent on the weather conditions.
8. **Pollution** Although known to occur, the detrimental effect of air pollution on secondary metabolites production, and particularly essential oil components, is **difficult to access**, since the consequences can be confused with the response to other types of stress.
9. **Diseases and pests** Several types of diseases and pests (mildew, rust and black spot, among other things) can **cause major detrimental effects** to the stability of the market supply. Some of these are a consequence of the introduction of new crops in places other than their original source.
10. **Edaphic factors** Several authors considered the type and composition of the soil as one of the determinant factors in secondary metabolites composition and that of volatiles in particular, in addition to other explanations for the differences found in oils of the same species.

11. **Geographic Variation** There are countless examples of the occurrence of geographic variations of the yield and composition of volatiles, determining, for several species, the existence of distinct chemotypes/chemical races.
12. **Genetic** Factors and Evolution Genetic and hybridization studies have shown that the composition of essential oils is under genetic control.
13. **Storage** The relative amounts of secondary metabolites may also be affected by the storage method. Although drying can give rise to a number of negative physical and chemical modi¼cations in½uencing the quality of the marketed plant, such as changes in appearance and aroma, due to the possible loss of volatile compounds, it may also reduce the growth of micro-organisms and prevent some negative biochemical reactions

Response to Secondary Metabolism to Light Irradiation

- The key factors related light radiation include **photo period (duration), intensity** (quantity), **direction and quality** (frequency or wavelength).
- In response to **light radiation**, plants are able to adapt to the changes of circumstances by the **release and accumulation** of various secondary metabolites including **phenolic compounds, triterpenoids and flavonoids,** and many of them, have high economic and utilization value due to the **well-known antioxidant property**.
- **Camptothecin (CPT)** class of compounds has been demonstrated to be effective against a **broad spectrum of tumours**.
- Their molecular target has been firmly established to be **human DNA topoisomerase I** (topo I).
- **Saponins**, including memory improvement, **wound and vein healing, antihistaminic, antiulcer and antilepsory treatments**, as an **antidepressant, and as antibacterial, antifungal, and antioxidant agents**.

Response of Plant SMs to Temperature

- The modulation of temperature to alkaloids accumulation was reported, and **high temperature preferable to induce the biosynthesis of alkaloids**.
- The total accumulation of alkaloids (morphinane, phthalisoquinoline and benzylisoquinoline) in dry Papaver somniferum was restricted at low temperature.
- In contrast, the total level of **phenolic acids and isoflavonoid** (genistein, daidzein and genistin) in soybean (Glycine max) roots increased after the treatment at low temperature for 24h, and among which the highest increase of about 310% was observed in genistin after the treatment at 10 °C for 24h, in comparison to the control.
- In women, aging and declining estrogen levels are associated with several cutaneous changes, many of which can be reversed or improved by estrogen supplementation.

Response of Plant SMs to Soil Water

- Water stress is **one of the most important environmental stresses** that can **regulate the morphological growth and development of plants** and alter their biochemical properties.
- Severe water deficit has been considered to **reduce the plants growth**, but several studies have demonstrated that **water stress may be possible to increase the amount of SMs in a wide variety of plant species**.

CHAPTER 3 Applications of microbial secondary metabolites

- ***Antibiotics***
- The discovery of penicillin initiated the researchers for the exploitation of microorganisms for secondary metabolite production, which revolutionized the field of microbiology.
- With the advent of new screening and isolation techniques, **a variety of β-lactam-containing molecules** and other types of antibiotics have been identified.
- About 6000 antibiotics have been described, 4000 from actinobacteria.
- In the prokaryotic group, unicellular bacteria *Bacillus* and *Pseudomonas* species are the most recurrent antibiotic producers.
- Likewise in eukaryotes, fungi are dominant antibiotic producers next to plants.
- In the recent years, myxobacteria and cyanobacteria species have joined these distinguished organisms as productive species.

Name of secondary metabolites	Source of secondary metabolites	Biological activities
Resistomycin	*S. corchorusii*	HIV-1 protease inhibitor
Himalomycins A and B	*Streptomyces* sp. B6921	Antimicrobial
Bonactin	*Streptomyces* sp. BD21–2	Antibacterial
Trioxacarcins	*S. ochraceus* and *S. bottropensis*	Antitumor and antimalarial
Chinikomycins A and B	*Streptomyces* sp.	Antitumor and antiviral
Daryamides	*Streptomyces* sp. CNQ-085	Cytotoxic polyketides
Resistoflavine	*S. chibaensis*	Antibacterial
Chalcomycin A and terpenes	*Streptomyces* sp. M491	Antibacterial
Napyradiomycin (C-16 stereoisomers)	*S. antimycoticus*	Antibacterial
Oxohexaene and Cephalaxine	*Streptomyces* sp. RM17*;* *Streptomyces* sp. RM42	Antibacterial
Citreamicin θ A, Citreamicin θ B, and Citreaglycon A	*S. caelestis*	Antibacterial
Spiramycin	*Streptomyces* sp. RMS6	Antibacterial
N-isopentyltridecanamide	*Streptomyces labedae* ECR 77	Antibacterial
Staurosporine	*Streptomyces champavatii* KV2	Antimicrobial
Coagulin	*B. coagulans*	Bactericidal, Bacteriolytic
Bacthurucin f4	*B. thuringenesis* sp.	Fungicidal sub sp., *kurstaki* BUPM4
Cerein	*B. cereus*	Bactericidal, bacteriolytic

Name of secondary metabolites	Source of secondary metabolites	Biological activities
Megacin	*B. megaterium*	,
Thuricin S	*B. thuringenesis*	,
Thuricin CD 19	*B. thuringenesis DPC6431* *B. anthracis*	,
Halobacillin 5b	*B. licheniformis*	Hemolytic, cytotoxic
Bacillomycin	*B. amyloliquefacins* FZB42, *B. subtilis*	Antifungal hemolytic
Bacilysocin	*B. subtilis*	Fungicidal, antibacterial
Bacilysin 1	*B. subtilis* 168, *B. pumilus* *B. amyloliquefaciens* GSB272	Antifungal, antibacterial
Pseudomonine	*P. stutzeri* KC	Competitive inhibition of phytopathogens
Hydrogen cyanide	*P. pseudoalcaligenes* P4	Antifungal
Lovastatin	*Monascus ruber;* *Aspergillus terreus*	Enzyme inhibitor
Limonene and guaiol	*Trichoderma viride*	Antimicrobial
Tubercullariols	*Tubercularia* sp. TF5	Anticancer
Oxaline	*Penicillium raistricki*	Anti-cell proliferation
Benzomalvin C	*Penicillium raistrickii*, *Penicillium* sp. SC67	Antimalarial
Roquefortine C	*P. roqueforti; P. crustosum*	Neurotoxin

VTU 3: How are the monoclonal antibodies produced using hybridoma technology

Chapter 4 Hybridoma Technology

- Hybridoma technology is a well-established method to produce **monoclonal antibodies (mAbs)** specific to antigens of interest.
- **Polyclonal antibodies** are made using several different immune cells, have the affinity for the same antigen but different epitopes (**bind to the same antigen, but different epitopes**)
- **Monoclonal antibodies** are made using identical immune cells that are all clones of a specific parent cell (**bind to the same epitope on a target antigen)**

- An **epitope** (also known as the **antigenic** determinant) is that part of the **antigen** to which **antibodies** bind.
- While the **antigen** evokes the antibody response **in the** host, the antibody doesn't bind to the entire protein, but only to that segment called the **epitope at paratope**.
- Polyclonal antibodies have limitations in **diagnostics and therapeutics, but monoclonal antibodies are identical and same specificity.**

Technique of monoclonal antibodies production is Hybridoma Techniques.

- Hybridoma cell lines are formed via fusion between
  - a short-lived antibody-producing B cell and an immortal myeloma cell.
    - B cells can produce **antibodies of single specificity**.
  - Myeloma cells or tumor cells are capable of **continuous division forming large number of cells**.
    - The fusion of two types of cells form cells called hybridomas.

- Hybrid cells will be having the property of B cells antibody production and tumour cells ability of continuous division.
- Hybrid cell ensures **antibody production continuously in dividing cells**.
- The mixture of cells are **cultured under conditions** which **permit growth of only hybridoma cells** .
- **Each hybridoma cell will produce a single type of antibody** against **a single epitope.**

- The hybridoma cells producing the desired monoclonal antibody are then cultured.
- Monoclonal antibodies are isolated and purified.
- Each hybridoma constitutively expresses a large amount of one **specific mAb**, and favored hybridoma cell lines can be cryopreserved for long-lasting mAb production.
- As a result, researchers usually prefer generating hybridomas over other mAb production methods in order to maintain a **convenient, never-ending supply of important mAbs**.

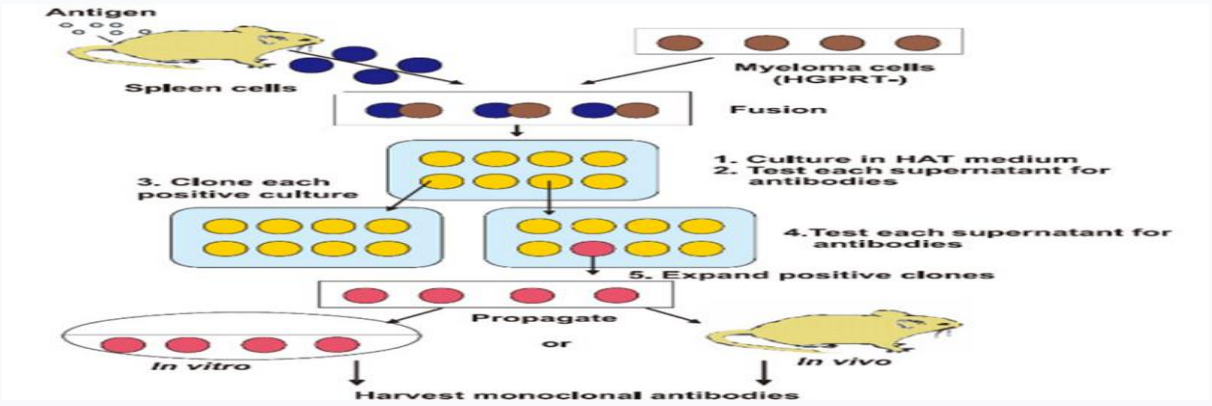
Fig 1. Hybridoma technology Inventor: **Georges Kohler and Cesar Milstein**

- Hybridoma technology was discovered in 1975 by two scientists, Georges Kohler and Cesar Milstein.
- They wanted to create **immortal hybrid cells** by fusing **normal B cells** from immunized mice with their **myeloma cells**.
- For **incidental reasons**, they had all the requirements fulfilled and it worked in the first attempt.

- By cloning individual hybrid cells, they established the first hybridoma cell lines which can produce **single type of antibody specific to the specific antigen**.
- Their discovery is considered one of the **greatest breakthroughs in the field of biotechnology**.
- For the past decades, hybridomas have fuelled the discovery and production of antibodies for a **multitude of applications**.

Steps Involved in Hybridoma Technology

- Hybridoma technology is composed of several technical procedures, including

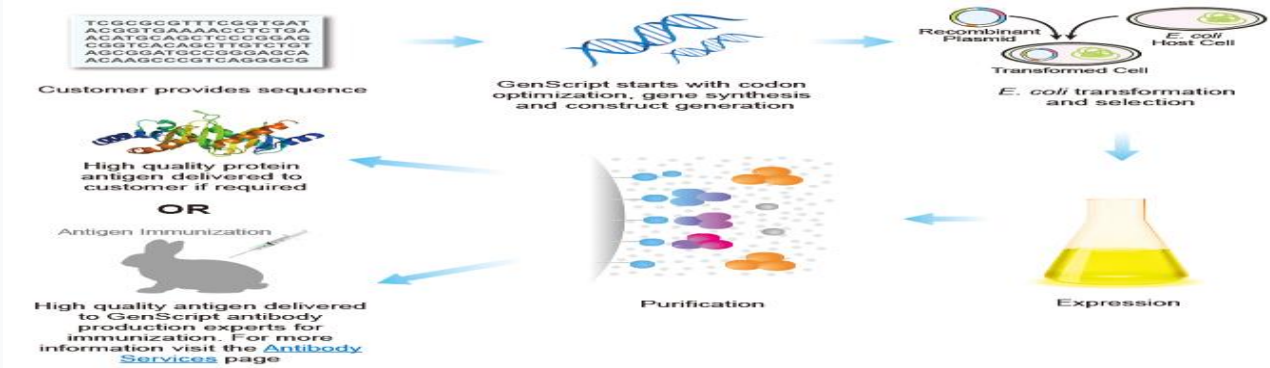


- Antigen Preparation,
- Animal Immunization,
- Cell Fusion,
- Hybridoma Screening and Subcloning,
- Characterization And Production Of Specific Antibodies.
- mAb generation by the hybridoma approach requires knowledge of multiple disciplines and practice of versatile technical skills, ranging from **animal handling, immunology to cellular and molecular biology**.
- Generation and identification of high-quality hybridoma clones is a **comprehensive and labor-intensive process**, and requires months of work during the time frame from immunization to specific hybridoma identification.

1. **Antigen Preparation**

Obtaining high-quality antigens is the key factor to successfully develop antibodies.

Schematic of Protein Antigen Production in *E. coli*

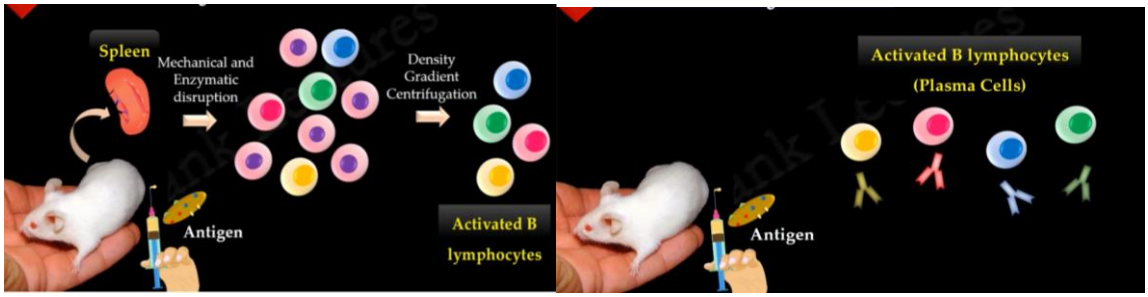


Protein Antigen Production in Mammalian System

- Mammalian antigen production **is often preferred** for the production of eukaryotic proteins.
- The advantage of a mammalian system is its **ability to confer the appropriate post translational modifications**, particularly, **glycosylation**.
- Combined with **its efficient protein folding system**, antigens produced in mammalian systems often **resemble native conformation and demonstrate better solubility**.
- Mammalian antigen production is therefore the system of choice for many proteins that are either eukaryotic, difficult to produce or required for antibody generation projects where detection of native antigen is absolutely critical.
- **Salvage pathway** refers to a short sequence of enzyme-catalyzed reactions in the purine and pyrimidine metabolism which uses preformed purine or pyrimidine bases, or nucleosides **to form nucleotides**.
- **De novo** synthesis refers to the **synthesis of complex molecules** from simple molecules **such as sugars or amino acids**, as opposed to recycling after partial degradation. ... **De novo** is a Latin phrase, literally translating to "from the new", but implying "anew", "from scratch", or "from the beginning."

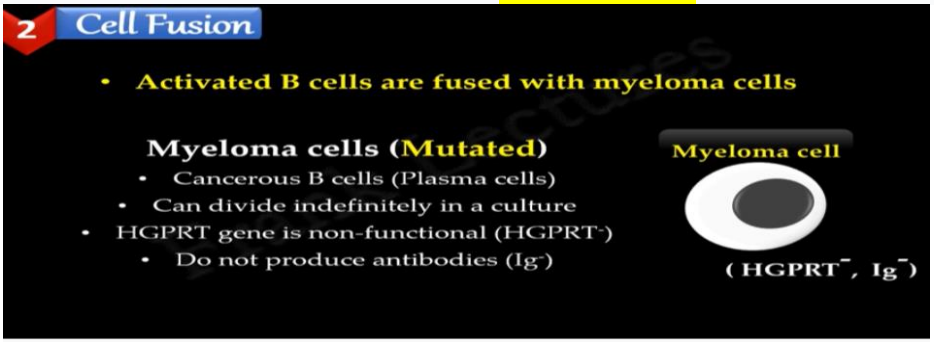
2. Animal Immunization of antigen

- Second step of the MABP is the **immunisation of the animals**.
- Usually the animal used is the **mouse**
- Mouse is immunised with **the antigens against which we need antibodies**.
- Say this is our antigen which has **four different epitopes**.
- Mouse is injected with antigen **several times**
- As a result mouse **B lymphocytes are stimulated against the epitope** or **the antigenic determinants of the injected antigen**.



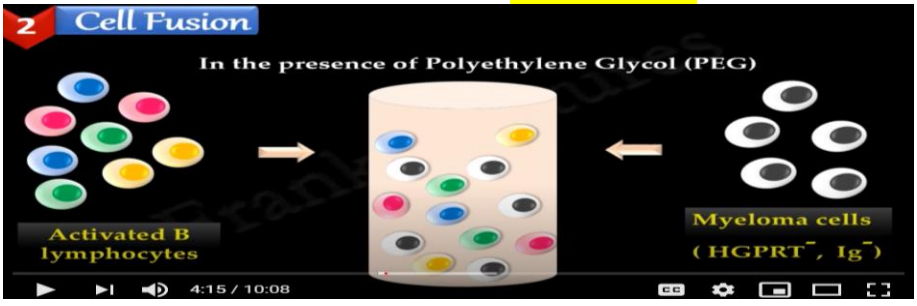
- After **several weeks when these beta lymphocytes reach optimal amount**, mouse is **sacrificed** and **spleen of the mouse is removed aseptically**
- It is known that the **Spleen is the secondary lymphoid organ**, we can **easily harvest activated beta lymphocytes from the spleen**.
- Spleen is subjected to **mechanical and enzymatic disruption**.
- This results in the release of cells.
- **Activated beta cells** are separated from the **normal spleen cells by density gradient** centrifugation.
- So at the end of this process we have **activated b cells** which are **capable of producing antibodies against the specific epitome present on the antigen**.

3. Cell fusion



- B-lymphocytes have **short lifespan in cell culture**.
- In this step activated **B cells are fused with Myeloma cells**.
- Here we need to know that the **Myeloma cells are mutated Myeloma cells**.
- Myeloma cells are **cancerous B cells** they **can devide indefinitely in a culture**.
- But their **two genes are mutated**, first is **HGPRT gene**: thus they are not able to synthesize **nucleotide by the salvage pathway**, and the **second gene is the Immunoglobuline gene**.
- As aresult of mutation these cells **cannot produce antibodies of their own**.
- These mutations are representaed ad $HGPRT^-$ and IG^-.
  Hypoxanthine-guanine phosphoribosyltransferase (***HGPRT***)

3. Cell fusion



- Cell fusion is done by **mixing two types of cells in the presence of chemical fusogen PEG**.
- **Polyethylene glycol (PEG) and** electrofusion are commonly used to induce cell fusion in hybridoma production.
- PEG fuses the **plasma membranes of adjacent myeloma** and/or **antibody-secreting cells**, forming a **single cell with two or more nuclei**.

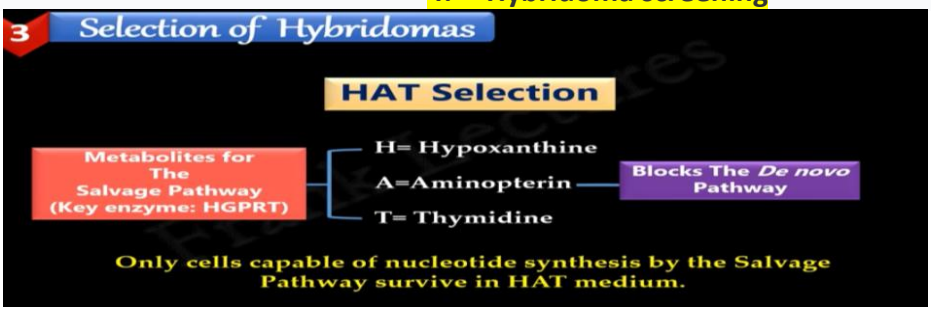
- This **heterokaryon** retains these nuclei until the nuclear membranes dissolve before mitosis.
- **Electrofusion** joins the membranes of neighboring cells by the **application of a pulsed electrical field**.
- **Electrofusion** is more **efficient than PEG** and the results are reproducible.

- Here , as a result of fusion of we will have 5 types of cells

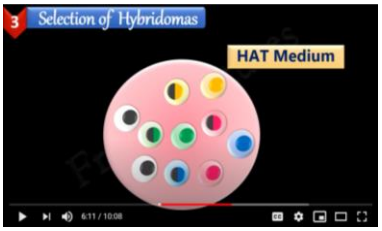
1. Unfused B cells.
2. Fused B cells
3. Unfused myeloma cells
4. Fused myeloma cells
5. Hybrid cells (Hybridoma) formed by activated B cells and Myeloma cells

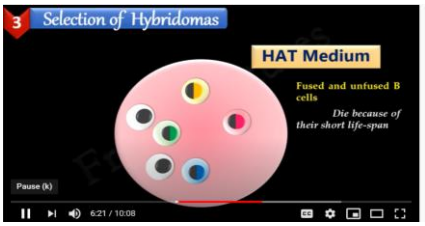
- In this figure we have **four types of activated B cells** each specific for one of the four epitome on the antigen: so hybrid cell will also be also be of four types.

4. Hybridoma screening

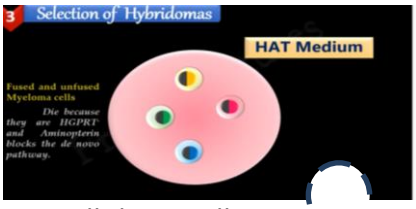


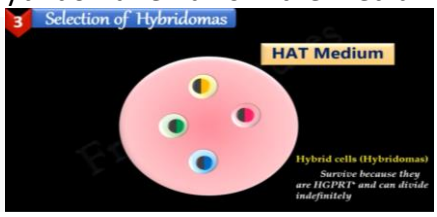
- **Our next aim is to select this hybridoma from the mixture of cells.**
- **Selection of hybridoma from the mixture of fused and unfused cells is done using HAT medium.**
- **HAT medium is the selection medium for mammalian cell cultures.**
- **Selection of the cells in this medium is done on the fact that there are two pathways of nucleotide synthesis in mammels (Salvage pathway and Denovo pathway).**
- **Aminopterine present in the Hat medium blocks the De Novo pathway.**
- **The only way a mammalian cell can survive in the Hat medium is by utilising Salvage pathway of nucletide synthesis.**
- **Hypoxanthine-guanine phosphoribosyltransferase is the key enzyme in the Salvage pathway.**
- **So if a cell has non functional HGPRT gene, cell die in the HAT medium.**
- **This is because for their cells both pathways of nucleotide synthesis will fail in HAT medium**
- In this step cells obtained after fusion is transferred to HAP medium.
- Fused and unfuded B cells die within few days, because of their shoret life span.
- They are not able to devide indefinitely in the medium.





- Fused and unfuded myeloma cells also die within few days, because of their HGPRT negative and **aminopterine present in HATM blocks de novo pathway**. Bur hybridoma survive in the medium because they are able to syntheise nucleotide **through salvage pathway**.
  - The functional HGPRT enzyme is contributed by activated B cell partner, hence desired hybridoma remains in the medium

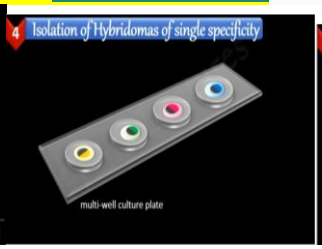




But still these cells are producing **antibodies of different specificities**. Each of these cells produce antibodies specific **to different epitopes on the same antigen**

- Even in the most efficient hybridoma fusions, only about **1% of the starting cells are fused**, and only about **1 in 10^5 form[illegible]e hybrids**.
- This **leaves a large number of unfused cells still in culture.**
- The cells from the immunized animal (antibody secreting cell) do not continue to grow in tissue culture and so do not confuse further work.
- However, the myeloma cells are **well adapted to tissue culture and must be killed,** which can be achieved by drug selection.
- Commonly, the myeloma cells have a **defective HGPRT enzyme** (hypoxanthine-guanine phosphoribosyl transferase), blocking their ability to use the salvage pathway.
- These cells containing a **non-functional HGPRT protein will die in HAT medium**.
- Only the **hybridoma cells have got the ability to divide and proliferate on the HAT medium** because genome from the B-lymphocyte makes them **HGPRT positive** and genome from the myeloma cells they **can divide indefinitely**.

- 6. **mAb production**



- Now our aim is to **select and propagate single antigen producing hybrid cell**.
- We need to **isolate these hybridoma and grow them individually**.
- So the next step (This step) is to **isolate hybridoma of single specificity**
- This is done by **limiting dilution method**: In this method cells are distributed in **multi culture well** in very low cell density so that each will contain single cell.
-

- In the next step hybridoma cells are **screened for secretion of antibodies of desired specificity**.
- Once the cells producing **desired antibodies are identified**, they are **isolated and cloned in the next step.**
- This techniques are done by two techniques **known as ELISA and RIA**
- So, Now we have separate clones of activated cells each capable of producing antibodies of each specificity, the monoclonal antibodies.
- 7. Characterization and storage

1.

In the final step these hybridomas and monoclonal antibodies are characterised and stewed in liquid nitrogen. Then the antibodies are used in treating and diagnosing diseases.

- Hybridoma antibodies can be produced in vitro and in vivo.
- For production of monoclonal antibodies in vitro, hybridomas are expanded by transfer to 24 well tissue culture plates followed by 25 cm^2 flask and a 75 cm^2 flask containing suitable medium.
- The cell density is maintained between 10^5 and 10^6 cells/ml.
- Typical culture supernatants yield up to l00µg/ml of antibody, the exact amount depending upon the cell density and rate of growth.
- Culture in vitro provides a more pure preparation of antibody.
- For producing monoclonal antibodies in vivo, mice are primed by intraperitoneal injection with 10^5 - 10^7 hybridoma cells.
- The rate of growth of the resulting ascites tumour is in general very variable and can be from less than two or more than five weeks.
- The ascites fluid can be collected from an anaesthetized mouse.
- It is possible to obtain 10 ml of ascites fluid or more from a mouse by regular tapping.
- Ascites fluid will be contaminated with mouse imunoglobulins to a small extent and if a very pure antibody is required this may prove inconvenient.

Applications of Hybridoma Technology

- **mAb therapeutics**
- Compared with other biologics, mAbs are able to maintain an extremely high affinity towards their target.
- Due to this high affinity and specificity, researchers began investigating the therapeutic potential of mAbs as metabolic activators, inhibitors and immuno-modulators.
- While the first few US FDA-approved mAb therapeutics, such as muromonab-CD3, were generated solely in mice, it became evident that in order to avoid immune rejection, future mAb-based therapeutics needed to undergo humanization.
- Since the approval of muromonab-CD3 in 1986, the FDA has approved approximately 80 more mAb therapeutics for diseases ranging from autoimmune disorders, to inflammatory diseases, HIV and cancer.

- Interestingly, despite the discovery of combinatorial display libraries in 1984 as an alternative mAb discovery platform, the majority of these mAb therapeutics were originally discovered using hybridoma technology in either fully murine or humanized mice.
- The reason for this preference is likely attributed to the natural ability of the murine immune system to generate highly specific mAbs that elicit strong constant domain functionality with limited immunoreactivity after humanization.

Chapter 5. Bioreactors considerations for animal cell cultures – Production of Monoclonal antibodies and therapeutic proteins

1. Introduction

- Monoclonal antibodies (mAbs) have been widely **used as a way to successfully achieve a broad range of extracellular targets with high specificity**.
- mAbs have various applications in **diagnosis and therapy** for several diseases such as **cancers, autoimmune diseases, sexually transmitted infections (STIs), and others**.
- In recent years, the use of mAbs has been expanded due to **significant advances in design**.
- The effect of **decreasing immunogenicity in humans**, **improvement in their bioavailability**, **optimizing the affinity and antigen-binding specificity**, and other **advances in protein engineering** are improving therapeutic mAb profiles (Figure 1).
- With the **advent of genetic engineering**, it has been possible to develop **new methods to obtain monoclonal antibodies**, both for improvement with regard to these **humanized antibodies** and **for production models**.
- Advances in **molecular and cell biology** for the development of **more efficient antibodies** have allowed advances in **diagnostic and therapeutic areas**.
- Such advances have **triggered improvements in production processes**, allowing for the **reduction of production costs** and **thus leading to an increase in the popularization of treatments with mAbs**.
- All process improvements provide a **consistent and reproducible production** of large quantities of mAbs at a moderate cost.
- **Large-scale production has revolutionized** the market for monoclonal antibodies by **boosting its production**, **making this a more practical method of production**.
- Production techniques **have only had a sizable breakthrough** due to molecular techniques
- In general, a **process of commercial production of mAb begins**
  - with the **generation of an mAb by immunizing an animal**
  - or **by molecular biology methods involving the**
    - **identification and optimization** of the coding DNA sequence
    - **construction and identification** of a stable high-producing clone.
- Improvements in cultivation are similar to those applied in other bioproducts that rely on

  - culturing microorganisms or cells,
  - Requiring the development of a well-designed culturing process comprising the full range of control
  - Associated operations that will support technical evaluations.
- mAbs production processes in **wave or single-use bioreactor (SUBs)** are characterized by **flexibility and low operating costs** when compared to the production processes in **fixed stainless steel vats**.
- The development of bioprocesses involving these production platforms can reap greater acceptance by the industry.
- **Drugs based on mAbs** have been controlled by **regulatory agencies around the world**.
- Therefore, it is necessary to elaborate regulatory protocols accompanying the increase in **production and the nuances of the characteristics of this class of drugs**.

2. mAbs production techniques

2.1. Hybridoma and phage display

- This technique consists of **creating a hybridoma**, a stable hybrid cell capable of producing a single type of antibody against a specific epitope present in an antigen.
- Hybridoma construction was initially **produced from murine models**.
- The technique consists of removing a pool of **activated B lymphocytes** from an immunized animal spleen and combining them with **immortalized myeloma cells** unable to produce the enzyme **hypoxanthine-guanine-phosphoribosyltransferase (HGPRT),** an important enzyme present in the ***salvage* pathway**, one of the pathways responsible for **nucleotide production**.
- To select **hybridoma cells**, the pool of cells resulting from the fusion (a mix of hybridoma cells and non-fused B lymphocytes and myeloma cells) are cultivated in a selective medium containing **aminopterin**, which inhibits the nucleotide de novo synthesis.
- Myeloma cells **lack the *salvage* pathway** for nucleotide production.
- When they are exposed to aminopterin present in selective medium, **the de novo synthesis is also blocked**, and as a result, myeloma cells are no longer viable since all major pathways for nucleotide production are blocked.
- In contrast, non-fused, activated B lymphocytes can survive as their *salvage* pathway works perfectly and they can continue nucleotide production even if the de novo pathway is blocked by aminopterin.
- However, these cells are not **immortalized** and can replicate only a limited number of times after which they eventually die.
- With this in mind, only cells **capable of replicating indefinitely and synthesizing** nucleotides through the *salvage* pathway can survive through selection conditions, and these cells are the hybridomas.
- In spite of the fact that the primary recombinant mAbs were delivered utilizing this innovation—including the first medication approved by the Food and Drug Administration (FDA) for therapeutic proposes (Table 1)—the great contribution of this

technology was mostly to elucidate immune response mechanisms and control in vitro antibody production.

- Therefore, mAb hybridoma production from murine sources exhibits a genuine downside in human therapeutics 2.2. Culture production factors

2.2.1. Cell lines

- One of the most critical steps in developing an mAb production system is to choose the cell line.
- The cells must be **stable** and **secrete the desired protein** with the correct conformation at high levels.
- Based on these requirements, **the mammalian cell is the most commonly** chosen expression system for mAb production.
- The **main advantage** of a mammalian expression system is that the **cellular machinery is adapted for the production, processing,** and **secretion of highly complex molecules**.
- The great majority of commercial mAbs are produced in **Chinese hamster ovary (CHO)** and murine myeloma ***cells*** **(NS0 cells)**, originating from plasmacytoma cells that were modified until IgG generation in nonsecreting B cells.
- Genetic modifications in CHO cells have generated cell lines capable of producing a high quantity of humanized mAbs.
- These cell lines were able to secrete up to **100 pg/cell/day**.
- Other modifications led to a high production of a **chimeric mAb**, ranging from **80 to 110 pg/cell/day**.
- NS0 modifications also have been made, leading to higher mAb production rates, ranging from **20 to 50 pg/cell/day**.
- In smaller quantities, hybridoma cell lines are also used in industrial mAb production. Some hybridoma strains are reported to have a production rate up to **80 pg/cell/day** [16].
- In spite of this, different mammalian cell lines and even more peculiar expression systems such as genetically modified plant cells, genetically modified insect cells, and genetically modified microorganism cells have also been used in mAb production and have gained space in the biopharmaceutical industry.
- Microorganisms modified by genetic engineering techniques have attracted much focus in industry, because these cells are simpler to handle and to modify when compared to animal cells.
- Other advantages of production methods using genetically modified microorganisms are that these cells have well-defined expression systems, and the production methodology is reproducible and easy to validate.
- Modified yeast cells, such as *Pichia pastoris* have a great potential for usage since these cells are known to achieve high secretion levels of heterologous proteins.
- Yeast cultivation systems for mAb production are easier scale-up and are cheaper when compared to mammalian cell cultivation systems.
- They can be cultivated in regular stirred tank bioreactors, in batch, or in feed-batch modes of operation.

- Generally, microorganisms do not have physicochemical and biological characteristics for the appropriate expression and posttranslational processing of mAbs.
- Modified plants have also gained attention since plants are easy to cultivate and propagate.
- Other cultivation advantages such as cheap medium, low maintenance cost, and high production yields make plant production a cheaper alternative when compared to mammalian cell cultures.
- However, there are some limitations—different glycosylation patterns and post-translational processing can also make plant cell utilization difficult.

2.2.2. Culture medium

- Cultivation media for mammalian cells must have a complex content of ingredients ranging from amino acids to trace elements.
- To supply the cellular demand of these nutrients, the culture medium uses serum in its composition, however, due to the emergence of diseases caused by defective prions, such as bovine spongiform encephalitis (BSE), there is a great incentive to remove any animal component of culture media composition, especially if the medium is used for industrial production of biopharmaceuticals products.
- This has led to the emergence of media free from any animal components, including well-defined media for CHO and NS0, the two most utilized cell types in mAb production.
- The development of a proper medium can be time consuming and very expensive.
- However, many companies prefer to develop their own production media to maintain the composition between production lots as well as develop an appropriate medium composition for the specific cell type that will be used and to achieve greater control over production.
- Added to this, the development of downstream processes that meet the requirement for high-purity products and tests to validate the final product quality raises the overall production cost of a drug based on monoclonal antibodies.
- Despite the complexity of developing a culture medium, much progress has been made in this area, allowing for greater cell growth and increasing cell conservation time in suitable conditions for the growth and production of molecules of interest.

2.2.3. Culture conditions

- Growing conditions can directly influence the cell growth and production levels of molecules of interest. Usually, mammalian cell culture conditions for mAb production are very well defined: 37 °C, pH 7.15, and dissolved O_2 (OD) levels at 30–60%. CO_2 level is monitored to mimic the physiological standard between 31 and 54 mmHg.
- However, changes in cellular conditions have shown great potential to change cellular metabolism toward cellular growth or molecule production and this can be used to increase mAb production. Bioprocesses can be designed to occur in two phases.
- First, cell growth is optimized to reach a certain cell density.

- Once this density is reached, the second phase begins and the bioreactor conditions are shifted so the cells continue to grow just at a maintenance rate and directing the metabolism toward monoclonal antibody production.
- Some CHO cell strains and hybridoma cells are sensitive to changes in temperature and pH. When subjected to temperature and pH values lower than those normally used, values between 30 and 35 °C and 6.7–7.0, respectively, cell growth metabolism is reduced and specific production increases.
- The growth metabolism reduction also contributes to lower production of some metabolic compounds which are toxic for cell cultures, allowing increased cell viability, which spend more time producing molecules of interest.
- A good way to monitor the growth stage of a cell culture for controlling changes in cultivation is watching the DO and pCO_2 levels, which can also be adjusted to maximize the production of proteins such as mAbs.

2.2.4. Production platforms

- The cell culture for mAb production can follow three different types of processes.
- The simplest of them is batch production, which consists of a closed system where a bioreactor is sterilized and prepared with a medium containing all the nutrients needed for cellular growth and product manufacturing and then, cells are inoculated.
- There is no feeding system with fresh medium or withdrawal of spent medium.
- As the process runs, nutrient concentration decreases and waste metabolites are produced, lowering cell viability.
- In spite of being a simple process, batch is not the most suitable type of production platform for mammalian cell cultures, as the environment inside the reactor quickly becomes unfavorable for cell growth and, at the same time, waste product concentration increases.
- Cultivation factors such as initial nutrient concentration and waste metabolite production directly determine the maximum concentration that cells can reach in a bath culture.
- Generally, this type of cultivation reaches a maximum density of $1–2 \times 10^6$ cells/mL, and then the cell viability drops rapidly.
- The production process lasts for 4–7 days, when productivity reaches certain concentration of interest.
- Supernatant is collected and the product is recovered by downstream processes.
- The time that each batch takes to finish also depends on the production kinetics.
- If the production is growth dependent (production occurs concomitantly with cellular growth), batch processes can be stopped as soon as cells reach the stationary phase.
- But if the product is not associated with growth (production only starts when the growth rate decreases), the culture needs to be carried for a longer period of time since production only starts at stationary phase.
- In contrast to batch, a second type of production process utilized is continuous fermentation.

- There are two types of continuous production: chemostat cultures and perfusion cultures.
- Concerning chemostat cultures, fresh medium is added to the bioreactor and fermented medium is removed along with cells at a constant flow rate so that the culture volume remains unchanged.
- The flow rate (dilution rate) controls cellular growth and when these two variables are equal, the bioreactor reaches equilibrium—cell concentration, nutrient concentration, and product concentration are held constant. In this context, the culture can be kept in equilibrium for several months reaching a cell density of 10–30 × 10^6 cells/mL.
- To avoid viable cell loss along with the constant outflow of the by-products of cell metabolism, many manufacturing plants have developed a cell-recycling system and thus, the perfusion culture method was developed where cells are kept inside the bioreactor
- The disadvantages of continuous fermentation are the use of a large amount of expensive culture media and the difficulty in recovering the product, which comes out fairly diluted.
- These two disadvantages are consequences of the constant medium flow rate.
- To work around the product dilution problem, some production manufacturing plants have ultrafiltration systems which retain the product inside the bioreactor.
- Another obstacle of this type of process is that the establishment of culture conditions for a stable industrial production plant can take months.
- For this to occur, the strain used must be very stable and have its physiological aspects clearly elucidated, such as growth rate, productivity, and response to certain stress conditions.
- It is not uncommon to hear that numerous attempts are made before the settlement of a stable production plant is achieved, but, once settled, this production process can bring many advantages, since it can be operated in smaller-volume bioreactors, and therefore have greater production flexibility.
- The third type of process for producing monoclonal antibodies is by far the most utilized at industrial scale, which is fed-batch process.
- In this process, the cell density reaches 8–12 × 10^6 cells/mL, and cell viability in the bioreactor is enhanced by controlled nutrient addition at specified intervals.
- The production process can take 12–20 days.
- Usually, the same medium used in the initial culture is also used for feeding, but in a more concentrated version.
- The feeding solution composition can be designed to supply the cells based on their metabolic state at different culture phases by analyzing and identifying the spent medium nutrients that are being more consumed.
- Furthermore, the medium used in feeding can be modified to promote cell growth or to stimulate molecule production, since different components may modify the behavior of cells, changing the metabolism for different purposes.
- The feed solution can also be designed to minimize the production of waste metabolites that cause cell stress when in excess.

- However, their production is not completely avoidable as they eventually reach harmful concentrations.
- It is relatively easy to scale up and operate this system. More summarized data about the advantages and disadvantages of each process for mAb production can be seen in .
- A lot of effort has been made to increase cell longevity in batch and feed-batch modes of operation.
- It is expected that the longer the cells are maintained viable, the greater the antibodies' production will be.
- So, in order to maintain cell viability, some culture parameters can be optimized, such as culture media, feed solution, and mAb secretion rates and by-product production.
- To improve mAb titers in the batch platform, the start medium can be supplemented with glucose and amino acids, increasing mAb production up to eightfold when compared with regular media.
- Improvements for the fed-batch platform can be achieved by adjustments in feed solution, as mentioned before.
- Feed solutions containing glucose and aminoacids/glutamine have been reported to increase mAb titers from two to fourfold, reaching production of up to 2 g/L, when compared with the batch production platform.
- The optimization of the antibody secretion rate can be achieved by high-density cell cultivation.
- On a fed-batch platform, a high cell cultivation culture can reach an mAb productivity rate of 0.94 g/L/day and a final titration of 17 g/L, while a continuous culture performed at high density conditions can reach final titration and productivity rates of 0.8 and 1.6 g/L/day, respectively.
- Optimizing mAb secretion highly depends on the cell line chosen for production.
- Each cell strain can be influenced by the manufacturing conditions and respond differently to increasing or decreasing mAb production and secretion.
- The accumulation of toxic by-products is a great bottleneck in manufacturing processes since they can inhibit cell growth and then directly affect mAb production.
- Although a few strategies to minimize this by-product accumulation have shown to be promising, some are not applicable for a large-scale production.
- Optimizing medium composition and feed solutions with substrates that reduce toxic compound production is the most common strategy used at industrial scales of production.
- Although most mAbs are produced by fed-batch process, there are tendencies indicating that in the future many bioprocesses will be operated in continuous platforms, especially for the production of biopharmaceuticals.
- On these platforms, the production system will be coupled to upstream and downstream processes. However, for this to actually happen, a great improvement in technological development still needs to be achieved.

2.3. Production systems

- The use of monoclonal antibodies as therapeutic drugs requires a large-scale production that far exceeds that of laboratory production (Figure 2).
- Various production systems have been developed and have evolved, while new alternatives are emerging.
- The production of mAbs at commercial scale can be performed with adherent cells or suspension cells, although the latter is by far the most used and is better established with more efficient production methods available for cells cultivation.
- Thus, scale-up using suspension cells is easier.
- Another advantage of the suspension production system is that a bioreactor with a large area for cell adhesion is not necessary since the cultivation of adherent cell productivity is directly linked to the bioreactor's area.
- Some cultivation issues and worries have arisen regarding the production scale increase, maintenance of product quality, contamination control, demand for oxygen supply, and control over DO and CO_2 removal, among others.
- Regarding suspension cell cultures, aeration is in part dependent on the agitation of the culture inside the bioreactor, which can lead to cell shear stress.
- To work around cultivation problems, major advances have been made in the process itself by developing better culture control and conditions, as well as the improvement and development of new bioreactors.

2.3.1. Production systems for cells in suspension cultures

- The different types of bioreactors commonly used for mAb production in submerged mammalian cells are stainless steel stirred tank bioreactors (STR), air-lift reactors, and disposable bioreactors.
- More details on each of these bioreactors are discussed below.

2.3.2. Stainless steel stirred tank bioreactors

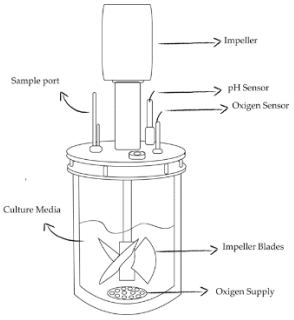


Figure 3.

- Schematic representation of a stainless steel stirred tank bioreactor. Showing the main components in a cell cultivation.
- Stainless steel stirred tank bioreactors are the most consolidated type of bioreactor used for industrial mAb production and consist of baffle-stirred tanks linked to rotor systems (Figure 3). It is a consolidated system, and there is a lot of knowledge and

experience surrounding this technology, acquired by its vast industrial use beyond production using mammalian cells.

- The cultivation in this bioreactor allows for wide flexibility of working volumes, ranging from 1.0 to 25.0 L, since this system is easily scalable to larger volumes due to its high control over production conditions and extensive handling knowledge.
- The mechanisms and cleaning and sterilization protocols are well defined. Additionally, cultivation parameters for this system, such as gas transfer coefficient, agitation, aeration, temperature maintenance, pH, and others are well controlled and regulated when compared to other production systems.
- Another advantage of the STR is that it can be used for cultivation of various cell types and in addition, the products obtained from the cultivation in this type of bioreactor are easily approved for therapeutic use, as regulatory terms are well defined for this type of production.
- However, the biggest disadvantage for the use of STR is the stress caused by shear. It can cause cell lysis and lead to loss in mAb productivity.

2.3.3. Air-lift reactors

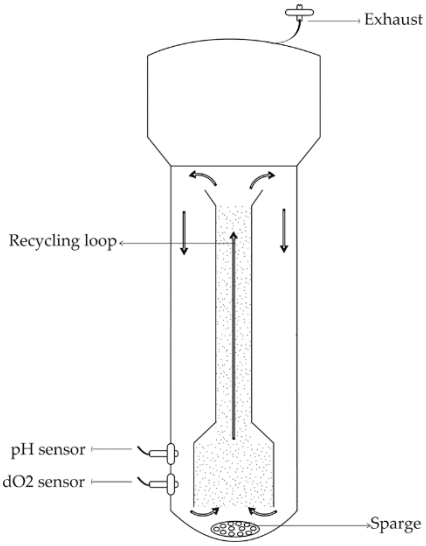


Figure 4.

- Schematic representation of an air-lift bioreactor. Showing the main components in a cultivation process.
- Air-lift reactors are also broadly used for the industrial production of mAbs.
- The reactor consists of tanks with a bubble column inside, and air is injected into the column base (Figure 4).
- The air flows through the column's length to the top of the bioreactor as degassed culture medium flows in the opposite direction to the reactor bottom.
- This creates a constant gentle mixing of the medium as well as proper culture aeration, annulling part of the shear stress caused by other stirring systems.

- Other advantages of this operation system are that it is easier to scale-up, contamination problems are more unlikely to occur, and the equipment is simpler.
- In spite of these advantages, this system is less utilized than STR reactors because the working volume ranges only from 2.0 to 5.0 L and the air-lift reactor handling is not so well elucidated.

2.3.4. Disposable bioreactors

- The first single-use bioreactors emerged in the late 1990s with the launch of a wave reactor system. After that, disposable stirred tank bioreactors were developed.
- This method brought many advantages for mAb manufacturing.
- At the end of the process, the bioreactor is discarded and replaced by a new clean and sterile one.
- This eradicates cross contamination between batches and decreases the time consumed with the equipment preparation between batches.
- When all the advantages of this process are taken in account, the savings made regarding production and investment capital are highly significant when compared with other process methods.
- The great disadvantage of this production system is the small work volume supported, ranging from 50 to 2000 L.
- The wave system consists of a sterile plastic bag lying on a rocking platform (Figure 5).
- The bag is half filled with cultivation medium and half filled with a gas mix of interest.
- The platform motion creates an undulation movement in the culture, ensuring efficient aeration and culture mixing without causing shear damage.

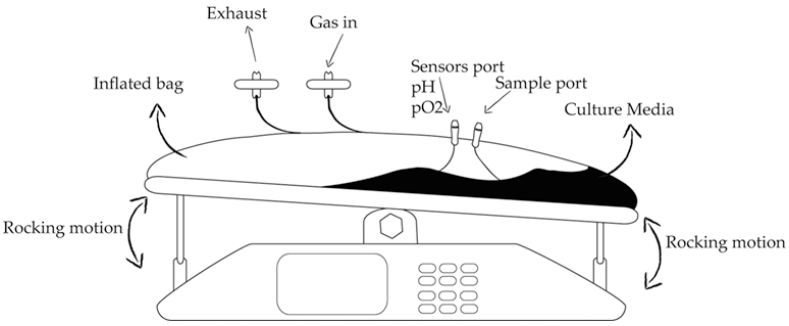


Figure 5.

- Schematic representation of a disposable wave bioreactor. Showing the main components in a cell cultivation process.
- The main features of SUBs are related to their technical characteristics similar to those of stainless steel bioreactors, that is, aeration rate, agitation, reactor geometry, and ease of monitoring internal conditions, a process similar to stainless steel bioreactors [9].
- SUBs are being widely used to replace many processes for the production of bioproducts. SUBs may be a cheaper and more efficient alternative from an industrial point of view, and its principle can easily replace any bioprocess to adapt the method to

the platform of interest to be replaced, such as large tanks and stainless steel or the motion rocking platforms.

- SUBs have been used in bioprocesses for monoclonal antibody production involving several expression systems, including mammalian cells, microorganisms, plants, mammary glands, etc. Animal cell culture technology is one of the oldest techniques for the production of mAbs.
- There is also the production of bottles known as roller bottles, consisting of mammalian cells growing in nutritional and physical conditions controlled in bottles which remain in rotational movement.

2.3.5. Roller bottles

- Roller bottles are a rotary motion system for growing cells and for the production of some bioproducts. It has been an alternative to other monoclonal antibody production systems (Figure 6).
- Roller bottles provide conditions that favor the transfer of oxygen and temperature control without aeration, agitation propellers, or circulation pumps.
- The bottle is mounted on a turntable which gives homogeneity of growth and aeration of the culture medium.

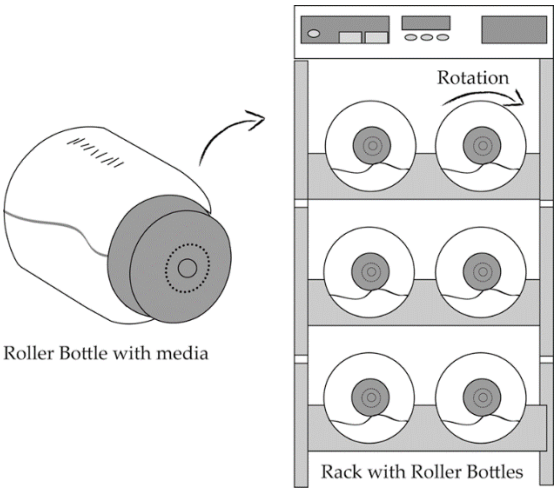


Figure 6.

- Schematic representation of roller bottles bioreactor and a rack with the rotational motion system in a cultivation for mAb production.
- For the production of monoclonal antibodies at commercial scale, the roller bottle technique can be adapted to racks containing tens of bottle in a production line.
- The advantages of this technique is the high growth potential linked to ease of handling and monitoring of certain conditions such as temperature and rotation. However, the scale of view requires a large physical footprint, which can make the process less economical