**Module 1.**

**Syllabus**
**FERMENTATION TECHNOLOGY:**

1. **Types of fermentation – submerged and solid state fermentation.**
2. **Modes of fermentation – Batch, continuous and fed-batch.**
3. **Microbial growth kinetics.**
4. **Development (from shake flask to 2L scale for 1st time) and Optimization of fermentation process – physiological and genetic strategies.**
5. **Production of primary and secondary metabolites.**
6. **Strategies to optimize product yield.**
7. Instrumentation and control.
8. Preservation of microbial products.
9. Production of antibiotics.
10. **Enumeration and screening of novel microbial secondary metabolites,**
11. **Strain improvement.**
12. **Process design criteria for various classes of byproducts (high volume, low value products and low volume, high value products),**
13. Microbiology of brewing (Distilled and non distilled beverages with examples).

**Course 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: TYPES OF FERMENTAION: SUBMERGED AND SOLID STATE FERMENTAION

1. **Explain different types of fermentation (VTU)**
2. **Differentiate between Solid-state fermentation and submerged fermentation**

**Anss: TYPES OF FERMENTAION**

- The term fermentation is used to indicate **microbial cell propagation** and **generation of products** under either aerobic, microaerobic, or anaerobic conditions
- Development of this fermentation techniques has leads to industrial level production of bioactive compounds such as **antibiotics, pigments, antioxidants, antitumor agent, bio-surfactants, bioactive peptides** etc.
- The **metabolism exhibited** by microorganism is different in SSF and SmF.
- The influx of nutrients and efflux of waste materials needs to be carried out based on the metabolic parameters.
- FERMENTATION is classified into two types based on the substrate used as follows

**1. Solid state fermentation (SSF)**

**2. Submerged fermentation (SmF)**

### 1. Solid state fermentation or surface fermentation:

- Solid state fermentation has been defined as "**the fermentation process occurring in the absence or near absence of free water utilizing the solid substrate**".
- **Moisture levels are vital** for the growth of filamentous fungi and the moisture content must be maintained at a specific level.
- **SSF bioreactor designs can be classified into four groups** which can be separated by **aeration and Agitation type**:
- Group 1: **Unforced aeration**, without agitation (static) **: Tray reactor**
- Group 2: **Forced aeration**, without mixing (static): **Packed bed reactor**
- Group 3: **Unforced aeration**, with continuous or intermittent agitation : **Rotating drum reactor**
- Group 4: Forced aeration, with continuous or intermittent agitation: **gas-solid fluidized-bed bioreactor**

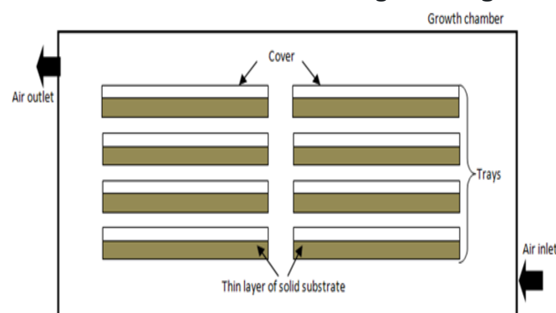



**Figure 1** Scheme of solid state ferntation.

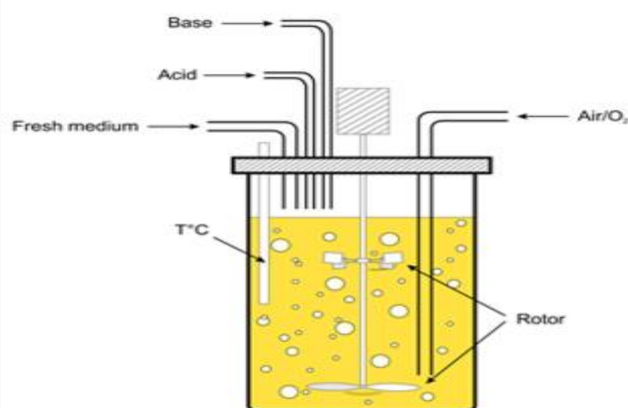
- Solid state fermentation is a manufacturing process used in the production of **fuel, food, cosmetic, fuel pharmaceutical, textile and industrial products**.
- These **biomolecules are mostly metabolites generated by microorganisms** grown on a solid support selected for this purpose.
- The solid state fermentation process involves a **solid matrix like rice bran** and placing it on a medium to alongside microorganisms create a substrate.
- Sterilization of the environment is not necessarily required when carrying out solid state fermentation, this is because inoculum prohibit other micro flora from growing.

- Inoculated substrate is stored is then stored in at a specific temperature, **between 5 and 95ºC /1-5 days.**
- It is **also subject to agitation** using constant or intermittent rotation.
- Solid state fermentation allows the growth of filamentous fungi in conditions which represent their natural environment.
- It allows air to come in contact with the mycelium by smearing the mycelium.
- The growth of mould is promoted by using substrates which have a reduced water level.
- It is necessary to monitor the rate of air flow as this has an effect on water and oxygen levels as well as any changes in temperature.

**2. Submerged fermentation / liquid fermentation**

- Submerged fermentation is a process involving the development of microorganisms in a liquid broth.
- This liquid broth contains nutrients and it results in the production of industrial enzymes, antibiotics or other products.
- Several types of submerged type of fermentors are known and they may be grouped in several ways: **shape or configuration, whether aerated or anaerobic and whether they are batch or continuous**. The most commonly used type of fermentor is the **Aerated Stirred Tank Batch Fermenter.**
- **Aerated stirred tank batch fermentor:**

- A *typical* fermentor of this type is an upright closed cylindrical tank fitted with one or more baffles attached to the side of the wall, a water jacket or coil for heating and/ or cooling, a device for forcible aeration (known as sparger), a mechanical agitator usually carrying a pair or more impellers, means of introducing organisms and nutrients and of taking samples, and outlets for exhaust gases.
- Modern fermentors are highlyautomated and usually have means of continuously monitoring, controlling or recording pH, oxidation-reduction potential, dissolved oxygen, effluent O2 and CO2, and chemical components.
- Further diagrams of stirred tank fermentors are shown below




- There are three common modes by which submerged fermentation takes place; they are **Batch, batch-fed fermentation and continuous fermentation**.

**Differentiate between Solid-state fermentation and submerged fermentation**

❖

| SSF | SMF |
|---|---|
| 1. There is no free water, and the water content of substrate is in the range 12-70% | 1. Water is the main component of the culture |
| 2. Microorganisms absorb nutrients from the wet solid substrates; a nutrient concentration gradient exists | 2. Microorganisms absorb nutrients from the liquid culture; there is no nutrient concentration gradient |
| 3. The culture system consists of three phases (gas, liquid and solid) and gas is the continuous phase | 3. The culture system mainly consists of liquid; the liquid is the continuous phase |
| 4. Inoculation size is large, more than 10% | 4. Inoculation size is small, less than 10% |
| 5. The required oxygen is from the gas phase; the process needs low energy consumption | 5. The required oxygen is from dissolved oxygen; there is a larger amount of dissolved oxygen |
| 6. Microorganisms attach and penetrate into the solid substrate | 6. Microorganisms uniformly distribute in the culture system |
| 7. At the end of fermentation, the medium is a wet state substrate, and the concentrations of products are high | 7. At the end of the fermentation, the medium is liquid and the concentrations of products are low |
| 8. High production rate and high product yield | 8. Low production rate and low product yield |
| 9. **Mixing is difficult or impossible**, some microorganisms are sensitive to mixing or agitation and the growth of microorganisms is restricted by nutrient diffusion | 9. Mixing is easy, and the growth of microorganisms is not restricted by nutrient diffusion |
| 10. Removal of metabolic heat is difficult | 10. Temperature control is easy |
| 11. Heterogeneity | 11. Homogeneity |
| 12. The fermentation parameters are hard to detect and control on-line | 12. The fermentation parameters can be detected and controlled on-line |
| 13. Extraction process is simple and controllable; little waste water | 13. Extraction process is usually complex; there is a large amount of waste water |
| 14. Low water activity | 14. High water activity |
| 15. Simple fermentation bioreactor | 15. High-tech design fermentation bioreactor |
| 16. Natural enrichment or artificial breeding systems | 16. Pure strains |
| 17. Energy consumption and equipment investment are high | 17. Energy consumption and equipment investment are low |
| 18. Low raw material cost | 18. High raw material cost |

## Chapter 2: Modes of fermentation

**Q 1: Write note on modes of fermentation (VTU)**
**Q 2: Illustrate Batch, continuous and fed batch fermentation**

- **Feeding and bleeding strategy** in bioreactor is the mode of providing nutrients into the bioreactor and removal of products from the reactor to achieve optimum metabolite production by microorganisms.
- **Microbial culture processes can be carried out in different ways.**
- **There are three modes of fermentation used in industrial applications: batch, continuous and fed batch fermentations.**

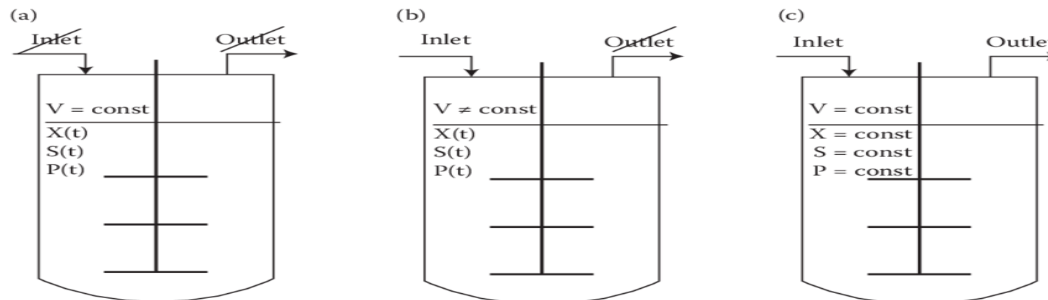



Figure 1. Fermentation process; x: biomass, s: substrate, p: product, t: time

### 1. BATCH FERMENTATION

- A batch fermentation system is a closed system, once seeded or inoculated, receive no further inputs of mass or energy and permit no outputs of waste materials.
- Batch reactors may be stirred or not stirred but in any case, conditions in the reactor are constantly changing.
- Nutrients and other materials like oxygen are declining and metabolic waste products are increasing.
- At time t=0, the sterilized nutrient solution in the fermenter is inoculated with microorganisms and incubation is allowed to proceed at a suitable temperature and gaseous environment for a suitable period of time.
- In the course of the entire fermentation nothing is added, except oxygen (in case of aerobic microorganisms), an antifoam agent, acid or base to control pH.
- The composition of the medium, the biomass concentration and the metabolite concentration generally change constantly as a result of metabolism of the cells.
- After the inoculation of a sterile nutrient solution with microorganisms and cultivation under physiological conditions, six typical phases of growth are observed

  Advantages of batch culture

  Batch culture systems provide a number of advantages:

  1 Reduced risk of contamination or cell mutation as the growth period is short.

  2 Lower capital investment when compared to continuous processes for the same bioreactor volume.

  3 More flexibility with varying product/biological systems.

  4 Higher raw material conversion levels, resulting from a controlled growth period.

  The disadvantages include:

1 Lower productivity levels due to time for filling, heating, sterilization, cooling, emptying and cleaning the reactor.
2 Increased focus on instrumentation due to frequent sterilization.
3 Greater expense incurred in preparing several subsultures for inoculation.
4 Higher costs for labour and/or process control for this non-stationary procedure.
5 Larger industrial hygiene risks due to potential contact with pathogenic microorganisms or toxins.

Common applications for batch cultures include:

1 Products that must be produced with minimal risk of contamination or organism mutation.
2 Operations in which only small amounts of product are produced.
3 Processes using one reactor to make various products.
4 Processes in which batch or semi-continuous product separation is adequate. Fig. 1

**2. CONTINUOUS FERMENTATION**

- In continuous fermentation an open system is set up.
- Sterile nutrient solution is added to the bioreactor continuously and an equivalent amount of converted nutrient solution with microorganisms is simultaneously taken out of the system.
- In a homogenously mixed bioreactor, we can have a chemostat or a turbidostat.
- In the turbidostat, cell growth is kept constant by using turbidity to monitor the biomass concentration and the rate of feed of nutrient solution is appropriately adjusted.
- In the chemostat, constant chemical environment is maintained, while in a turbidostat constant cell concentration is maintained.
- In a chemostat the growth chamber is connected to a reservoir of sterile medium.
- Once growth is initiated, fresh medium is continuously supplied from the reservoir.
- The volume of fluid in the growth chamber is maintained at a constant level by some sort of overflow drain.
- Fresh medium is allowed to enter the growth chamber at a rate that limits the growth of the bacteria.
- The rate of addition of fresh medium determines the rate of growth because the fresh medium always contains a limiting amount of an essential nutrient.
- There are several major advantages of using continuous cultures as opposed to batch cultures:
- Continuous reactions offer increased opportunities for system investigation and analysis.
- The constancy of the continuous process also provides a more accurate picture of kinetic constants, maintenance energy and true growth yields.
- Continuous culture provides a higher degree of control than a batch culture.
- Bioreactors operated as chemostats can be used to enhance selectivity for thermophiles, osmotolerant strains or mutant organisms with high growth rates.
- Because of the steady state of continuous culture, the results are not only more reliable but also more consistent leading to a better-quality product.
- It also results in higher productivity per unit volume, as time consuming tasks, such as cleaning and sterilization are unnecessary.
- The ability to automate the process makes it more cost-efficient and less sensitive to the impact of human error.

Disadvantages include:

1 The control of the production of some non-growth related products is not easy.

2 Wall growth and cell aggregation can also cause wash-out or prevent optimum steady-state growth.
3 The original product strain could be lost over time if a faster growing one overtakes it.
4 The viscosity and heterogenous nature of the mixture can also make it difficult to maintain filamentous organisms.
5 Long growth periods not only increase the risk of contamination but also dictate that the bioreactor must be extremely reliable and consistent, incurring a potentially larger initial expenditure in higher quality equipment.

## 3. FED-BATCH FERMENTATION

- The fed batch method is characterized by the addition of small concentrations at the beginning of the fermentation and these substances continue to be added in small doses during the fermentation process.
- Despite the apparent similarity between the fed batch reactor model and the continuous culture model, they are very different.
- Whereas the chemostat process (continuous culture) for biomass accumulation is composed of a growth and removal process, the fed batch procedure is composed of a growth and dilution process.
- The concept of steady state cannot be easily applied to a fed batch reactor.
- It is significantly more difficult to maintain a specific growth rate in a fed batch system than in continuous culture.
- As cells are not removed during the fermentation, fed batch cultures are well suited for the production of compounds produced during very slow or zero growth.
- Unlike a continuous culture, the feed does not need to contain all the nutrients needed to sustain growth.
- The feed may contain only a nitrogen source or a metabolic precursor.
- Contamination and/or mutation will not have the same dramatic effect on a fed batch fermenter.
- The feed can also be manipulated to maximize product formation. During fermentation, the feed composition and feed flow rate can be adjusted to match the physiological state of the cells.
- Fed batch reactors can maintain low nutrient and substrate concentrations .
- They are very useful for the production of vinegar and amylase.

Advantages of fed batch systems:

1 Higher yield, resulting from a well-defined cultivation period during which no cells are added or removed.
2 Increased opportunity for optimizing environmental conditions of the microorganisms in regard to the phase of growth or production and age of the culture.
3 Nearly stationary operation, important with slightly mutating microorganisms and those at risk for contamination.

Disadvantages include:

**1 Lower productivity levels due to time for filling, heating, sterilization, cooling, emptying and cleaning the reactor.**

**2 Higher costs in labour and/or dynamic process control for the process**

# Difference between batch, fed- batch and continuous culture technique

## Difference between batch, fed-batch and continuous culture technique

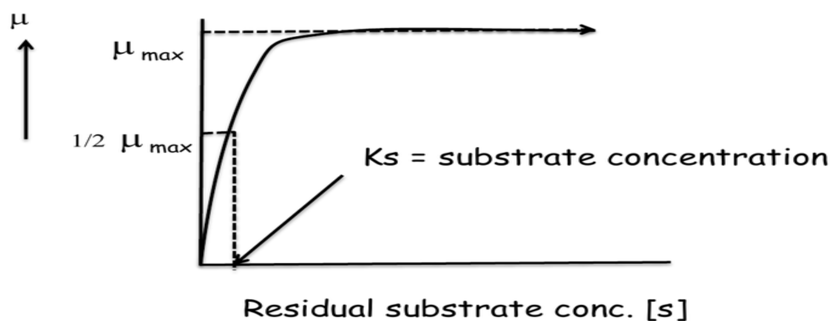
| Characteristics | Batch culture | Fed-batch culture | Continuous culture |
|---|---|---|---|
| Cultivation system | Closed type | Semi-closed type | Open type |
| Addition of fresh nutrition | No | Yes | Yes |
| Volume of culture | Constant | Increases | Constant |
| Removal of wastes | No | No | Yes |
| Chance of contamination | minimum | Intermediate | Maximum |
| Growth phase | Lag, log, stationary and decline phase | Lag, log , stationary and decline phase | Lag and log phase |
| Log phase | Shorter | longer | Longest and Continuous |
| Density of bacteria | Change with time | Change with time | Remain same |
| Product yield | Low | Medium | High |

## Chapter 3: Microbial growth kinetics

**Question 1: Explain batch growth kinetics of microorganisms (VTU)**

### 1. Microbial growth kinetics

**Microbial growth kinetics** is the relationship between the **specific growth rate (μ)** of a microbial population and the **substrate concentration (*s*)**




### 2. Growth curve of batch reactor

- A **growth curve is** a graphical representation of how a particular quantity increases over time.
- **Growth curves are** used in statistics to determine the type of **growth** pattern of the quantity—be it linear, exponential, or cubic
- Cell growth implies **increase in its mass and physical size** controlled by physical, biological and chemical environments.

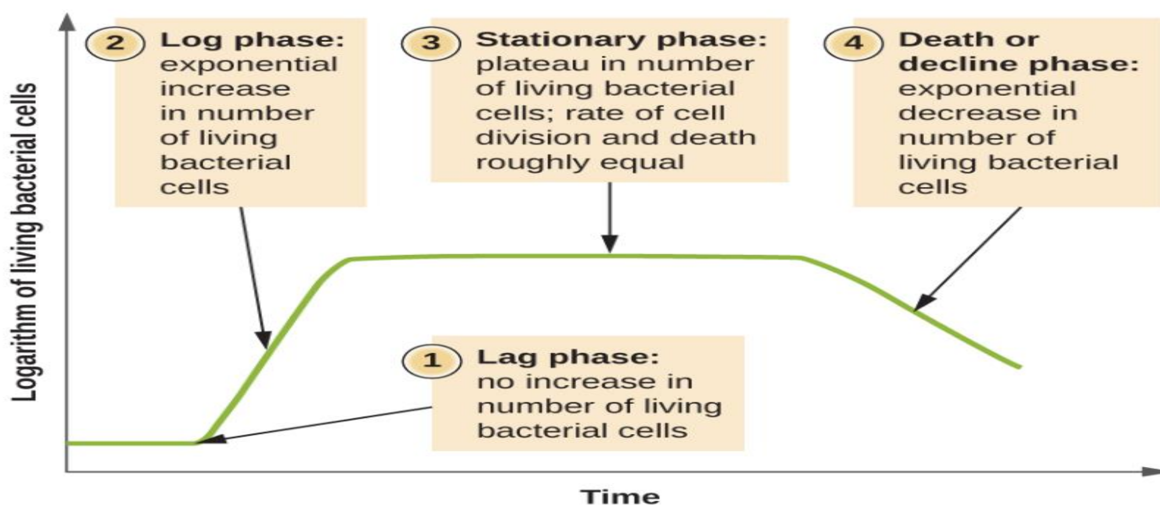



Figure 4. The growth curve of a bacterial culture is represented by the logarithm of the number of live cells plotted as a function of time. The graph can be divided into four phases according to the slope, each of which matches events in the cell. The four phases are lag, log, stationary, and death.

Fig 1. Conceptual plot of plotting the viable cell concentration, X, versus time is growth curve

- Microbial growth is **quantified by increase in the macromolecular and chemical constituents** of the cell and **growth pattern of each microbe is unique**.
- During the lag phase dX/dt and dS/dt are essentially zero.
- However as exponential growth phase begins it is possible to measure dX/dt and dS/dt values which are very useful for defining important microbial kinetic parameters.

Yield coefficient

$$Y = \frac{dX}{dS} = \frac{\text{mass of new cells}}{\text{mass of substrate consumed}}, \text{[dimensionless]} \quad (1)$$

Specific growth rate

$$\mu = \frac{dX}{X_0 dt} = \frac{\text{mass of cells produced}}{\text{original mass of cells} \cdot \text{time}}, \left[\frac{1}{\text{time}}\right] \quad (2)$$

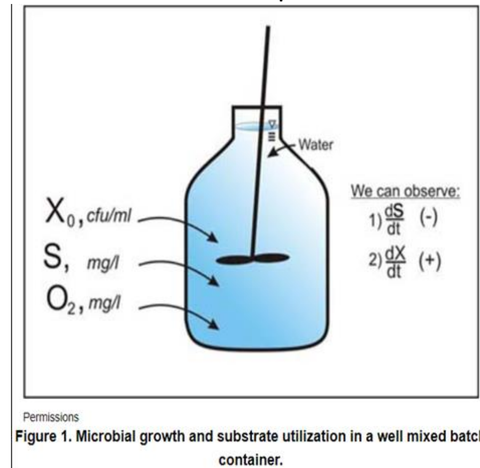



Figure 1. Microbial growth and substrate utilization in a well mixed batch container.

Fig 2 Nutrients + microbial cells > cell growth + energy + reaction products

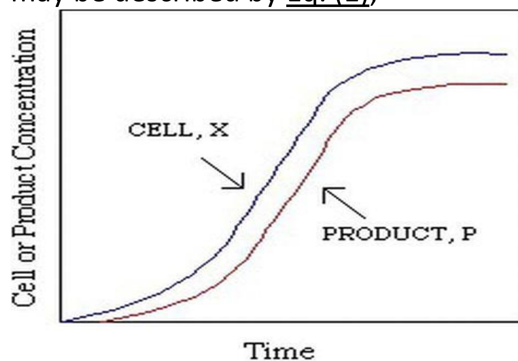
- Using corresponding observations of dS/dt (Substrate utilisation rate) and dX/dt (Cell growth rate) obtained just after the onset of exponential growth phase in we can compute the yield coefficient $Y_{XS}$ (substrate-to-biomass yield )and the specific growth rate μ.
- The yield coefficient, commonly referred to as the substrate-to-biomass yield, is used to convert between cell growth rate dX/dt and substrate utilization rate dS/dt.

Classified based on the relationship between product synthesis and energy generation in the cell:

- Growth associated
- Non-growth associated
- Mixed-growth associated

**2.1 Growth associated**

Growth linked products are formed by growing cells and hence primary metabolites. Figure 1 clearly shows that product is formed simultaneously with growth of cells. That is product concentration increases with cell concentration. The formation of growth associated product may be described by Eq. (1);



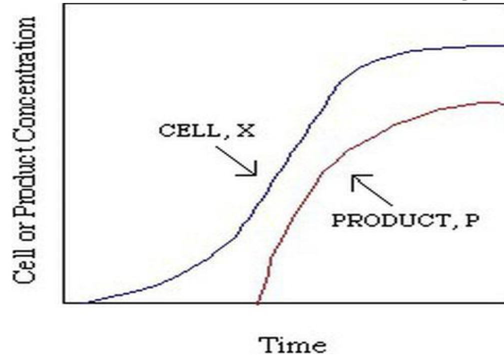

$$\frac{dP}{dt} = r_p = q_p X$$

Figure 1 clearly shows that product is formed simultaneously with growth of cells.

where *P* = concentration of product, *qp* = specific rate of product formation, *X* = biomass concentration.

**2.2 Non-growth associated**

They are formed by cells which are not metabolically active and hence are called secondary metabolitesThe formation of Non-growth associated product may be described by Eq. (2);



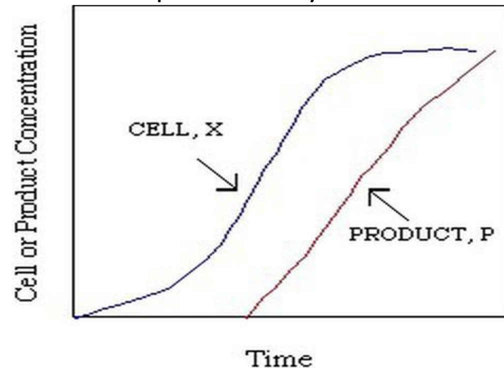

$$q_p = \beta = constant \qquad E2$$

. Figure 2 clearly shows that product formation is unrelated to growth rate but is a **function of cell concentration**.

Figure 2. Non-growth associated.

**2.3 Mixed-growth associated**

The product formation from the microorganism depends on both growth and Non-growth associated. It takes place during growth and stationary phases. The formation of Mixed-growth associated product may be described by Eq. (3);




$$q_p = \alpha_\mu + \beta$$

*In Figure 3, **product formation** is a combination of **growth rate** and **cell concentration**.*

*Figure 3. Mixed growth associated.*

## 3. Growth kinetic relationships

- The yield coefficient and the specific growth rate used to develop three types of microbial growth kinetic relationships; Monod , first order ,and zero order kinetics.

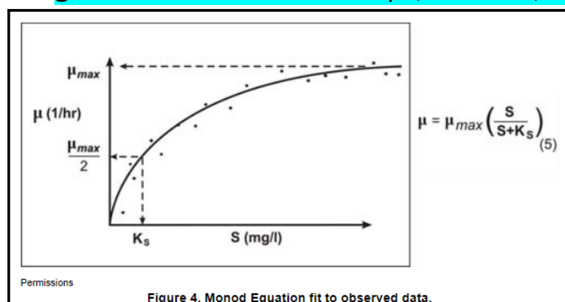
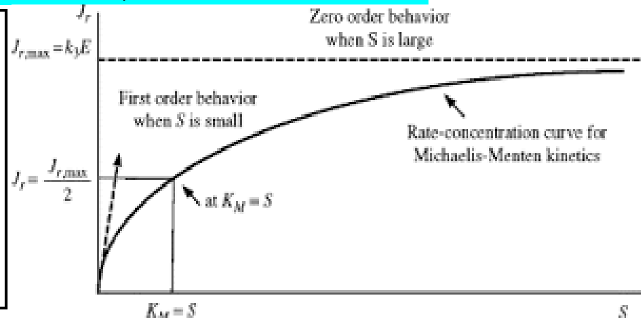



Figure 4. Monod Equation fit to observed data.



## Chapter 4: Development and optimization of Fermentation (Bioprocess optimisation)

**Question 1:** Establish a design strategy to develop and optimize fermentation relevant for bioprocessing. 10 marks

### 1. Introduction to process development and optimisation

- Fermentation Process optimization strategies are important for sustainable development and global security.
- Optimized processes require fewer resources (Fig 1).
- Developing an optimized process help in developing cost-effective fermentation process.
- Fermentation processes are involved in the production of many of the biotechnological products in use today.
- Fermentation has wider application, as fermentation is used in a variety of processes from food and energy production to environmental clean-up
- Hence, Developing and Optimizing fermentation required for **reliable**, **sustainable and cost-effective process and product**.

### a. Process Development

- The objective of process development is the **timely production of sufficient product** to meet market demand using a **cost-effective and reliable process**, which also **meets safety and quality requirements**.

- The broad stages of process development are
  1. **Product Identification:** Product identification is a set of activities to identify the potential product starting from its metabolic pathway involved and its manipulation, physicochemical properties of product relevant for recovery and its pharmacokinetic properties.
  2. **Process Identification:** Process identification is a set of activities aiming to systematically define the set of **processes** of a fermentation and establish clear criteria for prioritizing them
  3. **Product Validation: Process** to demonstrate that the product is safe and efficacious for biotherapeutic and diagnostic purposes
  4. **Scale-Up : Turning a laboratory process into a robust practical commercial process**
  5. **Process Validation:** Establishing documented evidence which provides high degree of assurance that a specific process will consistently produce a **product** meeting its predetermined Quality specifications and Quality characteristics.

### b. Process Optimization

- The **economic objective of process optimization** is to produce the **greatest quantity of saleable products at the least cost to maximize profit**.
- To affect this optimization requires **a process model** spanning **upstream and downstream processes**, which can be used to define strategies for optimizing sub-processes.
- However, **process optimization is applied to the major sub-processes** which have the **greatest impact on the competing priorities of quality, productivity and cost (i.e. efficiency)**. **Optimizing yield** is a preferred strategy to **reducing operating costs**.

- **Optimization strategy at design stage is best option for process development**.
- The potential benefits are **the economic benefits** and **a more robust and more reliable process**.
- Process development and process optimization are thus all part of the same process improvement continuum.
- Optimized processes require fewer resources (Fig 1).

i. Bioprocess design and economic analysis

- During the early stages of development, analysis of Cost-of-Goods (COG) identifies the principle "cost drivers" to the process and quantifies the **sensitivity of COG** to the **impact of the values of these drivers**.
- In any process it is very important to **understand the cost breakdown critically**, so that we can **identify the location of cost drivers** so as to achieve the biggest saving potential.
- The major cost drivers in the bioprocess industries are raw material, utilities, labor and fixed costs.

ii. Comparison of bioprocess alternatives

- Critically analyze the unit steps of Fermentation process and compare with other process alternatives in term of its productivity, efficiency, product stability and cost.
- Simulate the process alternatives by mathematical models to determine if the potential cost advantages of process alternatives are worth the development effort, and to set the production target required for the process alternatives to be economically advantageous.

iii. Bioprocess facility design, engineering and construction

- We need to have accurate and detailed COG simulation to determine the budget for bioprocess plant engineering and construction, to provide detailed specifications of the equipment manufacture, to generate efficient floor plan, utilities and equipment requirements.
- Results obtained from the process simulators can be recommended to for appropriate project execution and provide turnkey project execution resources for facility design, engineering, construction and validation so that the project is implemented in time to reduce the capital cost.
- This is very important as the operational burdens can be reduced, but unnecessary money spent on capital cost cannot be reduce once the project is implemented.

**e. PD (Process development) and PO(Process optimisation) Strategies should incorporate:**
**Isolation of microorganisms of potential industrial interest**

- Classical methods of isolation and selection of microbial strains are expensive, time consuming and without any clear objectives.
- Cost can be reduced considerably by isolation of microorganisms from variety of sources, with desired characteristics, which gives a selective edge over existing strain.
- Important characteristics, such as
  c. Growth on simple growth medium,
  d. Growth at ambient temperature
  e. Better resistant to contaminants,
  f. Which will be of economic significance -may be screened and selected.

## g.Optimization Techniques

- **Response surface methodology (RSM):** RSM, which uses factorial designs to optimize the production processes of the desired metabolites.
- **Artificial neural network:** An artificial neural network (ANN) is a mathematical or computational model that is influenced by the structural and/or functional aspects of the biological neural networks.
- **Genetic algorithm (GA):** A trained mathematical model serves as a fitness function in the determination of optimum concentration of the medium components using GA.
- **Nelder Mead (NM) simplex method** is another statistical technique, which has been found to be helpful in reducing the expenses of classical optimizations and gives satisfactory results.

## Chapter 5: PRODUCTION OF PRIMARY AND SECONDARY METABOLISM PRODUCTION

**Question 1: Optimize the strategy to optimize primary and secondary metabolite production (VTU)**

**Introduction**

• Applications of microbes for industrial production of primary and secondary metabolites - Industrial Microbiology.

• Metabolism in microorganisms involves two pathways: Primary metabolic pathways (PMPs, produced during the growth phase of the organism) produce too few end products , while secondary metabolic pathways (SMPs, produced during the stationary phase) produce a variety of products.

• There are some similarities between the pathways that produce primary and secondary metabolites:

θ The product of one reaction is the substrate for the next and

θ The first reaction in each case is the rate-limiting step.

θ Also the regulation of secondary metabolic pathways is interrelated in complex ways to primary metabolic regulation.

**Industrial fermentation based on the end-product application, can be categorized into four types:**

1**. Biomass:**The end-product is viable cellular material eg, single cell protein, baker's yeast, probiotic cultures.

**2. Extracellular metabolites:** Chemical compound intermediates of microbial biochemical pathways are produced and can be divided two groups:

a. Primary metabolites (produced during the growth phase of the organism, eg, ethanol, citric acid, glutamic acid, lysine, vitamins and polysaccharides)

b. Secondary metabolites (produced during the stationary phase, eg, penicillin, cyclosporin A, gibberellin, and lovastatin).

**3. Enzymes and other proteins (intracellular components):** A key component of this process is lysis of cells at the end of fermentation. Proteins are typical end products and need to be purified and crystallized.

**4. Substrate transformations:** Raw material is biologically transformed into a finished product. Generally used for steroid transformations, food fermentations and sewage treatment.

**Primary metabolites**

- Involved in growth, development and reproduction. Hence, essential for survival and existence of the organism and reproduction.
- Formed at the same time as new cells. Production curve follows the growth curve.
- Formed in trophophase during exponential growth as normal end products of primary metabolism.
- Also called central metabolites as these maintain normal physiological processes. Cells maintain optimum concentration of all macromolecules (proteins, DNA, RNA etc.).
- Produced in adequate amount to sustain cell growth for example vitamins, amino acids, nucleosides etc.
- 

- Overproduction can be genetically manipulated. Auxotrophic (auxo, "increase," and trophos, ''food'') mutants having a block in steps of a biosynthetic pathway for the formation of primary metabolite . Growth rate slows down due to limited supply of any other nutrient.
- Metabolism does not stop but product formation stops. Industrially important for example ethanol, acetone, lactic acid, $CO_2$.
- Common food supplements, L-glutamate and L-lysine, are produced and purified via the mass production Corynebacterium glutamicum.
- Citric acid, commonly used in pharmaceutical and cosmetic industries is produced by Aspergillus niger.

**Secondary metabolites**

• Secondary metabolites are not produced until the microbe has largely completed its logarithmic growth phase and entered the stationary phase of the growth cycle. Period of production is called idiophase and metabolites as idiolites.

• In the idiophase, cells do not divide but are metabolically active.

• Idiolites are organic compounds produced only after considerable number of cells and a primary metabolite have accumulated (end or near the stationary phase of growth). Rather it can be said that these are produced under sub-optimal concentrations of $O_2$ , deviations of pH or when primary nutrient source is depleted.

• Though idiolites are a characteristic feature of fungal, yeast, actinomycetes and bacterial growth but are not produced by a few strains of E. coli.

• In some strains secondary metabolite are produced by further conversion of a primary metabolite..

• Not necessary for growth, development, and reproduction like primary metabolites. Their production is influenced by environmental factors.

• Secondary metabolites are synthesized for a finite period by cells that are no longer undergoing balanced growth.

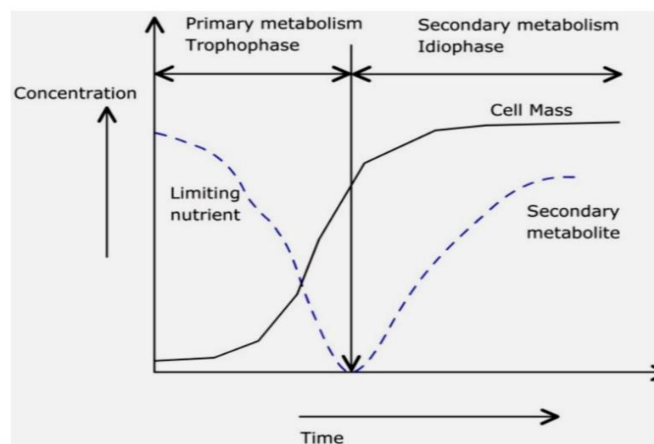
• A single microbial type can produce very different metabolites.

• Their **production is regulated by complex biochemical pathways** and some strains can produce a variety of idiolites. For example a strain of ***Streptomyces* can produce a variety of 35 anthracyclines.**

• **Overproduction of secondary metabolites can be achieved by manipulating larger number of genes** (gene cassettes).

Typical examples include antibiotics, toxins and pigments to name a few.

Primary and secondary metabolism.




During the trophophase, the cell mass increases logarithmically but as the resources become limiting, growth rate drops and production stops.

Question 6. What is a Product Development Strategy? Develop an efficient effective product design strategy in fermentation process

- There was a time in in the industry when a company could create a notable product and **reap the benefits of that product for many, many years**.
- Unfortunately, today's market is far more competitive than it used to be, and companies now face the prospect of **market saturation for single product designs,** causing revenues and profits to falter or fall.
- Savvy companies are now understanding **how product evolution** or **product diversification** is needed to stay at the top of the game in their industry.
- To do that, you need a **product development strategy**.

### Product Development Strategy Definition

- A <u>product development strategy</u> is a strategy based on **developing new products** or **modifying existing products** so they appear new, and offering those products to current or new markets.
- These strategies typically come about when **there is little to no opportunity** for new growth in a company's current market.
- At that point, a company has one of three choices: **create an updated product** for a current product in a current market, **enhance an existing product** for a new market, or **simply move away from the product altogether, and cease growth**.
- Most competitive companies **won't opt for the third choice so a strategy** is therefore **designed to either evolve a product for its existing market**, or **enhance it to introduce into a new market**.

### Updating a Product – Product Evolution

Depending on the product a company offers, there may be **little opportunity to introduce that product into new markets**.

- With that limitation, a company must instead look at **updating an existing product** for its current market. This is known as **<u>product evolution</u>**.

- Product evolution is used by companies **who have the vision to not only see a product idea, but how that product can evolve over time**.
- It's the idea of mapping out, often before the first product is even manufactured, what **future iterations of a product** might be as it improves and grows.
- Another term used for this is "**product modification**". Product modification strategies are generally aimed at existing markets, although a side benefit may be the capturing of new users for the new product.

### Creating a New Product for a New Market

- This approach is actually much more common than most people may think.
- This is the **idea of a company going outside its existing business model** or **approach and developing new product for a new market**.
- **Creating or enhancing a product for a new market** requires steps similar to designing a brand new product.
- Steps to create a new product (or enhance an existing one) for a new market may include all or some of the following steps: **product design, product analysis, design documentation, prototyping, and of course product production**.
- Regardless of which path a company chooses to take, **a product development strategy is critical to the ongoing growth and success of a company**.

### Why biotech is special in product development?

- An essential element for fermentation processes, whether for cell mass or product, is the **efficient growth of the organism**.
- **Optimizing the environment for growth** requires balancing the **economic feasibility of maintaining a suitable chemical and physical environment**, while meeting the physiological needs of the organism.
- In biomass production, objectives are **productivity optimization** and **yields conversion**, but key operating variables are **carbon source** and **oxygen**.
- In enzyme production, objective are **maximum total activity** and **maximum productivity**, and important variables are **growth rate** and **composition and concentration of nutrients** in the environment.
- For metabolite production, objectives are **rapid accumulation of cell mass**, **conditioning of the cell to maximize the rate** and **extent of product formation but variables are growth rate**.
- An **analysis of strategies to meet the metabolic demands** provides insight into ways to improve the organism through genetic engineering, as well as the process through bioreactor design.

## 7. Instrumentation and control of fermentation process

INSTRUMENTATION AND CONTROL OF A BIOREACTOR

Bioreactor Control

- ¨ Temperature
- ¨ Dissolved oxygen ¨
- pH ¨ Foam ¨
- Agitator speed ¨
- Nutrient addition rate ¨
- Level control

**Temperature Control ¨**

- Most fermenters operate around 30-36°C.
- ¨ Requires water jackets or pipe coils.
- ¨ In many small systems there is a heating element, 300 to 400 W capacity is adequate for a 10 L fermenter, and a cooling water supply which are on or off depending on the need for heating or cooling.
- In exothermic reactions, the fermentation vessel requires cooling.
- ¨ The heating element should be as small as possible to reduce the size of the 'heat sink' and resulting overshoot when heating is no longer required.
- For small-scale use, a unit will pump recirculating thermostatically heated water through fermenters for up to 10L capacity and give temperature control of ±0.1ºC. ¨
- In large fermenters, a regulatory valve at the cooling – water inlet may be sufficient to control the temperature.
- There may be provision for circulation of refrigerated brine if excessive cooling is required.
- ¨ Steam inlets to the coil and jacket must be present if a fermenter is being used for batch sterilization of media.
- Temperature is measured using glass thermometers, thermocouples, thermistors, resistance thermometers. ¨ Thermocouples – cheap and simple to use, but they are low in resolution and require a cold junction.
- ¨ Thermistors – are semiconductors, which exhibit a change in electrical conductivity with temperature.
- Very sensitive and inexpensive.
- They give a highly nonlinear output. ¨ Platinum resistance thermometers are usually preferred as standard.
- To avoid contamination, the thermometers are usually fitted into thinwalled stainless steel pockets, which project into the bioreactor.
- The pockets are filled with a heat-conducting liquid to provide good contact and to speed the instrument response.
- The resistance thermometer works on the principle that electrical resistance changes with temperature.

**Dissolved Oxygen Control ¨**

- In most aerobic fermentation, it is essential to ensure that the dissolved oxygen concentration does not fall below a specified minimal level.
- ¨ The most common method of measuring DO is based on electrochemical detector.
- Two types of detector are available: galvanic and polarographic detectors.
- Both use membranes to separate electrochemical cell components from the both.
- ¨ The membrane must be permeable to oxygen only and not to other chemicals.
- Oxygen diffuses from the media, across the permeable membrane to the electrochemical cell of the detector, where it is reduced at the cathode to produce a measurable current or voltage, which is proportional to the rate of arrival of oxygen at the cathode.
- The probe has to be calibrated for accurate measurements.
- ¨ In the galvanic detector, it has a lead anode, silver cathode and employ potassium hydroxide, chloride, bicarbonate or acetate as the electrolyte.
- The lead anode is in the electrolyte solution, which is oxidised.
- Therefore, the probe life depends on the surface area of the anode.

**pH Control**

¨ Carried out using a combined glass electrode.
¨ The electrode maybe silver/silver chloride with potassium chloride as an electrolyte. ¨ Occasionally calomel/mercury electrodes are used.
¨ The electrode is connected via leads to a pH meter/controller.
¨ A recording unit maybe wired to the meter to monitor the pH pattern throughout the process cycle.

## Chapter 8: Preservation of Microbial Strains

### Question 8: How to Preserve Industrially Useful Microorganisms: Top 7 Methods and Techniques

- Isolation, preservation and detection of industrially useful microorganisms is a time consuming and very expensive process.
- Therefore, it is essential to keep the isolated organisms in a viable condition so that it retains the desirable characters and it can be used whenever required for industrial production.
- This is done by storing it by creating certain special environmental conditions by which it remains in a viable condition but in an inactive state.
- This phenomenon is called as preservation of culture.
- The preservation of culture should be done in such a way that it eliminates the genetic changes, prevents contamination and retains the viability.

**Though there are several methods or techniques of preservation of industrially useful organisms, a description of only some important methods are given below:**

***(1) Repeated Sub-Culturing:***

- This is the most common, simplest and routine method of preservation of microorganisms. Selected microorganisms are initially grown on agar slants.
- After sufficient growth has taken place, they are transferred to fresh medium before they lose their viability.
- The appropriate time period for such transfer ranges from a week to few months (generally four to eight months).
- Though an organism may be kept viable by this method but there is a probability of occurrence of mutations in the organism, which may lead to strain degeneration and subsequent uselessness of the organism for commercial usage.
- That is why it is less frequently used for preserving microorganism.

***(2) Storage under Liquid Nitrogen:***

- This method is also called as cryogenic storage method, because a cryoprotective agent in the form of 10% glycerol is used.
- Industrially useful microorganisms are stored under very low temperature ranging from 150°C – 196°C.
- In this method ranging, low temperatures are created by employing liquid nitrogen.
- Metabolic activities of microorganisms are reduced considerably at this low temperature.
- This method is generally employed for the preservation of fungi, bacteriophages, viruses, algae, yeasts, animal and plant cells, and tissue cultures.
- This technique involves growing the desired microorganism in sufficient quantity either in the form of cells or spores or fragments of fungal mycelium.
- The grown up culture is suspended in 10% glycerol. The suspension is then introduced in to small ampoules at the rate of approximately 0.5 ml each. The ampoules are usually made up of borosilicate glass.
- The ampoules containing culture suspension are frozen and sealed hermitically.

- Freezing is done either by directly dipping the ampoules into the liquid medium or hanging the ampoules initially over the column of liquid nitrogen for some time and finally dipping into the liquid.
- The frozen ampoules are then dipped one above the other on small aluminum containers at the rate of six ampoules per can.
- The cans are then packed in aluminum boxes, 20 per each box.
- The perforations allow free flow of liquid nitrogen.
- There may be loss of viability in few cells during freezing process but there is virtually no loss of viability during storage phase.

***(3) Employment of Dried Cultures:***

- This technique has been used extensively for the storage of fungi and actinomycetes particularly for sporulating mycelial organisms.
- Moist soil is generally used as a preservating medium.
- Moist soil is first sterilized and then inoculated with a desired culture and incubated for several days to allow some growth to occur.
- The soil with growing organism is dried at room temperature for a period of two weeks.
- The dried soil is then stored in a dry atmosphere or in a refrigerator.
- Silica gel and porcelain beads may be used alternatively for soil. It is possible to preserve a culture for more than 20 years.
- **method about 50% were viable, after 20 years storage.**

***(4) Lyophilization:***

- It is one of the best methods for long-term preservation of microorganisms.
- It is generally used for the preservation of fungi, viruses, bacteria, enzymes, toxins, sera and other microorganisms.
- It is a convenient method for the preservation of large number of cultures.
- Lyophilization, which is also called as freeze drying involves freezing of a culture followed by its drying under vacuum which results in the temporary inhibition of metabolic activities of microorganisms.

**(5) With Mineral Oil:**

- This is one of the cheap and easy methods of preservation. Many microbes can be successfully preserved for longer time.
- In this method tubes with sterile agar slants are inoculated with a given culture. The tubes are incubated till sufficient growth of the given microbe takes place.
- The grown up culture is covered with a suitable mineral oil to a depth of about 1 cm above the top of the slanted surface using sterile technique.
- Thus, over laid cultures can be stored at room temperature or preferably at low temperature by about 15°C.
- Paraffin oil of specific gravity 0.865 to 0.890 is generally used in this method.
- The oil is sterilized either in McCartney bottles for 15 minutes at $103.41 \times 10^3$ $Nm^{-2}$ pressure or in an autoclave at $103.41 \times 10^3$ $Nm^{-2}$ pressure for 2 hours and then dried in an oven at 170°C for 1-2 hours, before it is used.
- Maintenance of viability of a culture under this treatment largely varies with the species and generally ranges from 10-20 years.

*(6) Storage in Soil:*

Spore suspension in sterile water is poured into a culture bottle containing twice autoclaved loam soil (20% moisture). Fungal growth is allowed for few days and then stored with loose caps in a refrigerator. Fungi like Khizopus, Altemaria, Aspergillus, Penicillium and Fusarium which have long viability and stability can be maintained by this method.

***(7) Silica Gel Storage:***

- Mc Cortney bottle is filled partly with medium grain non-indicating silica gel and sterilized by dry heat.
- The bottles are kept in a tray of water to the depth above the level of the gel.
- The water is frozen by placing the tray in a deep freeze with the temperature 17°C to 102°C.
- Spore suspensions are prepared in sterilized and cooled 5% skimmed milk and added to silica gel crystals in the tray of frozen water using Pasteur pipette and wetted three quarters to avoid over saturation.
- The gel bottles are left in the ice bath for about 20 minutes until the ice around them have melted a little.
- The crystals are agitated to ensure thorough dispersion of the suspension. Bottles are dried with the caps loose for 10-14 days at 25°C until the silica gel crystals separate.
- Bottles are reversed down from tightly and stored over indicator silica gel in air tight container at 4°C. The indicator gel requires replacement once or twice in a year.

## Chapter 9: **Production of secondary metabolite: Antibiotic Eg: Penicillin**

- Penicillin is produced by a fermentation process in a stirring tank batch reactor at temperature of 25 °C with continuous supply of sterile air.
- Most of the antibiotic biosynthetic pathways are well known along with their **relevant enzymes and gene location**. With this knowledge, we will be able to **improve the strains and optimize the productivity of fermentation**. Structure of the penicillin G and penicillin V is as follows.
  Both of these nuclei are bulk products and are used for chemical synthesis of the semi-synthetic penicillin.
- Penicillin G and V are fermented products from fungus ***Penicillium chrysogenum*** and bulk of these products are used as starting material for production of the **active β-lactum nucleus**, 6-aminopenicillianic acid
- Penicillin G can also be **ring expanded chemically to the cephalosporin nucleus** which after **enzyme hydrolysis**, yield the **active nucleus 7-aminodesecetoxycephalosporanic acid (7-ADCA)**.
- Penicillin improvement program have been in existence for over 50 years.
- By using **conventional mutation and selection**, the original **titres of less than 0.1 mg $ml^{-1}$ have been increased by 400-folds**.
- Further gains have been realized by media modification and engineering developments
- Rate limiting steps in the synthesis have been identified and attempts made to increase the productivity of the rate limiting enzymes. For example, extra genes coding for **cyclase (pcbC) and acyl transferase (penDE)** have been inserted to *Penicillium chrysogenum.*
- Strain improvement is very much important as high concentration of penicillin increases the **volumetric productivity, increases the extraction efficiency, decreases the proportion of unwanted products, makes purification easier** and **reduces the cost of product**.
- Genetic engineering opened up other alternatives to the present-day strain improvement techniques.
- This can be used to express select enzymes both in recombinant *Escherichia coli* and in the producing microorganism.
- **Additional copies of the genes encoding these rate-limiting enzymes are incorporated to strengthen the weak links**.
- New enzymes can also be added to produce new metabolites.
- Penicillin G or Penicillin V can be produced by the addition of the precursor molecule, **phenyl acetic acid or phenoxy acetic acid** respectively to the fermentation of *Penicillium chrysogenum.*
- For the optimum production, *Penicillium chrysogenum* is grown on **batch medium of corn steep liquor or soy flour along with minerals and fed carbohydrate** as corn syrup thought the fermentation cycle.
- Along with this, precursor and ammonium sulphate is fed to maintain critical concentration of these components needed for biosynthesis of penicillin.

**3 Fermentation**

- **Stirring tank reactor (STR)** has been used in bioprocess industries since 50 years with extensive scale up studies and design correlations.
- Large-scale fermentation method is always designed after some work in the **laboratory and scale up to production level** by various approaches like **rule of thumb, scaling according to one specific parameter, geometric similarity and scale-down method**.
- Initially we need to calculate the number of fermenters required based on the input from the annual production target.

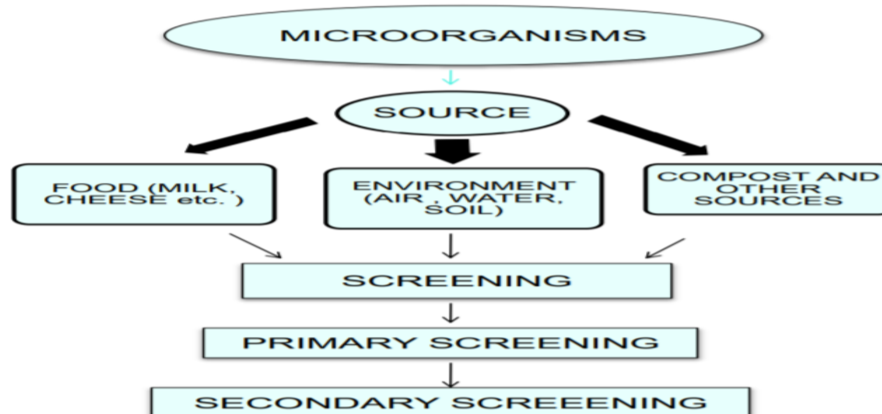
## 0. Enumeration and screening of microbial metabolites

### Enumeration and screening of novel microbial secondary metabolites

- The isolation and subsequent screening of bacteria from diverse habitats has **led to the discovery of many novel and useful secondary metabolites**.
- Surprisingly, the approach to the search for potentially valuable bacteria has been **largely empirical** and restricted to sampling a tiny fraction of the microbial community found in natural habitats.
- In essence, many representatives of a **few well-established bacterial taxa are isolated** and then screened in the hope that something useful turns up.
- However, it is now **essential to develop new and more objective procedures** for the selective isolation of **uncommon and novel** (that is, previously undiscovered) microorganisms in order to improve the biological quality of the material screened.
- **Taxonomical expertise** is needed to apply rapid and reliable methods to differentiate between **microbial taxa in order** to reduce the rediscovery of known bioactive compounds to an acceptable minimum.

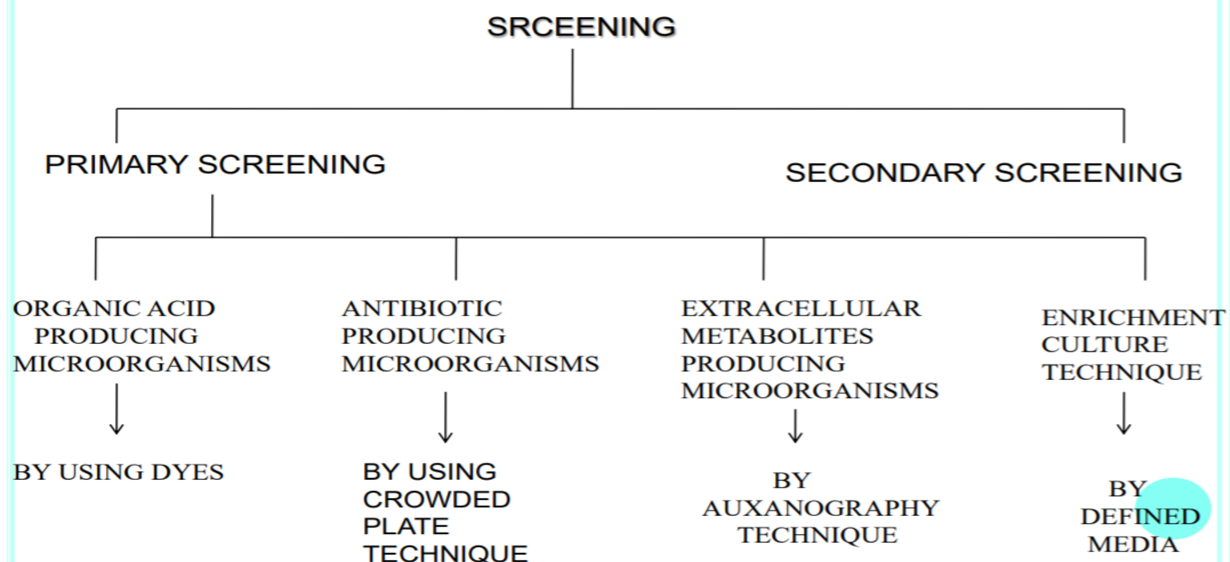
**SCREENING**

The procedure of isolation, detection , and separation of microorganisms of our interest from a mixed population by using highly selective procedures is called SCREENING



## IMPORTANT THINGS TO BE CONSIDERED WHILE SCREENING :-

- 1.) CHOICE OF SOURCE - Samples from screening is taken from soil, water, air, milk, compost etc.
- 2.) CHOICE OF SUBSTRATE -Nutrients and growth factors should be supplied for growth of desired microorganism.
- 3.) CHOICE OF DETECTION - Proper isolation and detection of desired microorganisms is important




**1)Primary Screening:**

- This consists of some elementary tests required to detect and to isolate new microbial species exhibiting the desired property.
- The techniques involved are:

i)**The crowded plate technique:**

- Used to detect and isolate antibiotic producers.
- Serial dilutions of soil sample are made and they are plated on nutrient agar.
- After incubation the ability of the organism to exhibit antibiotic activity is indicated by the presence of a zone of growth inhibition around the colony.
- **Such cultures are then selected, purified and maintained as pure culture.**

**ii)Auxanography:**

- This technique is largely employed to for detecting microbes able to produce growth factors extracellularly.
- The two major steps are:
- A filter paper strip is put across the bottom of petridish.
- The nutrient agar is prepared and poured on the paper disc and allowed to solidify.
- Soil sample is diluted and proper dilutions are inoculated and incubated.

  **a)Preparation of first plate:**
- **b)Preparation of second plate:**
- A minimal medium lacking the growth factor is prepared and seeded with the test organism. T
- he seeded medium is poured onto a fresh petridish and the plate is allowed to set.
- The agar in the first plate is then carefully lifted with the spatula and placed on the second plate without inverting.
- The growth factors produced by the colonies present on the surface of the first layer of agar can diffuse into the lower layer containing the test organism.
- The zones of stimulated growth of the test organism around the colonies Is an indication that the organism produce growth factor extracellularly.

**iii)Enrichment culture technique:**

- This was first designed by Beijerinck to isolate the desired microorganism from a heterogenous microbial population
- Nutrient broth is inoculated with the microbial source material and incubated.
- A small portion of the inoculums is plated on to the solid medium and well isolated colonies are obtained Suspected colonies from the plate are subcultured on fresh media and subjected for further testing.
- iv)Use of an indicator dye:
- This method is used to detect microbes capable of producing organic acids or amines which changes the colour of the medium according to pH.
- Examples of such dye are neutral red, bromothymol blue, etc.

**2)Secondary screening:**

- This helps in detecting in really useful microorganisms in fermentation process.
- This is accomplished by performing experiments in agar plates, in flasks or in bioreactors containing liquid media. Example: antibiotic producing Streptomyces sp is taken.
- Streptomycal isolate is streaked as a narrow band on nutrient agar plates and plates are incubated.
- Test organisms are then streaked from the edge of the plates without touching the streptomycal isolate and the plates are then incubated.
- At the end of incubation, growth inhibitory zones for each organisms are measured in millimeters.
- Such organisms are again subjected to further testing by growing the culture in sterilized liquid media and incubated at constant temperature in a mechanical shaker.
- Samples are withdrawn at regular intervals under aseptic condition and are tested in quality control laboratory.

The tests to be done include:

i)checking for contamination
ii)cheking for pH
iii)estimation of critical nutrients
iv)assaying of the antibiotic. Some other determinations include: i)screening of fermentation media in which high yield is obtained. ii)determining whether the antibiotic is new
iii)determination of number of antibiotics accumulated in the broth. iv)toxicity tests are to be done in mice.
v)the streptomycete is characterized and is classified into species.

**3)Detection and assay of fermentation products:**

Primary and secondary screening requires the use of good detection and assay procedures for fermentation products.
These procedures must be quick, simple, reliable and accurate.
These assays usually fall into 3 categories:

**i)Physical-chemical assay:**

Three methods are employed

**a)Titration and gravimetric analysis:**

- Amount of organic acid such as lactic acid produced during fermentation is determined by adding a pH indicating dye such as bromothymol blue,to sample of fermentation broth followed by titration with alkali of known strength. Electrometric titrations are also employed.

**b)Turbidity**

- Analysis and cell yield determination: Turbidity analysis is used to measure the cell yield of a fermentation. Cells suspended in growth medium are diluted to a turbidity range that can be measured as optical density with a calorimeter. Portions of fermentation broth with their cells are centrifuged in graduated centrifuge tubes and the volumes of sedimented cells are measured in cubic centimeter for cell yield determination. If the medium contains sediment other than microbial cells , plate counting procedure is done.
- c)Spectrophotometric Assay: It is used to measure the amount of absorption of visible light by colored solutions at specific wavelength, quantity of UV light absorbed by a compound or intensity of fluorescence emitted when a compound is exposed to UV.

**ii) Biological Assay:**

- These are more difficult to perform, provide greater error and less reproducible than chemical or physical assays. This assay falls into 4 groups.

**a)Diffusion assay:**

- Carried out on solid medium and the compound to be assayed is allowed to diffuse through medium in a radial fashion. The diameter of the area reflects the concentration of compound being assayed and compared with standards.

**b)Turbidimetric and growth assays:**

- These are the method in which the effect of compound under test in liquid culture is measured associated with turbidity showing growth rate or total growth of

microorganisms. Turbidity can be determined using spectrophotometer. Readings are made as OD/absorbance.

**c)Endpoint determination assays:**

- A fermentation product that inhibits growth, such as antibiotic is inoculated with test organism. Tubes are incubated and observed for growth. Relative amount of fermentation product in original fermentation broth is determined by amount of dilution which the fermentation product can withstand in the assay tubes and still able to inhibit growth of test organism.
- d)Metabolic response assays: Used for assays of metabolic reactions such as acid production, Co2 evolution, oxygen absorption and enzyme dehydrogenase activity.

**e)Enzymatic assay:**

- Highly specific and quantitatively detect minute amounts of fermentation products. Eg: L-glutamic acid can be assayed by adding washed cells of certain strains of E.coli which contains the glutamic acid decarboxylase.

**iii)Chromatographic Partition Assay:**

- This allows detection of compounds in either pure/impure states. Paper and thin layer chromatography are examples of partition chromatography.
- Solute/sample is partitioned continuously between a stationary phase, such as paper or silica gel of thin layer plates and a mobile phase, consisting of mixture of solvents, as these solvents migrate across the paper/silica gel layer.
- Paper chromatography utilizes a good grade of chemically clean filter.
- Thin layer chromatography requires thin layer of silica gel, aluminium oxide or others applied to the surface of glass plate.
- A binding compound is used such as calcium sulphate/starch to bind the layer to the glass. Sample is placed as spot on the paper/glass and allowed to dry.
- After drying, paper or glass containing the sample spot is called as chromatogram.
- A mixture of solvents is allowed to migrate across the chromatogram, but not completely. During solvent migration individual components of the spot also moves along the stationary phase.
- The ratio of the distance from the origin that each compound has traveled to the total distance traveled by the solvent provides ‘Rf value’ which is characteristic of each compound. Solvent migration must be carried out in closed tanks
- The compounds being separated will be observed when they are colored. Eg: aminoacids are detected by spraying the chromatograms with ninhydrin to yield purple colored spots.
- Antibiotics can be detected by placing the chromatogram for a short period on the surface of inoculated agar.
- Antibiotic diffuses from the chromatogram to the agar and shows inhibition of growth of test organism.

## Chapter 11 :Strain improvement: Genetic strategy to process and product optimization

### 11. Design a microbial strain improvement programme in cost effective and sustainable manner

- **Microbes generate a wide range of products that are of interest to human beings.**
- **These products are produced in low concentrations.**
- **Because microbe regulates its expenditure of energy, efficiently to maximize its growth.**
- **By using metabolic and genetic engineering techniques these products can be overproduced.**
- **Strain improvement has been a strategy of industrial processes for a long time.**
- **Because it is necessary to make the industrial processes more efficient and cost effective.**
- **Overproduction of primary metabolites and secondary metabolites is a complicated process.**
- **Therefore a prerequisite of strain improvement is**
  - **Having knowledge of microbe's physiology**
  - **knowledge of pathway regulatory controls.**
- **However, current advances in gene Strain improvement techniques have made it easier to modify the strain.**
- **A strain is a genetic variant or subtype of a microorganism.**
- Strain improvement **is the tailoring of microbial strains for specific biotechnological purposes.**
- Strains may be genetically modified in various ways in order to
  - **Improve existing desirable capabilities,**
  - **Eliminate undesirable qualities**
  - **Add new properties**
- **Conventionally**, strain improvement has been achieved through
  - **mutation,**
  - **selection,**
  - **genetic recombination**.
- Successful development of improved strains requires
  - **Knowledge of physiology, pathway regulation and control**,
  - **Design of creative screening procedures**,
  - **Mastery of the fermentation process for each new strain**
  - **Sound engineering know-how for media optimization**
  - **Fine-tuning of process conditions**.

**Strain Improvement of Micro-organisms Used in fermentation.**

- In industries the micro-organisms are **selected by using various screening procedure.**
- The strain which is selected on industrial scale for commercial production of a product should be **able to produce high yield of product constantly**.
- This constant high yield of product **makes the fermentation economic** as well as **face the competition with other industries**.
- For obtaining high yield of product the **industries carry out strain improvement** as well as **strain selection programs continuously**.
- During the strain improvement program **various parameters are adjusted to increase product yield.**

- The parameters like pH, temperature, media components, aeration, agitation, inoculum levels are adjusted and variation in this levels are tested for increasing yield of product.
- But however this **change in parameters doesn't give large yield and effective results** by microbial strains used.
- Hence constant **strain improvement program** and **selection of the most efficient strain for production** of product on industrial scale is required.
- The strain improvement of micro-organism used in fermentation process is done by **altering the genetic make up of strain** and **selecting the most efficient strain** from various improved strain and result in increased yield of product.
- After the strain improvement program the **selected strain should be genetically stable**.
- The selected strain **should produce desirable product in large amount** and **undesired product in less quantity**.
- Before caring out the mutation program the selected strain **should be efficient under all optimum fermentation condition**.
- The selected strain is **exposed to various strain improvement programs** and the strain giving high yield of product **is selected among all tested strains**.
- The strain that is selected should be **able to produce high amount of yield** as **compared to the original unaltered parent strain**.

**The genetic make-up of selected strain can be changed by using following methods:-**

1. Genetic recombination or gen transfer.
2. Mutation
3. Genetic engineering

**1] Genetic recombination or gene transfer**

- Transfer of a gene from one type of strain to other type of strain of micro-organism takes place.
- This gene transfer takes place by
  - **transformation:** Donor DNA free in the environment/DNA is transferred as naked DNA
  - **transduction:** Donor DNA transfer is mediated by a virus
  - -**conjugation:** Transfer involves cell-to-cell contact and a conjugative plasmid in the donor cell
- Gene transfer can be carried out by **protoplast fusion** of two different efficient strains and new improved strain can be developed.
- The fungal strain undergo **para sexual cycle** and **mitotic cell division** which is very important and of great value in strain improvement.
- **Genetic Breeding** is also possible in different genera of yeast.
- Thus by using **genetic recombination** and **gene transfer technique** it is possible to combine the desired characters from two different strains of same species by inter strain breeding and obtain a efficient strain that is able to produce high yield of product on industrial scale .

**2] Mutation**

- Mutation can be defined as **change in genetic structure of micro-organism**.
- The **ability** of micro-organism to produce a desired product can be **enhanced by a mutation process**.

- In mutation process the **most stable and efficient strains** are exposed to mutagenesis by using **different mutagenic agents**.
- The mutagenic agents like ionization, ultraviolet radiation, acids, and alkalies are used in mutation.
- Result of mutation obtain should be **increased in yield of desired product** and **decreased yield of undesired product**.
- The mutagenic agents are **used in such concentration** that maximum number of cells die due to mutagenic agents and **only the that are capable to carry out mutation and have the capacity to tolerate the levels of mutagenic agents are able to survive**.
- From the survived cells the cells which are undergone mutation and have the **capacity to produce high yield of fermented product** is selected.
- Whenever the microbial cells are subjected to mutation there are two possibilities and that are the mutation occurred may be **major mutation** or **minor mutation**.
- The strain undergone desired mutation should be carefully **selected, isolated** and **maintained** properly.
- The selected mutant strains with **altered morphological and biochemical characters** are now the **strain that produce high yield of desired product**.
- The improved selected strain should be **tested with laboratory scale** experiments followed by **pilot plant experiments** and then further used on **commercial scale.**
- The **finalized strains are selected, purified and maintained**.
- All the test and experiments should be carried out with **high accuracy** as these procedures are time consuming and expensive.
- Further when these strains are used on **industrial scale a proper record of yield produce should be maintained** as minor changes should be noted.

**3] Genetic engineering**

- Genetic engineering technique is the most successful technique used for **strain improvement programs** on industrial scale.
- It is also called as **Recombinant DNA technology** and in this process there is **alteration of genetic characters of cells** and hence result in change in **phenotypic character of cell**.
- In this technique a desired type of **gene is introduced from one microbial cell to other microbial cells** by using **cloning vectors**, like plasmids, phages or cloning vectors.
- **A specific and desired character of a microbial cells** can be introduced in the selected strain **to improve the yield of product.**

**Strain improvement**

- Mutation/selection program without any knowledge of biosynthetic pathways are time consuming, labor intensive and very random.
- Adoption of strain improvement using mutation/selection program in the existing or proposed process can be a very cost effective.
- Mutation/selection program can be initiated or adopted only if it can be justified on the financial ground to improve overall economy of the process.
- With the proper knowledge of the biosynthetic pathways, activity of the undesirable enzymes can be blocked to push the biosynthetic path way in a direction which is economical and efficient in terms of optimal utilization of resources and time.

- Cost effective strain improvement programs include the selection of the strains with improved yield, increased production rate, strain stability, resistant to phage infection, response to dissolved oxygen, tolerance to medium components, production of foams and morphological forms of organisms.

- Table 4.2 Main target of the strain improvement and its impact on the process and product

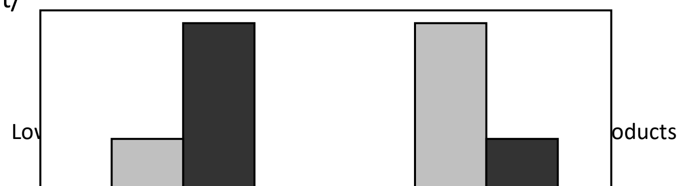
| Target | Impact on process or product |
|---|---|
| 1. Improved productivity | Reduced production cost.<br>Optimum capacity utilization.<br>Lower capital cost.<br>4. Increased bioseperation efficiency. |
| 2. Increased product yield | Cut the substrate cost.<br>Reduces the production of heat and $CO_2$.<br>Reduces the temperature control cost.<br>4. Reduces pollution and wastes. |
| 3. Changed catabolic capability | Shift to more favorable substrate alternatives.<br>2. Avoid production of polysaccharides and hence omission of pretreatment steps. |
| 4. Changed physicochemical features relevant to bioseperation. | Added characteristics such as flocculation behavior, mycelial structure, sporulation, foaming and strain stability that reduces capital and operation cost, and improves the separation characteristics.<br>2. Less number of problems in inoculum preparation and scale up of process. |
| 5. Improved product characteristics | Reduced production cost due to less number of impurities.<br>2. Less prone to enzymatic degradation. |
| 6. Modified product | Improved extractability due to the addition of side chains.<br>2. Changed thermal stability due to altered protein structure. |
| 7. Changed product location | Product recovery improvement with the omission of cell disruption techniques.<br>2. Reduced stress and sheer related denaturation due to cell disruption techniques. |

## Chapter 12: Process design criteria for various classes of bioproducts

### Question 12: Analyze criteria s required to design a fermentation process to produce low value and high value bioproducts of industrial importance

- Wide varieties of commercially important bioproducts are generally considered either as '**low value high volume product**' or as **'high value low volume products'**, based on **market volume and market price** or concentration in fermentations broth and purity requirement.
- '**Low value-high volume products**' such as citric acid, ethanol, antibiotics, proteases and amylases are bulk industrial products required in relatively low purity with relatively low cost, and on the other hand '**high value-low volume products'** such as therapeutic enzymes, interferon, growth factors and diagnostic enzymes are required in purest form and are generally expensive materials.
- Objectives of designing bioprocess unit steps are two extremes, as **low value-high volume product** are to be produced in **cost effective manner** in large quantity, while **high value-low volume product** are to be produced in **purest form** without taking into consideration of the cost of processing.
- Hence, **we cannot generalize** the **fundamental concepts, industrial practices** and **theoretical relationships** for designing a unit downstream processing steps for both 'low value-high volume product' and 'high value-low volume products', as the objectives are entirely different.
- Either internal or external process engineering team based on the input from the customer does the design aspects involving multidisciplinary team.
- Process design exercise done after taking an example each from low value-high volume product and high value-low volume product.

Purity requirement/
Selling price



- Figure 1 Major categories of commercially important bioproducts
- The process design starts with **certain inputs** from **consumer requirements, research and analysis,** and **process development**.
- Based on the **consumer requirement** or **anticipated demand,** process is developed to fit the **consumer need and company objectives**.
- This follows **some research development** or process development using **marketing inputs**.
- The marketing inputs **influence the design and technical data**.
- **Technical data** is required for designing of the plant.
- Raw material analysis data is required for **fermentation design, selection of ion exchange columns, and final product purity**.
- Ingredients corrosion data helps us to select suitable material of construction for all unit process.
- Solubility data is required to select proper solvent, evaporator and crystallizer.
- Product specification is required to select number and type of unit steps to achieve the goal. Health, safety and environmental data are required to formulate an overall design.
- Product flow characteristic data is required to design storage hoppers and bagging equipment's.
- During the initial phase of design, it is very much important to **compile a process data book**, so that we can use it as standard.

- Inputs from the process description and the mass balance are critical for **developing the process flow diagram (PFD)** at the preliminary design phase. Usually backward calculation gives mass balance.
- Inputs from **research and development are critical** for the development of process description.
- **Project scope defining** is very much important prior to the design team designs the actual process.
- **Strategic importance of the project, process advantage, performance specification, outline design requirement, utility requirement, availability of suppliers, statutory requirements of environmental impact study, statutory requirements of the law of the land, access to facility, labor welfare, geographical data of the location, and availability of resources, manpower, utilities etc. are critical for evaluating the scope of the project**.

1. **Process design criteria for 'low value - high volume' products**

- Even though few of the lifesaving antibiotics are considered as high value-low volume product, penicillin produced by fermentation is still considered as bulk commodity product, as most of it is been used as a raw material for the production of life saving medicines.
- Since penicillin is a '**low value high volume product'**, downstream unit process has to be **designed in a cost-effective manner**.
- Cost effectiveness of the process can be achieved by adopting following strategies.
- Adopt fermentation and recovery unit steps **that are already adopted and proved** that cost effective.
- **Standardize the variable cost of processing steps**, as it is a key economic factor.
- Get the **benefit of economy-of–scale principle** by increasing capacity or by optimizing capacity of utilization.
- **Design a process centered around** readily, easily and cheaply available raw material, ingredients, resources, and work force all along the year without much transportation cost

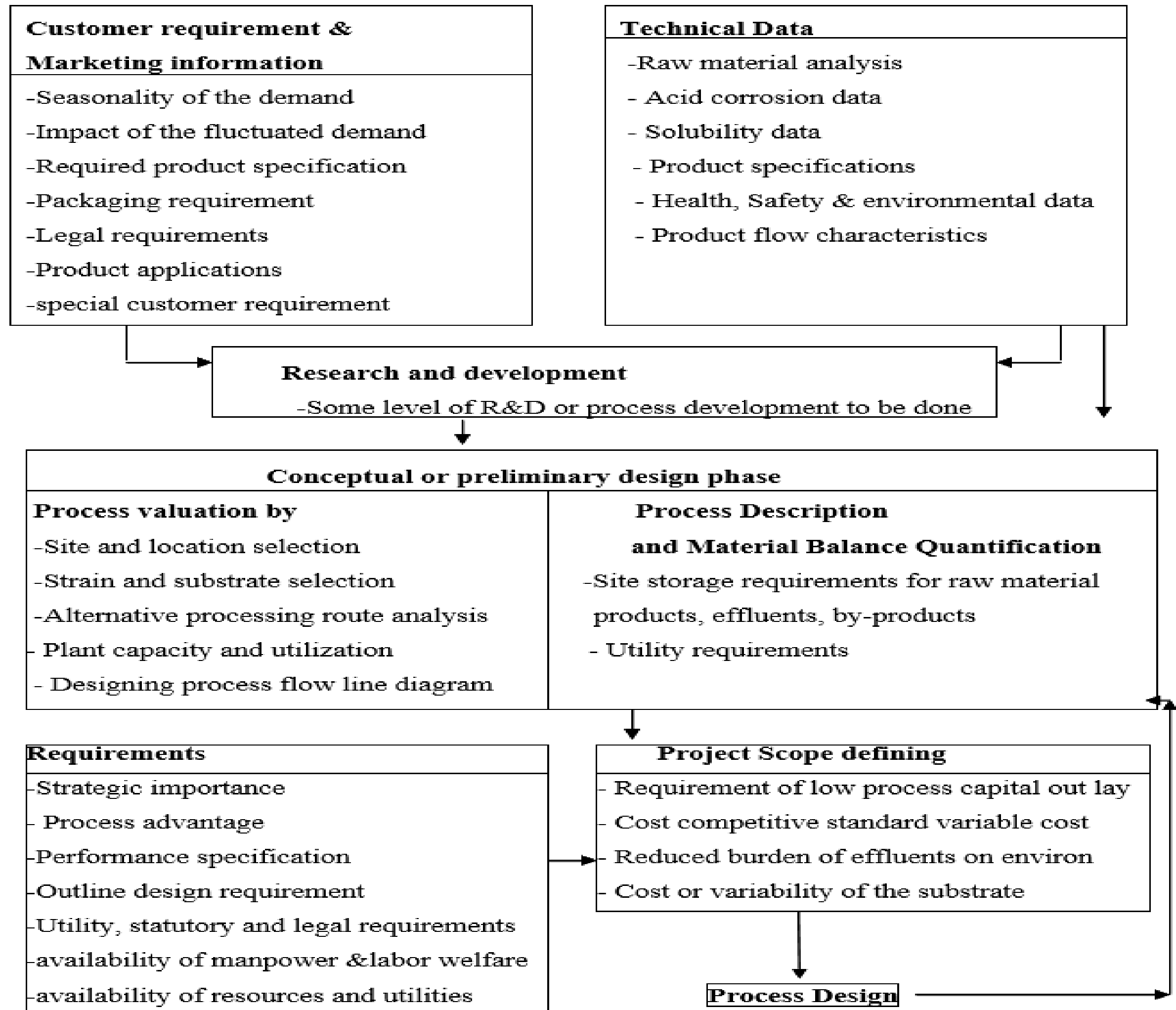
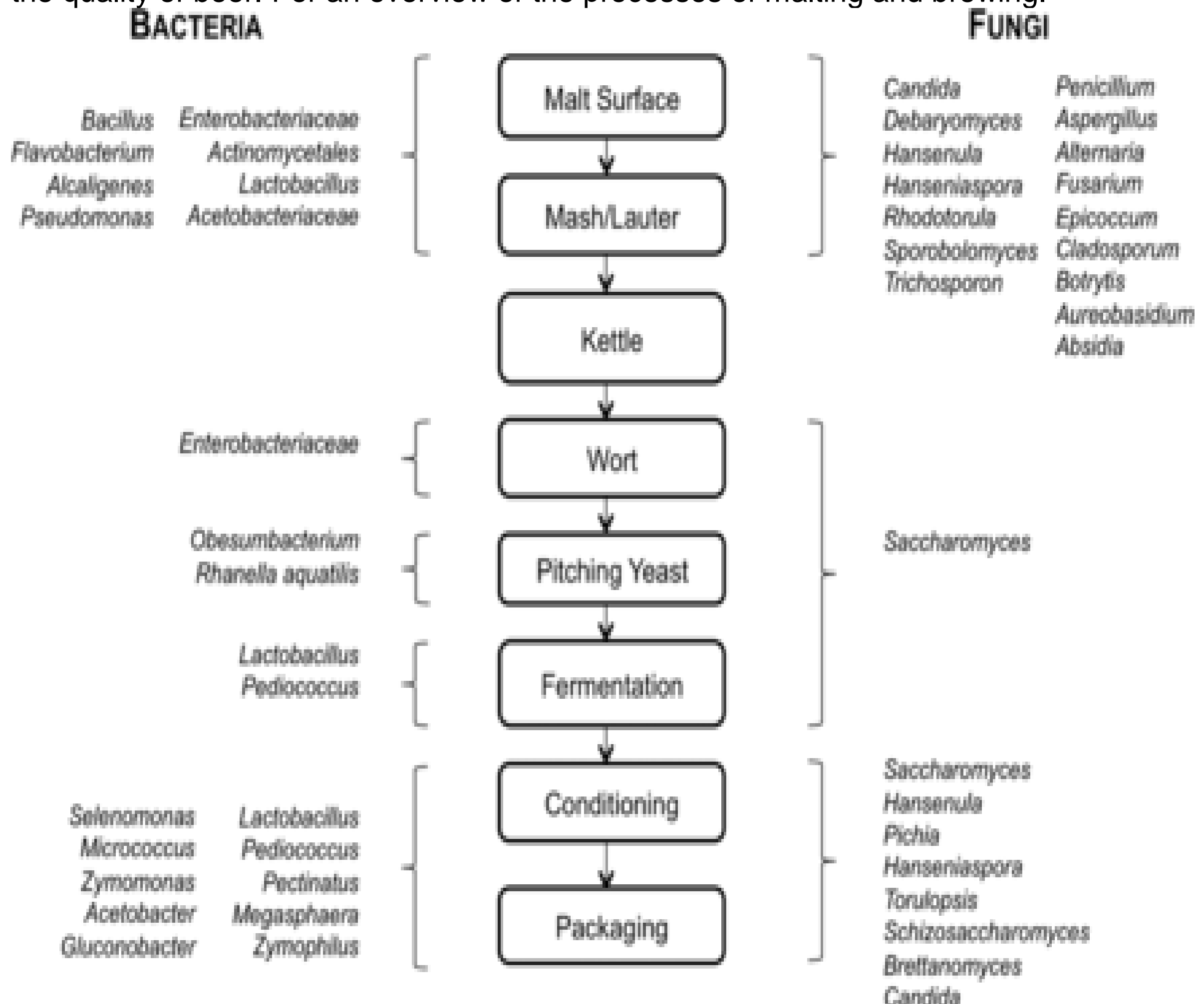



Figure 5.2 Schematic representation of the sequence of project design

Chapter 13: Microbiology of brewing

- Brewing beer involves microbial activity at every stage, from raw material production and malting to stability in the package.
- Most of these activities are desirable, as beer is the result of a traditional food fermentation, but others represent threats to the quality of the final product and must be controlled actively through careful management, the daily task of maltsters and brewers globally.
- Beer, like any fermented food, is an immutably microbial product. Microbial activity is involved in every step of its production, defining the many sensory characteristics that contribute to final quality.
- While fermentation of cereal extracts by *Saccharomyces* is the most important microbial process involved in brewing, a vast array of other microbes affect the complete process.
- Microbial interdiction at every step of the barley-to-beer continuum greatly influences the quality of beer. For an overview of the processes of malting and brewing.




- 

lthough all strains of *Saccharomyces* will produce ethanol as a fermentation end product, in practice the strains employed in the production of beers worldwide are classified into the categories of ale and lager yeasts.