MARUDHAR KESARI JAIN COLLEGE FOR WOMEN (AUTONOMOUS)

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IInd M.Sc. Biotechnology – Semester - III

E-Notes (Study Material)

Core Course 9-: BIOPROCESS TECHNOLOGY
Unit: 1 Introduction to fermentation. General requirements of fermentation. Microbial growth kinetics of batch and continuous culture. Solid substrate, slurry fermentation and its application. Microbial cell culture. Immobilization of cells and enzymes.(20 Hours)
Learning Objectives: Outline the basis of Bioprocess Engineering
Course Outcome: Basic principles of fermentation, including the biochemical processes involved in the conversion of raw materials into desired products.

Fermentation:

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Introduction to Fermentation

Fermentation technology is a field which utilizes microorganisms and enzymes to produce compounds that find use in pharmaceutical, chemical, energy, material and food industries. Biotechnology can be defined as the amalgamation of natural sciences and engineering techniques to utilize organisms, cells and their parts for industrial products and services. Thus, fermentation is a biotechnological tool that uses microorganisms to produce several industrial products.

Fermentation is a very ancient technique that has been in use for thousands of years. It was used for the first time in the production of beer. Today, biotechnology has intersected with fermentation technology to create value-added products such as **hormones, enzymes, antibiotics and other metabolites.**

History of Fermentation

Louis Pasteur was the first scientist to study fermentation back in the middle of the 19th century. He showed that living cells like yeast could demonstrate fermentation. He also showed the process of lactic acid fermentation in living cells.

Later in 1907, Eduard Buchner bagged a Nobel Prize for demonstrating the process of fermentation in yeast cells. In 1929, another pair of scientists, namely Arthur Harden and Hans Euler-Chelpin, won a Nobel Prize for their investigation of how enzymes cause fermentation. By the 1940s, the production of antibiotics by fermentation was established.

Fermenters were first developed by a British scientist, Chain Weizmann, for the production of acetone in WW1. However, first, large-scale fermenters were developed in 1944 for the production of yeast. Finally, an industrial level fermenter with aseptic conditions, good agitation and aeration was developed by Hindustan Antibiotic Ltd., Pimpri, Pune, in 1950 in India.

Types of Fermentation

1. Solid State Fermentation (SSF)

Solid state fermentation is the growth of microorganisms on a moist solid substrate in the absence or minimum water between the particles. The moisture content of the solid medium is between 12-80%. This type of fermentation is largely used for agricultural products and foods, such as soybean by *Aspergillus* and *Rhizopus*.

The six different types of solid state fermenters used are:

(i) Rotary drum,

(ii) Tray,

(iii) Swing solid state,

(iv) Packed-bed,

(v) Air-solid

(vi) Stirred vessel.

The application of SSF includes:

- Enzyme production such as pectinase, cellulase, protease, amylase and phytase.
- Production of organic acids such as gallic acid and lactic acid.
- Various secondary metabolites can be produced by SSF, such as gibberellic acid and antibiotics.
- Production of unsaturated fatty acids and biocontrol agents.

The advantages of SSF include high product titre, low use and waste of water, no hassle of foaming, low expenditure and reduced energy requirements. There are, however, several disadvantages of SSF, such as difficulty in controlling moisture of the solid substrate, poor oxygen availability, no method to control pH during fermentation, and only limited species that can be used in SSF.

2. Submerged Fermentation (SmF)

Submerged fermentation involves the cultivation of microorganisms in an enriched liquid broth. This type of fermentation is mainly employed for industrial applications. This procedure involves growing a microbe in a closed container containing broth rich in nutrients with high levels of oxygen. The production medium is an important component of the submerged fermentation that is optimised according to the microbe and target molecule.

There are three modes in which SmF is carried out:

(i) Batch mode,

(ii) Fed-batch mode and

(iii) Continuous mode.

a. Batch Mode

It is a simple mode of fermentation where all the prerequisites for the process are added in a container, and nothing is added in between except air. The prerequisites include sterilisation of the fermenter and production medium and addition of inoculum. The fermenter is run in a closed manner, and the process is terminated when either the nutrient is exhausted or the target molecule has reached its maximum concentration.

Advantages of batch mode include:

- Simple to use.
- Less chances of contamination.
- It can be handled by a relatively inexperienced operator.

b. Fed-batch Mode

As the name suggests, the fed-batch mode is a type of fermentation where the system is not run in a closed manner. In this mode, substrates, nutrients, or inducers are added to the system when required. This addition of products increases the productive phase of the microorganism.

Advantages of fed-batch fermentation are:

- High cell density is achieved.
- It leads to increased production of metabolites.
- The growth rate of the organism and its oxygen requirement can be controlled.

c. Continuous Mode

In this mode, the organism is fed with fresh nutrients along with the removal of spent medium and cells so as to maintain the volume, substrate concentration, product and biomass at a constant rate.

Advantages of continuous mode include:

- It gives a longer period of productivity.
- The high density of cells is achieved.
- Culture physiology can be studied in this mode.

Types of Fermenter

There are six types of fermenters or bioreactors that are used in industries. They include:

1. Continuous stirred tank fermentor
2. Airlift fermentor
3. Bubble column fermenter
4. Fluidised bed fermenter
5. Packed bed fermenter
6. Photo bioreactor

Application of Fermentation in Biotechnology

Fermentation technology is widely being used in various industrial products, such as:

a. **Production of cells or biomass:** The fermentation process involves producing the cells in a large amount that can be used for the extraction of metabolites. When an inoculum of microorganisms is grown in a properly enriched production medium, it reaches its

maximum growth rate. The biomass obtained can be downstream to extract the target product.

b. **Production of metabolites:** Fermentation technology is used to produce both primary and derived metabolites from microorganisms. Primary metabolites are produced in the growth phase of the microorganism, and examples include ethanol, citric acid, tryptophan, lysine and threonine.

   Secondary metabolites are produced by microorganisms during the stationary phase of their life cycle. Examples of secondary metabolites include antibiotics like penicillin and bacteriocins.

c. **Modification of compounds:** Fermentation technology can be applied to alter the metabolic pathways using cultivation-based or molecular approaches.

d. **Production of recombinant products:** Recombinant proteins, vaccines and hormones can be produced by fermentation that are used widely by pharmaceutical companies.

General requirements of Fermentation

The basic requirements of an industrial fermentation process are given below:-

1. Microorganisms: Selection and usage of a suitable organism plays a major role in fermentation process. Different varieties of organisms are used by isolating from different sources like soils, lakes, oceans, river, plant, animal, air, non-living objects.

2. Sterilization of medium for microbial growth

3.Water: All fermentation process, except solid state fermentation require vast quantities of water

4. Oxygen: Depending upon the amount of oxygen required by theorganism, it may be supplied in the form of air containing 21%v/v oxygen.

5. Buffers: Buffers are used to control drastic changes of pH .for example:-protein, peptides, amino acids act as good buffers at neutral pH.

6.Growth factors: Crude media constituents provide enough amounts of growth factors so no extra addition of growth factor is required. If there is a lack of any kind of vitamins or nutrients, growth factor can be added to media. for example:-Yeast extract and beef extract

7.Chealators: Many media can be prepared withoutprecipitation during autoclaving for example:-EDTA and citric acid

8.Production fermenter:-large model

9.Equipment:-

- Drawing the culture medium
- cell separation
- collection of cell
- product purification
- Effluent treatment

Additional Resource:

https://youtu.be/HPxxfoYpYcU?si=wpaaVY8THHp2yMb_

Practice Question:

1. Define fermentation and explain its significance in various industries.
2. Describe the general process of fermentation, including the role of microorganisms and substrates involved.
3. Compare and contrast aerobic and anaerobic fermentation processes.

Microbial Growth Kinetics of Batch Culture:

Growth kinetics is, *"The study of the growth of bacterial cultures does not constitute a specialised subject or branch of research: it is the basic method of Microbiology" J Mood, 1949*

Batch Fermentation

Definition

It refers to a technique in which microbial cells grow and multiply to convert substrates into products. Batch fermentation is performed using the **stirred tank fermentor**.

Batch fermentation itself clears that it involves **batch-wise** fermentation of the specific media. Microbial cells harness the added nutrients. Thereafter, they ferment raw materials into desired products within a predetermined fermentation time.

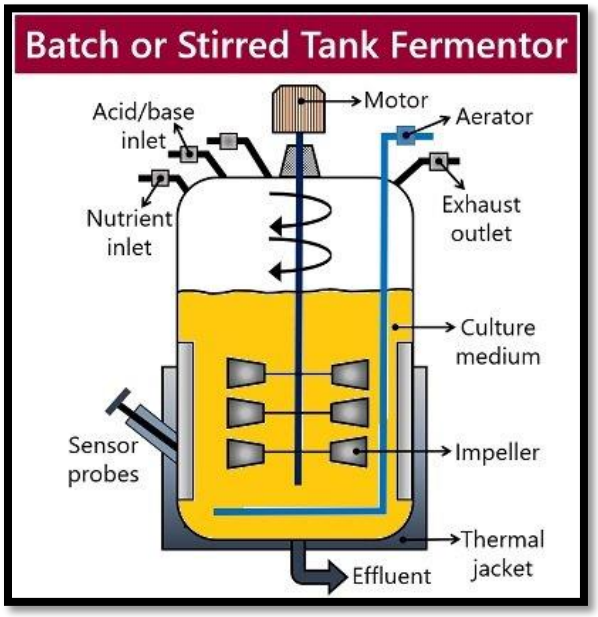
The factors affecting the fermentation:

- Type of substrate you are adding
- Environmental conditions
- Inoculum concentration
- The product of interest

Batch culture depends upon the closed system fermentation. The **culture volume** remains the **same**.

There is no addition or elimination of any content during the process.

After every batch, one can harvest the fermented broth culture for product recovery.



Principle

Batch culture follows a closed system fermentation. You should add all the required materials before the fermentation starts. The incubation of culture media proceeds for a specific **fermentation period**.

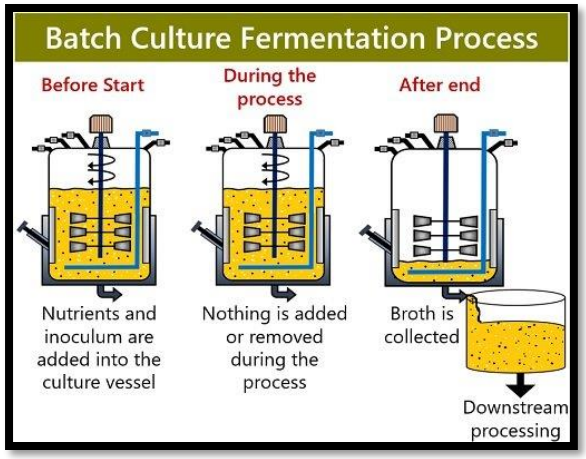
During this period, you can neither add fresh media nor discard spent media. Batch culture fermentation involves the commercial production of various products. This process takes place in **separate batches**.

Here, other factors become limiting, as we supply **limited nutrients** for microbial growth. As a result, the bacteria show a **sigmoid growth curve** with four distinct phases.

We can summarize the process into three consecutive stages:

Before starting

- Add sterile **nutrient broth** and other additives into the bioreactor through the nutrient inlet.
- Then, add inoculum (containing living microbial cells) into the bioreactor. This stage is called "**Seeding**".
- Incubate the culture medium for a definite **fermentation period** under optimal physiological conditions.



During the Batch Fermentation

- ✓ **Aerator** provides a continuous supply of oxygen in the form of air. To control the **pH** inside the culture vessel, you can add acidic or alkaline solutions.
- ✓ The **agitator** allows uniform mixing of the culture broth. Also, it maintains a constant temperature. The **foam sensor** senses the froth formed in the bioreactor. And, to break the froth, we can add antifoaming agents.

After the end of Batch Fermentation

The obtained broth culture contains the desired product, cell debris and toxins. Finally, **harvest** the entire broth culture into the container. Then, you need to settle the fermented broth culture for **downstream processing**. This involves four successive stages:

1. **Separation** of microbial cell suspension through filtration, centrifugation and sedimentation methods.
2. The **concentration** of remaining media through evaporation and precipitation methods.
3. You can **purify** the desired product through the following techniques:
   - Chromatography
   - Adsorption
   - Precipitation
   - Crystallization methods.
4. **Drying** of final product and waste disposal.

Microbial Growth Kinetics in Batch Culture-

In the Batch culture, the nutrients are supplied only once and there is no scope of removal the waste or toxic molecules. Due to which the bacteria shows peculiar pattern of growth.

Observing their growth pattern, we have divided it into four phases- lag phase, log phase, stationary phase and decline/death phase.

- **Lag phase** – There is visible no bacterial growth. Bacteria uses this time in order to get adapt to the new environment.
- **Log phase** – There is exponential increase in bacterial population.
- **Stationary phase** – In this phase, the number of producing cells is equal to number of death cells due to which there is no change in bacterial population. Hence, called as stationary phase.
- **Death phase** – Due to the nutrient exhaustion and accumulation of toxic molecules, the bacteria starts dying. Hence, there is decline in the bacterial population.

When bacteria multiplies by binary fission, it produces two daughter cells from a mother cell. Therefore, the bacterial population increase in the pattern of 2, 4, 8, 16, 32…

The growth pattern can also be mathematically written as 2^n, where n is the number of generation.

$2^1 = 2$

$2^2 = 4$

$2^3 = 8$ and so on

To find final bacterial cell number or biomass, we should be be aware of initial bacterial population. suppose, X_0 is the initial bacterial population then the final population or biomass (X) can be calculated by

$X = X_0 2^n$

Growth Kinetics of Lag, Log, Stationary and Death phase –

Lag phase – As there is no noticeable growth in lag phase, we don't need to calculate the rate of increase in bacterial population.

Log phase – In log phase, there is constant change in bacterial biomass or population is observed. And this change of biomass is directly proportional to cell biomass concentration This can be represented as –

$$dx/dt \quad \alpha \quad X$$

Equation no. 1

Solving the directly proportional relationship, we get

$$\frac{dX}{dt} = \mu X$$

Equation no. 2

Where X – mass or number of bacterial cells (mass/volume)

t – Time in hours

μ – is specific growth rate constant (1/time)

Equation no. 2 can be used to calculate the generation time as well as the specific growth rate.

Rearranging the equation no. 2

$$\frac{dX}{X} = \mu dt$$

Equation no. 3

Integrating equation no. 3, We integrate it from 'zero' to 't' because the growth is measured from zero time to final time t. After integrating equation no. 3 we obtain

$$\int_0^t \frac{dX}{X} = \mu \int_0^t dt$$

Equation no. 4

$$\left[\log x\right]_0^t = \mu \left[t\right]_0^t$$

Equation no. 5

$$\text{Log } x_t - \text{Log } x_0 = \mu (t - t_0)$$

Equation no. 6

At initial time $t_0 = 0$ (zero), hence equation no. 6 would become

$$\text{Log } x_t - \text{Log } x_0 = \mu t$$

Equation no. 7

$$\text{Log } x_t = \mu t + \text{Log } x_0$$

Equation no. 8

Equation no. 8 is similar to y = mx +c (it is logarithmic equation for linear type of reaction. The bacterial growth in log phase is also a linear type. Hence, 'm' slope of logarithmic equation is similar to μ of equation 8. The μ represent the rate of change of bacterial growth in logarithmic

phase and it can be calculated from slope of line obtained from semi log plot of exponential phase.

$$\frac{x_t}{x_0} = e^{\mu t}$$

Equation No. 9

Rewriting equation no. 9

$$x_t = x_0 e^{\mu t}$$

Equation No. 10

Stationary phase –

It is the third phase in which there is no change in bacterial population because of rate of cell production is equal to rate of cell death. This situation can be expressed mathematically as

$$\frac{dX}{dt} = 0$$

Equation No. 11

The cells die because of the exhaustion of nutrients and accumulation of waste. The cells that multiply in stationary phase by utilizing the left out nutrients and they also feed on dead cells. The bacterial growth on dead cells is called as endogenous metabolism.

Death Phase –

This is the last phase of bacterial growth curve of the batch culture . In this phase, bacteria are still multiplying but at very slow rate. The death rate is much more than production of cells, i.e. more number of cells are dying than producing. Hence, this situation can be mathematically represented as

$$\frac{dX}{dt} = -k_d X$$

k_d is specific death rate

Significance of Bacterial growth kinetics –

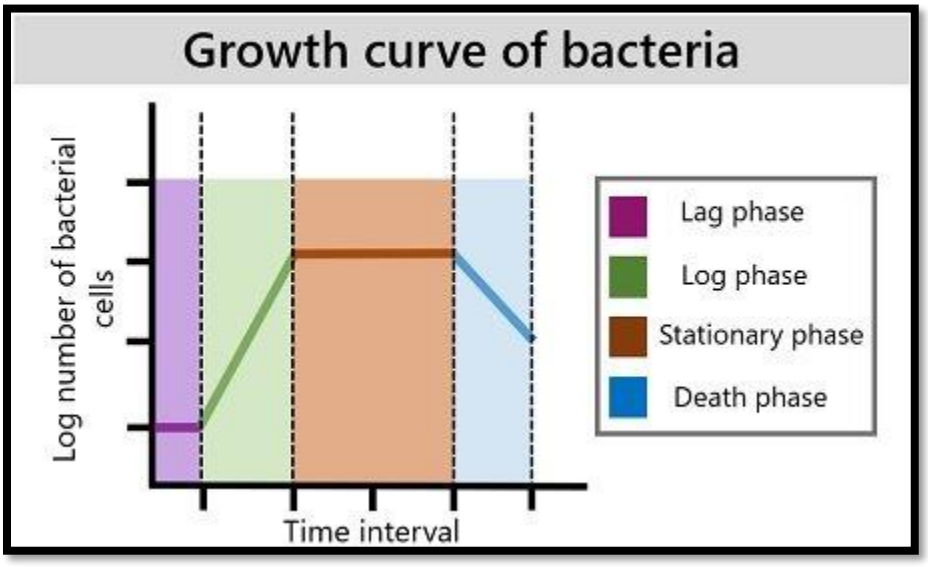
- The bacterial growth kinetics explains the change of biomass production in different phases of bacterial batch culture.
- It can be used to study the role of environmental factors affecting the change of biomass.

Growth Curve in Batch Culture

We can study the standard growth of bacteria in batch culture. Bacteria grow in a geometric fashion with typical **four phases**:

1. Lag phase
2. Log phase
3. Stationary phase
4. Decline phase

Also, you can determine bacterial generation time through a standard **growth curve of bacteria**



Properties	Lag Phase	Log Phase	Stationary Phase	Death Phase
Meaning	It is the preparatory phase in which cells adjust according to the changing environmental conditions	It is an exponential phase in which cells grow and divide to double their population	The stagnant phase is where the cell population remains constant, and cells neither grow nor multiply	It is the decline phase where the number of viable cells decreases exponentially
Cell number	Cells increase in size, but not in number	Cells exponentially increase their population	The number of viable cells becomes equal to	The number of nonviable cells increases

			nonviable cells	
Cell size	Small	Large	Small	Small
Occurrence	It occurs due to cells' vigorous metabolic activity	It occurs when cells divide rapidly to double their population	It mainly occurs due to nutrient depletion	Cell density declines due to the accumulation of toxic wastes
Cellular activity	Low, cells adapt to new conditions	Very high	Very low	No cellular activity
Growth rate	Zero	Constant	Zero	Negative
Production	Cells prepare or adapt themselves to new environmental conditions	Cells are at active growth, and they produce primary metabolites like amino acids to make up the essential macromolecules	After active growth, cells form secondary metabolites like toxins, drugs etc.	Cells lyse and release the cellular content or cell debris

Advantages

- Its setup is **easy** to make and run.
- It has wide applicability in laboratories and industries.
- Due to a closed fermentation system, there is **less chance of contamination**.
- It is easier in comparison to the continuous culture. Here, you can **reinstall** the setup, if contamination occurs.
- Complete conversion of a substrate into a product is possible.

Disadvantages

- Product isolation is difficult. This is because, fermentation broth contains nutrients, products, reagents, cell debris and toxins.
- It involves **high downtime** between two consecutive batches.
- It requires high labour cost and involves batch to **batch variability**.

Applications

1. The technique helps microbiologists to observe the metabolic activity of the bacteria. Also, it helps in studying the cell physiology of microbes.
2. It is efficient for the production of secondary metabolites such as antibiotics.

Additional References-

- https://booksite.elsevier.com/samplechapters/9780123705198/Sample_Chapters/04~Chapter_3.pdf
- http://rpdata.caltech.edu/courses/Physiology%20Matlab%202014pdf

Practice Question-

1. Define batch fermentation and describe its significance in microbial processes.
2. Outline the typical phases observed in microbial kinetics during batch fermentation.
3. Describe the role of mathematical models, such as Monod kinetics, in understanding and predicting microbial kinetics in batch fermentation.
4. What characterizes the stationary phase in batch fermentation? How does it differ from the exponential growth phase?
5. Explain the importance of product formation kinetics in batch fermentation. How can product formation be optimized?

Continuous Culture

Definition

Continuous culture is a continuous process where nutrients are continually added to the bioreactor and the culture broth (containing cells and metabolites) is removed at the same time. The volume of the culture broth is constant due to a constant feed-in and feed-out rate (i.e consumed nutrients are replaced and toxic metabolites are removed from the culture).

Principle of Continuous Culture

- A continuous flow system consists of a reactor into which reactants are pumped at a steady rate and from which products are emitted.
- The factors governing their operation are:
    - how material passes through the reactor (which depends upon its design);
    - the kinetics of the reaction taking place.
- In continuous culture, growth-limiting nutrients can be maintained at steady-state concentrations, which permits microorganisms to grow at submaximal rates.

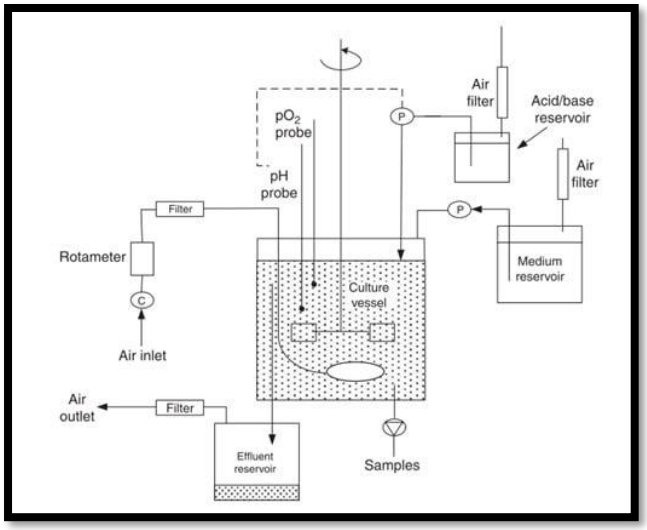
- In a steady-state, the cellular growth rate and environmental conditions, like the concentrations of metabolites, stay constant
- Moreover, in continuous culture, parameters such as pH, oxygen tension, the concentration of excretion products, and population densities can easily be monitored and controlled.

Process of Continuous Culture

- In continuous culture, an open system is set up in which one or more feed streams containing the necessary nutrients are fed continuously, while the effluent stream containing the cells, products, and residuals is continuously removed.
- A steady-state is established by maintaining an equal volumetric flow rate for the feed and effluent streams.
- The culture volume is kept constant, and all nutrient concentrations remain at constant steady-state values.
- During this process, the exponential growth phase is prolonged and the formation of byproducts is avoided.
- Continuous fermentation is monitored either by microbial growth activity or by-product formation, and these processes are referred to as-

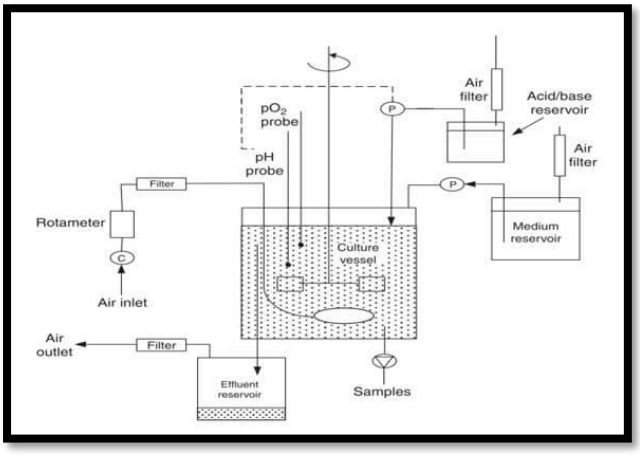
A. Turbidostat method

- Cell growth is controlled and remains constant in this system, but the flow rate of fresh media varies.
- Cell density is controlled based on the set value for turbidity, which is created by the cell population while fresh media is continuously supplied.



B. Chemostat method

- In a chemostat, the nutrients are continuously supplied at a constant flow rate, and the density of the cells is adjusted according to the supplied essential nutrients for growth.
- In a chemostat, the growth rate is determined by adjusting the concentration of substrates like carbon, nitrogen, and phosphorus.

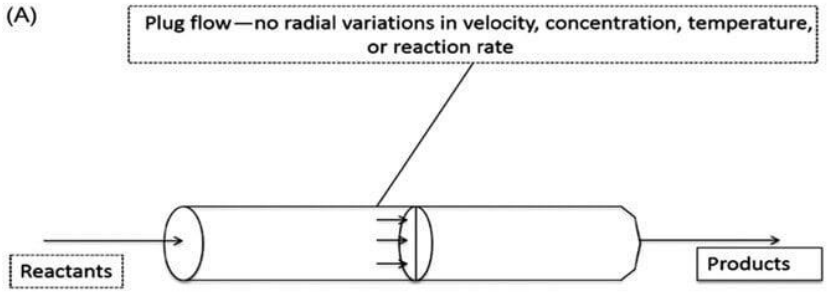


The continuous cultures of chemostat and turbidostat systems have the following criteria:

- Medium and cells are continuously changing
- The cell density is constant
- Steady-state growth
- Open system

C. Plug-flow reactor

- In this type of continuous culture, the culture solution flows through a tubular reactor without back mixing.
- In a plug flow reactor, nutrients (reactants) enter the reactor in the form of "plugs," which flow in an axial direction through the reactor.
- The culture medium flows steadily through a tube and the cells are recycled from the outlet to the inlet.



<u>Advantages of continuous culture are:</u>

1. In continuous culture, keeping the working volume constant simplifies culture scale-up based on a constant- power-to-volume strategy.
2. It is possible to set up the optimum conditions for maximum and long-term product synthesis.
3. Ability to obtain stable product quality (the steady-state consists of homogeneous cell culture with a constant biomass and metabolite concentration).
4. It als results in higher productivity per unit volume, as time-consuming tasks, such as cleaning and sterilization are unnecessary.
5. Cultures in a steady-state can last for days, weeks, or even months, thus greatly reducing the downtime and making the process more economically competitive.

<u>Application of Continuous Culture</u>

- Continuous culture fermentation has been used for the production of single-cell protein, organic solvents, starter cultures, etc.
- It has been used in the production of beer, fodder yeast, vinegar, baker's yeast, etc.
- In the industrial production of secondary metabolites (such as antibiotics from a *Penicillium* or a *Streptomyces* sp.)

- Continuous culture has been tested for L-lysine-producing *C. glutamicum* mutant B-6
- It has been used in municipal waste treatment processes.

Limitations Continuous Culture

- In the long-term cultivation process, sterility maintenance can be tricky, and downstream processing can prove challenging.
- Controlling the production of some non-growth-related products is not easy Because of this, continuous culture often requires fed-batch culturing as well as continuous nutrient supply.
- As a result of the viscosity and heterogeneous nature of the mixture, it may be challenging to maintain filamentous organisms.
- If a faster-growing strain overtakes the original product strain, it may be lost over time.

Additional References

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2. Ghosh, B., Bhattacharya, D., & Mukhopadhyay, M. (2018). Use of Fermentation Technology for Value-Added Industrial Research. *Principles and Applications of Fermentation Technology*, *August*, 141–161. https://doi.org/10.1002/9781119460381.ch8

Practice Question

1. Define continuous culture and explain its significance in microbial processes.
2. Compare and contrast continuous culture with batch culture and fed-batch culture.
3. Describe the components of a continuous culture system, including the chemostat and turbidostat.
4. Explain the principle of nutrient limitation in continuous culture and its role in controlling microbial growth.
5. Discuss the concept of dilution rate in continuous culture and its impact on cell growth and substrate utilization.

Solid State Fermentation (SSF)

- ✓ **Solid State Fermentation (SSF)** is a fermentation method used by several industries like the pharmaceuticals, food, textile etc., to produce metabolites of microorganisms using solid support in place of the liquid medium.
- ✓ It is defined as the growth of microbes without free-flowing aqueous phase.
- ✓ The SSF is alternative to submerged fermentation for production of value-added products like antibiotics, single cell protein, PUFA's, enzymes, organic acids, biopesticides, biofuel and aroma production.
- ✓ The support used is especially grain brans, de-oiled oil seed cakes, and other substances alike.
- ✓ Initially, mostly fungi were used in such fermentation (as these microorganisms were considered to be very optimally active in very low water activity). Later, many bacterial species and yeasts were used to carry out such fermentation also.
- ✓ The microbiological process of SSF has generated great interest in recent years because it can be used for a variety of purposes, supported by some authors who have even indicated numerous advantages over their liquid counterparts (submerged fermentation).

Organisms Used in Solid State Fermentation(SSF)

- ➢ The microbiological components of SSF can occur as single pure cultures, mixed identifiable cultures or totally mixed indigenous microorganisms.
- ➢ Some SSF processes e.g., tempeh and ontjom production, requires selective growth of organisms such as molds that need low moisture levels to carry out fermentation with the help of extracellular enzymes secreted by fermenting microorganisms.
- ➢ However, bacteria and yeasts, which require higher moisture content for efficient fermentation, can also be used for SSF, but with a lower yield.

Steps in SSF

SSF is normally multistep processes involving the following steps:

1. Pre-treatment of substrate raw materials either by mechanical, chemical or biochemical processing to enhance the availability of the bound nutrients and also to reduce the size of the components, e.g., pulverizing straw and shredding vegetable materials to optimize the physical aspects of the process.

2. Hydrolysis of primarily polymeric substrates, e.g., polysaccharides and proteins.

3. Utilization (fermentation) of hydrolysis products.

4. Separation and purification of end products.

Applications of SSF

1. **Microbial Products:** SSF is used for the production of a wide range of microbial products. It has been successfully applied in the production of enzymes, including amylases, cellulases, proteases, lipases, and many others. These enzymes find applications in various industries such as food processing, textile, detergent manufacturing, and biofuel production. SSF is also used to produce organic acids, flavoring compounds, and other valuable metabolites.
2. **Feed Production:** SSF is utilized for the production of feed additives and animal feed. Solid substrates, such as agricultural by-products, can be converted into nutritional supplements and feed ingredients using SSF. This helps in utilizing agricultural waste and improving the nutritional value of feed for livestock.
3. **Biofuels**: SSF has shown promise in the production of biofuels. Lignocellulosic materials, such as agricultural residues and forestry waste, can be fermented using SSF to produce bioethanol and other biofuels. This application contributes to the development of sustainable and renewable energy sources.
4. **Food and Beverage Industry:** SSF plays a crucial role in the production of various fermented food and beverage products. Examples include traditional fermented foods like idli, dosa, dhokla, and bread. SSF is also employed in the production of fermented beverages like sake, soy sauce, and certain types of beer. Fermentation enhances the nutritional value, digestibility, and flavor of these food products.
5. **Bioremediation and Bioleaching:** SSF has been used for bioremediation purposes, particularly in the degradation of pollutants in solid waste or contaminated soil. Microorganisms capable of degrading pollutants are grown on solid substrates to carry out the remediation process. SSF is also utilized in bioleaching, which involves the use of microorganisms to extract valuable metals from ores and industrial waste.
6. **Industrial Chemicals and Pharmaceuticals**: SSF is employed in the production of various industrial chemicals and pharmaceutical products. It offers an efficient and cost-effective method for producing compounds such as organic acids, antibiotics, bioactive compounds, and other high-value chemicals. SSF can be used to cultivate specific microorganisms that

produce these compounds, which can then be extracted, purified, and utilized in the manufacturing of pharmaceuticals and other industrial products.

Overall, the applications of SSF span a wide range of industries, contributing to the production of microbial products, feed additives, biofuels, fermented foods, bioremediation, and industrial chemicals. Its versatility and potential for sustainable production make SSF an attractive option in various bioprocesses.

Advantages of Solid State Fermentation

- **Environmental Friendliness:** SSF produces minimal waste and liquid effluent, making it less damaging to the environment compared to liquid fermentation methods. This is beneficial for sustainable production and reduces the overall ecological footprint.
- **Simple and Low-Cost Process:** SSF employs natural solid substrates as media, often using agro-industrial residues or agricultural waste. It is a low-technology and low-energy process, requiring less capital investment compared to other fermentation techniques. The use of readily available and inexpensive raw materials contributes to cost-effectiveness.
- **Reduced Contamination:** SSF has a lower risk of microbial contamination compared to liquid fermentation since there is no need for sterilization. The solid substrate provides a physical barrier against contaminants, resulting in a cleaner fermentation process.
- **Easy Downstream Processing:** SSF facilitates easy downstream processing and product recovery. The concentrated nature of the solid substrate simplifies extraction and purification steps, reducing the need for large volumes of solvents or chemicals.
- **Utilization of Agro-Industrial Residues:** SSF allows for the utilization of underutilized or non-utilized agro-industrial residues as substrates. This provides an alternative avenue for value addition to these residues, contributing to waste reduction and resource efficiency.
- **High Yield and Productivity:** SSF can achieve reasonably high product yields due to the concentrated source of nutrients provided by the solid substrates. The controlled conditions and specific microorganisms used in SSF can lead to efficient conversion of substrates into desired products.
- **Simple Bioreactor Design**: Bioreactors used in SSF are typically small in volume and compact, resulting in a simpler design compared to large-scale liquid fermentation

systems. Aeration and effluent treatment processes are also relatively straightforward in SSF.

- **Versatile Substrate Options:** SSF can utilize a wide range of domestic, industrial, and agricultural wastes as substrates. This flexibility in substrate choice allows for the utilization of various natural materials, providing an economical and sustainable approach to fermentation.
- **Economic Feasibility:** SSF has proven to be economically feasible due to its low-cost operation, utilization of inexpensive substrates, simplified processing steps, and high product yields. It presents an attractive option for industries looking for cost-effective fermentation processes.

Limitations of Solid State Fermentation

- The microorganisms that tolerate only low moisture content can be used.
- Precise monitoring of SSF (e.g., O2 and CO2 levels, moisture content) is not possible.
- The organisms grow slowly and consequently, there is a limitation in product formation.
- Heat production creates problems, and it is very difficult to regulate the growth environment.

Additional Reference

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Practice Question

1. What is solid-state fermentation (SSF), and how does it differ from submerged fermentation (SmF)?
2. Describe the basic principles underlying solid-state fermentation processes.
3. What are the advantages of solid-state fermentation over submerged fermentation?
4. Discuss the applications of solid-state fermentation in various industries.
5. What are some examples of substrates commonly used in solid-state fermentation processes?

Immobilization of Enzymes and Cells: Methods, Effects and Applications

Immobilization of enzymes (or cells) refers to the technique of confining/anchoring the enzymes (or cells) in or on an inert support for their stability and functional reuse. By employing this technique, enzymes are made more efficient and cost-effective for their industrial use. Some workers regard immobilization as a goose with a golden egg in enzyme technology. Immobilized enzymes retain their structural conformation necessary for catalysis.

- **There are several advantages of immobilized enzymes:**
  - Stable and more efficient in function.
  - Can be reused again and again.
  - Products are enzyme-free.
  - Ideal for multi-enzyme reaction systems.
  - Control of enzyme function is easy.
  - Suitable for industrial and medical use.
  - Minimize effluent disposal problems.
- There are however, certain disadvantages also associated with immobilization.
  - The possibility of loss of biological activity of an enzyme during immobilization or while it is in use.
  - Immobilization is an expensive affair often requiring sophisticated equipment.
- Immobilized enzymes are generally preferred over immobilized cells due to specificity to yield the products in pure form. However, there are several advantages of using immobilized multi-enzyme systems such as organelles and whole cells over immobilized enzymes. The immobilized cells possess the natural environment with cofactor availability (and also its regeneration capability) and are particularly suitable for multiple enzymatic reactions.

Methods of Immobilization:

The commonly employed techniques for immobilization of enzymes are—adsorption, entrapment, covalent binding and cross-linking.

Adsorption:

- ✓ Adsorption involves the physical binding of enzymes (or cells) on the surface of an inert support. The support materials may be inorganic (e.g. alumina, silica gel, calcium

phosphate gel, glass) or organic (starch, carboxymethyl cellulose, DEAE-cellulose, DEAE-sephadex).

- ✓ Adsorption of enzyme molecules (on the inert support) involves weak forces such as van der Waals forces and hydrogen bonds . Therefore, the adsorbed enzymes can be easily removed by minor changes in pH, ionic strength or temperature. This is a disadvantage for industrial use of enzymes.

Entrapment:

- ✓ Enzymes can be immobilized by physical entrapment inside a polymer or a gel matrix. The size of the matrix pores is such that the enzyme is retained while the substrate and product molecules pass through. In this technique, commonly referred to as lattice entrapment, the enzyme (or cell) is not subjected to strong binding forces and structural distortions.
- ✓ Some deactivation may however, occur during immobilization process due to changes in pH or temperature or addition of solvents. The matrices used for entrapping of enzymes include polyacrylamide gel, collagen, gelatin, starch, cellulose, silicone and rubber. Enzymes can be entrapped by several ways.

1. Enzyme inclusion in gels:

This is an entrapment of enzymes inside the gels

2. Enzyme inclusion in fibres:

The enzymes are trapped in a fibre format of the matrix

3. Enzyme inclusion in microcapsules:

In this case, the enzymes are trapped inside a microcapsule matrix (Fig. 21.4C). The hydrophobic and hydrophilic forms of the matrix polymerise to form a microcapsule containing enzyme molecules inside. The major limitation for entrapment of enzymes is their leakage from the matrix. Most workers prefer to use the technique of entrapment for immobilization of whole cells. Entrapped cells are in use for industrial production of amino acids (L-isoleucine, L-aspartic acid), L-malic acid and hydroquinone.

Microencapsulation:

Microencapsulation is a type of entrapment. It refers to the process of spherical particle formation wherein a liquid or suspension is enclosed in a semipermeable membrane. The

membrane may be polymeric, lipoidal, lipoprotein-based or non-ionic in nature. There are three distinct ways of microencapsulation.

i. Building of special membrane reactors.
ii. Formation of emulsions.
iii. Stabilization of emulsions to form microcapsules.

Microencapsulation is recently being used for immobilization of enzymes and mammalian cells. For instance, pancreatic cells grown in cultures can be immobilized by microencapsulation. Hybridoma cells have also been immobilized successfully by this technique.

Covalent Binding:

Immobilization of the enzymes can be achieved by creation of covalent bonds between the chemical groups of enzymes and the chemical groups of the support .This technique is widely used. However, covalent binding is often associated with loss of some enzyme activity. The inert support usually requires pretreatment (to form pre-activated support) before it binds to enzyme. The following are the common methods of covalent binding.

1. Cyanogen bromide activtion:

`The inert support materials (cellulose, sepharose, sephadex) containing glycol groups are activated by CNBr, which then bind to enzymes and immobilize them

2. Diazotation:

Some of the support materials (amino benzyl cellulose, amino derivatives of polystyrene, aminosilanized porous glass) are subjected to diazotation on treatment with $NaNO_2$ and HCI. They, in turn, bind covalently to tyrosyl or histidyl groups of enzymes

3. Peptide bond formation:

Enzyme immobilization can also be achieved by the formation of peptide bonds between the amino (or carboxyl) groups of the support and the carboxyl (or amino) groups of enzymes .The support material is first chemically treated to form active functional groups.

4. Activation by bi- or poly-functional reagents:

Some of the reagents such as glutaraldehyde can be used to create bonds between amino groups of enzymes and amino groups of support (e.g. aminoethylcellulose, albumin, amino alkylated porous glass).

Cross-Linking:

The absence of a solid support is a characteristic feature of immobilization of enzymes by cross- linking. The enzyme molecules are immobilized by creating cross-links between them, through the involvement of poly-functional reagents. These reagents in fact react with the enzyme molecules and create bridges which form the backbone to hold enzyme molecules There are several reagents in use for cross-linking. These include glutaraldehyde, diazobenzidine, hexamethylene diisocyanate and toluene di- isothiocyanate.

Glutaraldehyde is the most extensively used cross-linking reagent. It reacts with lysyl residues of the enzymes and forms a Schiff's base. The cross links formed between the enzyme and glutaraldehyde are irreversible and can withstand extreme pH and temperature. Glutaraldehyde cross- linking has been successfully used to immobilize several industrial enzymes e.g. glucose isomerase, penicillin amidase. The technique of cross-linking is quite simple and cost-effective. But the disadvantage is that it involves the risk of denaturation of the enzyme by the poly-functional reagent.

Choice of Immobilization Technique:

The selection of a particular method for immobilization of enzymes is based on a trial and error approach to choose the ideal one. Among the factors that decide a technique, the enzyme catalytic activity, stability, regenerability and cost factor are important.

Immobilization of L-amino acid acylase:

L-Amino acid acylase was the first enzyme to be immobilized by a group of Japanese workers (Chibata and Tosa, 1969). More than 40 different immobilization methods were attempted by this group. Only three of them were found be useful. They were covalent binding to iodoacetyl cellulose, ionic binding to DEAE-Sephadex and entrapment within polyacrylamide.

Stabilization of Soluble Enzymes:

Some of the enzymes cannot be immobilized and they have to be used in soluble form e.g. enzymes used in liquid detergents, some diagnostic reagents and food additives. Such enzymes can be stabilized by using certain additives or by chemical modifications. The stabilized enzymes have longer half-lives, although they cannot be recycled. Some important methods of enzyme stabilization are briefly described.

Solvent Stabilization:

Certain solvents at low concentrations stabilize the enzymes, while at high concentrations the enzymes get denatured e.g. acetone (5%) and ethanol (5%) can stabilize benzyl alcohol dehydrogenase.

Substrate Stabilization*:*

The active site of an enzyme can be stabilized by adding substrates e.g. starch stabilizes a-amylase; glucose stabilizes glucose isomerase.

Stabilization by Polymers:

Enzymes can be stabilized, particularly against increased temperature, by addition of polymers such as gelatin, albumin and polyethylene glycol.

Stabilization by Salts:

Stability of metalloenzymes can be achieved by adding salts such as Ca, Fe, Mn, Cu and Zn e.g. proteases can be stabilized by adding calcium.

Stabilization by Chemical Modifications:

Enzymes can be stabilized by suitable chemical modifications without loss of biological activity. There are several types of chemical modifications.

- Addition of poly-amino side chains e.g. polytyrosine, polyglycine.
- Acylation of enzymes by adding groups such as acetyl, propionyl and succinyl.

Stabilization by Rebuilding:

Theoretically, the stability of the enzymes is due to hydrophobic interactions in the core of the enzyme. It is therefore, proposed that enzymes can be stabilized by enhancing hydrophobic interactions. For this purpose, the enzyme is first unfold and then rebuilt in one of the following ways

1. The enzyme can be chemically treated (e.g. urea and a disulfide) and then refolded.
2. The refolding can be done in the presence of low molecular weight ligands.
3. For certain enzymes, refolding at higher temperatures (around 50°C) stabilize them.

Stabilization by Site-Directed Mutagenesis:

Site-directed mutagenesis has been successfully used to produce more stable and functionally more efficient enzymes e.g. subtilisin E.

Immobilization of Cells:

Immobilized individual enzymes can be successfully used for single-step reactions. They are, however, not suitable for multi-enzyme reactions and for the reactions requiring cofactors. The whole cells or cellular organelles can be immobilized to serve as multi-enzyme systems. In addition, immobilized cells rather than enzymes are sometimes preferred even for single reactions, due to cost factor in isolating enzymes.

For the enzymes which depend on the special arrangement of the membrane, cell immobilization is preferred. Immobilized cells have been traditionally used for the treatment of sewage. The techniques employed for immobilization of cells are almost the same as that used for immobilization of enzymes with appropriate modifications. Entrapment and surface attachment techniques are commonly used. Gels, and to some extent membranes, are also employed.

Immobilized Viable Cells:

The viability of the cells can be preserved by mild immobilization. Such immobilized cells are particularly useful for fermentations. Sometimes mammalian cell cultures are made to function as immobilized viable cells.

Immobilized Non-viable Cells:

In many instances, immobilized non-viable cells are preferred over the enzymes or even the viable cells. This is mainly because of the costly isolation and purification processes. The best example is the immobilization of cells containing glucose isomerase for the industrial production of high fructose syrup.

Limitations of Immobilizing Eukaryotic Cells:

Prokaryotic cells (particularly bacterial) are mainly used for immobilization. It is also possible to immobilize eukaryotic plant and animal cells. Due to the presence of cellular organelles, the metabolism of eukaryotic cells is slow. Thus, for the industrial production of biochemical, prokaryotic cells are preferred. However, for the production of complex proteins (e.g. immunoglobulin's) and for the proteins that undergo post- translational modifications, eukaryotic cells may be used.

Additional Reference

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2.https://youtu.be/sL_iEOuvK80?si=IHzkybz8rOlMg35p

Practice Question

1. Define enzyme immobilization and explain its significance in biotechnology.
2. Describe the different methods used for enzyme immobilization.
3. What are the advantages of enzyme immobilization over free enzymes?
4. Discuss the various supports/materials commonly used for enzyme immobilization.
5. Explain the mechanisms involved in enzyme immobilization on solid supports.
6. Compare and contrast the characteristics of immobilized enzymes with those of free enzymes.
7. How does enzyme immobilization affect enzyme stability and activity?