Solid State and Submerged Fermentation

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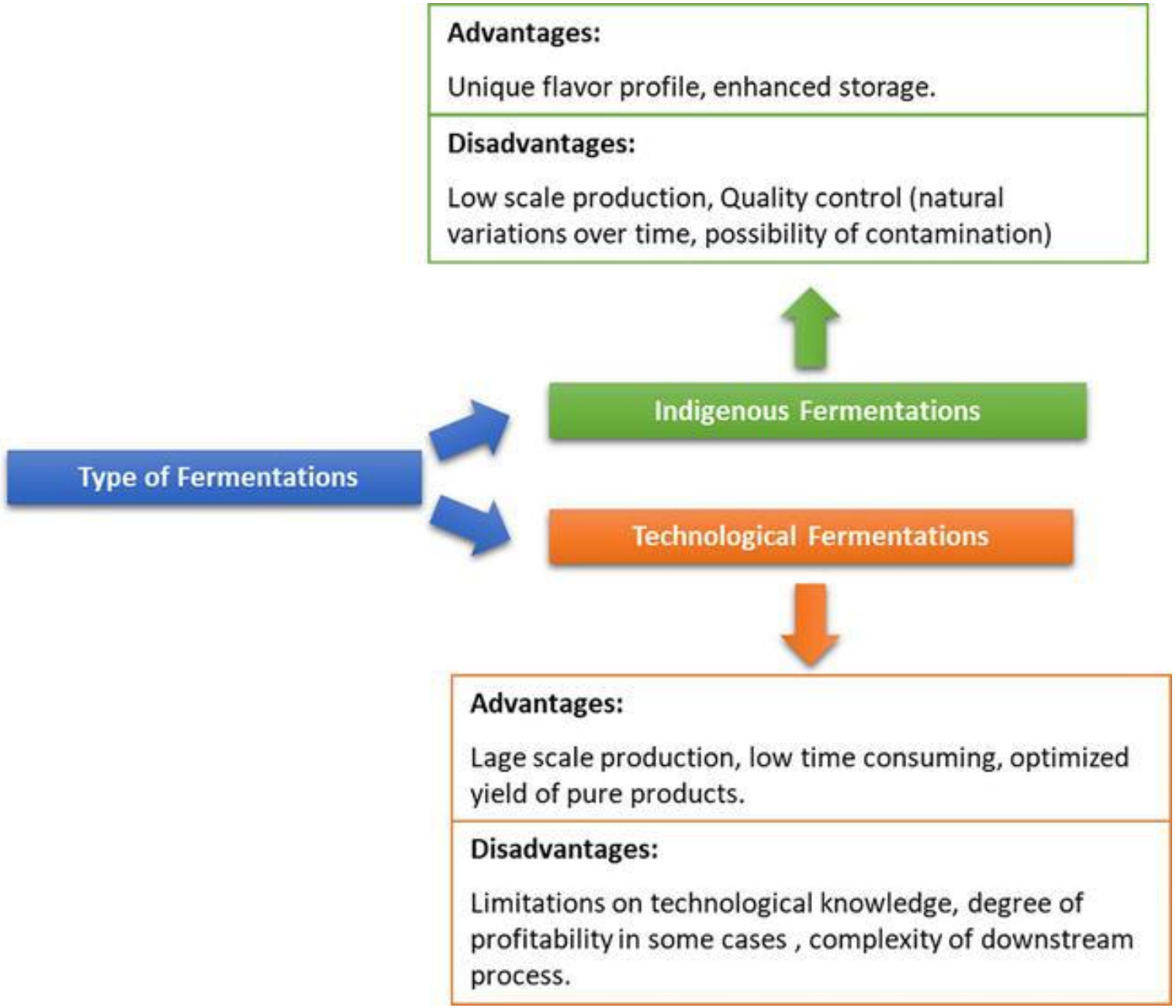
Fermentation

- Fermentation is the process involving the biochemical activity of organisms, during their growth, development, reproduction, even senescence and death.
- The term ‘fermentation’ is derived from the Latin verb “fervere’, to boil, thus describing the appearance of the action of yeast on extracts of fruit or malted grain.
- Fermentation technology is the use of organisms to produce food, pharmaceuticals and alcoholic beverages on a large scale industrial basis.
- The production of alcohol by the action of yeast on malt or fruit extracts has been carried out on a large scale for very many years and was the first ‘industrial’ process for the production of a microbial metabolite.
- Industrial microbiologists have extended the term fermentation to describe any process for the production of product by the mass culture of a micro-organism.
- Brewing and the production of organic solvents may be described as fermentations in both senses of the word but the description of an aerobic process as a fermentation is obviously using the term in the broader sense.


Advantages:
Unique flavor profile, enhanced storage.
Disadvantages:
Low scale production, Quality control (natural variations over time, possibility of contamination)
Indigenous Fermentations
Type of Fermentations
Technological Fermentations
Advantages:
Lage scale production, low time consuming, optimized yield of pure products.
Disadvantages:
Limitations on technological knowledge, degree of profitability in some cases , complexity of downstream process.

Fermentation Technology

- Fermentation Technology could be defined simply as the study of the fermentation process, techniques and its application.
- The basic principle involved in the fermentation technology is that organisms are grown under suitable conditions, providing raw materials meeting all the necessary requirements such as C,N, salts, trace elements and vitamins.
- The end products formed as a result of their metabolism during their life span are released into the media, which are extracted for use by human being and that have a high commercial value.
- The intentional use of fermentation technology for the large scale production of microbial biomass or metabolites is **called industrial fermentation.**

The component parts of a fermentation process

- Regardless of the type of fermentation an established process may be divided into six basic component parts:

(i) The formulation of media to be used in culturing the process organism during the development of the inoculum and in the production fermenter.

(ii) The sterilization of the medium, fermenters and ancillary equipment.

(iii) The production of an active, pure culture in sufficient quantity to inoculate the production vessel.

(iv) The growth of the organism in the production fermenter under optimum conditions for product formation.

(v) The extraction of the product and its purification.

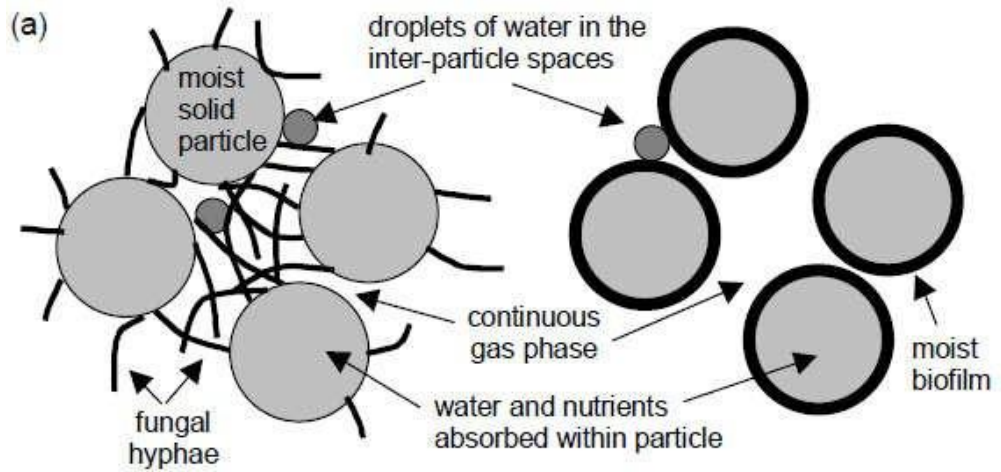
(vi) The disposal of effluents produced by the process.

Fermentation Techniques

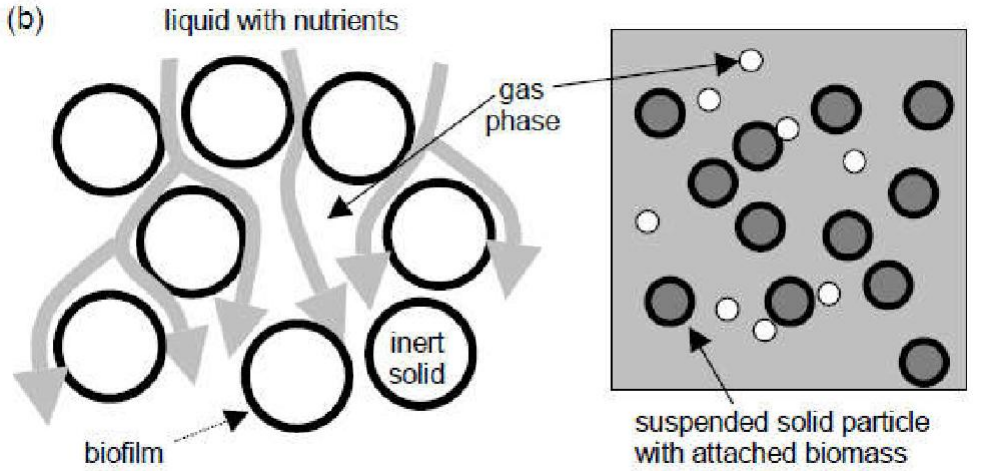
- Two broad fermentation techniques have emerged as a result of this rapid development:
  - Solid State Fermentation (SSF)
  - Submerged Fermentation (SmF)
- At the research level, both SSF and SmF have been used; however, some techniques yielded better results than others.

What is SSF?

- Solid-state fermentation (SSF) involves the growth of microorganisms on moist solid particles, in situations in which the spaces between the particles contain a continuous gas phase and a minimum of visible water.
- The more general term “solid-substrate fermentation” is used to denote any type of fermentation process that involves solids, including suspensions of solid particles in a continuous liquid phase and even trickling filters.
- The majority of SSF processes involve **filamentous fungi**, although some involve bacteria and some involve yeasts.
- SSF processes may involve the pure culture of organisms, or the culture of several pure strains inoculated simultaneously or sequentially, while in some processes a “self-selected” microflora arises from the original microflora (e.g., in composting) or from a specially prepared traditional inoculum.
- The majority of SSF processes involve aerobic organisms.
- The substrates used in SSF processes are often products or byproducts of agriculture, forestry or food processing.
- Typically the source of nutrients comes from within the particle, although there are some cases in which nutrients are supplied from an external source.
- Usually a polymer gives the solid structure to the particle and this polymer may or may not be degraded by the microorganism during the fermentation.
- There are also some cases in which artificial or inert supports are used, with a nutrient solution absorbed within the matrix.

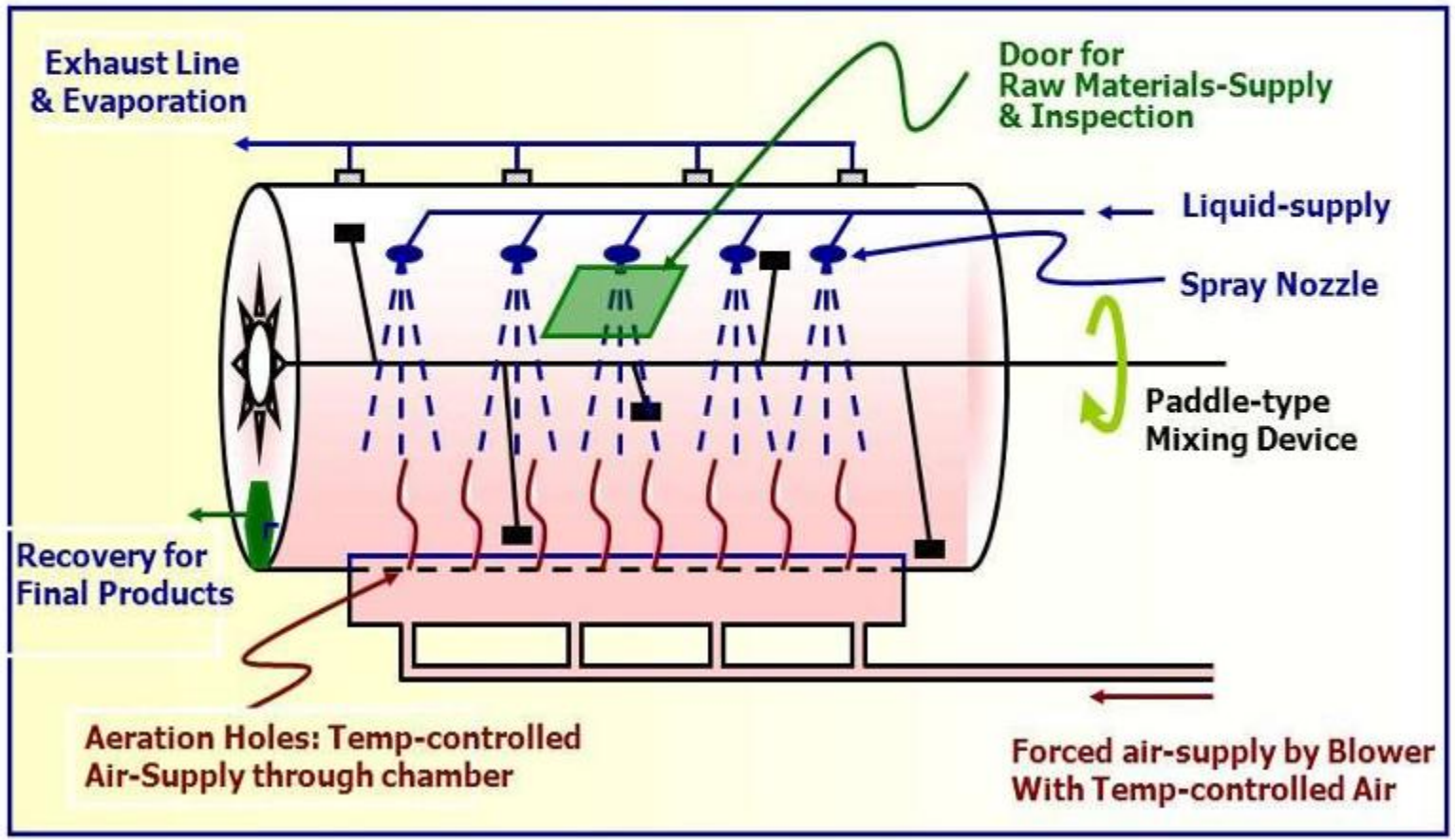


The arrangement of moist solid particles and the continuous gas phase in SSF systems involving a filamentous fungus (left-hand side) and a unicellular organism (right-hand side).



Other systems that involve growth on solids, but which are not defined as SSF due to the large amount of water in the inter-particle spaces. The left-hand diagram represents a trickling-filter type system while the right- hand diagram represents a suspension or slurry system

Design of Solid-state Fermentor



The environment in SSF can be quite stressful to the organism. For example:

- 1.fungal hyphae are exposed to an air phase that can desiccate them;
- 2.temperatures can rise to values that are well above the optimum for growth due to the inadequate removal of waste metabolic heat. In other words, the temperature to which the organism is exposed can vary during the growth cycle;
- 3.O2 is typically freely available at the surface of the particle, however, there may be severe restrictions in the supply of O2 to a significant proportion of the biomass that is within a biofilm at the surface or penetrating into the particle;
- 4.the availability of nutrients to the organism may be poor, even when the average nutrient concentration within the substrate particle, determined after homogenizing a sample of fermenting solid particles, is high. In other words, there tend to be large concentration gradients of nutrients within the particles;
- 5.movement of the particles of the solid substrate can cause impact and shear damage. In the case of fungal processes the hyphae can suffer severe damage;
- 6. it may be difficult to provide pH control.

However, despite being more problematic SSF may be appropriate in many instances for eg:

- 1.when the product needs to be in a solid form (e.g., fermented foods);
- 2.when a particular product is only produced under the conditions of SSF or, if produced in both SLF and SSF, is produced in much higher levels in SSF. For example, certain enzymes are only induced in SSF and some fungi only sporulate when grown in SSF, in which the hyphae are exposed directly to an air phase. If it is desired to use genetically unmodified organisms in a process for the production of such a product, then SSF may be the only option;
- when the product is produced in both SLF and SSF, but the yield is much higher in SSF. For example, Monascus pigment and many fungal spores are produced in much higher yields in SSF;
- 4.when socio-economic conditions mean that the fermentation process must be carried out by relatively unskilled workers. Some SSF processes can be relatively resistant to being overtaken by contaminants;
- 5.when the product is produced in both SSF and SLF, but the product produced in SSF has desirable properties which the product produced in SLF lacks. For example, spore-based fungal biopesticides produced in SSF processes are usually more resistant to adverse conditions than those produced in SLF, and are therefore more effective when spread in the field;
- when it is imperative to use a solid waste in order to avoid the environmental impacts that would be caused by its direct disposal. This is likely to become an increasingly important consideration as the ever- increasing population puts an increasing strain on the environment.

General Steps in SSF Process

- Inoculum preparation
- Substrate preparation
- Bioreactor preparation
- Inoculation and loading
- Bioreactor operation
- Unloading
- Downstream processing
- Waste disposal

Substrate preparation

- The substrate may need to be cut, milled, cracked, or granulated in order to obtain particles of an appropriate size.
- It may be necessary to add water and nutritional supplements or to cook or pre-treat the substrate to increase the availability of nutrients.
- The substrate might be sterilized, or at least pasteurized, outside the bioreactor.
- Alternatively, it may be possible and preferable to do this step with the substrate inside the bioreactor.
- **Inoculum preparation**
- The type and method of inoculum preparation depends on the microorganism involved.
- Many SSF processes involve filamentous fungi and therefore spore- based inocula may be used.
- The aim of this step is to develop an inoculum of sufficient size and high viability.
- The inoculum can often be prepared in one of various forms.
- For a fungal fermentation it may be possible to produce a suspended mycelial inoculum by SLF, or to undertake a solid-state fermentation followed either by suspension of spores in a liquid or by drying and grinding of the solid to produce a powder than can be used as the inoculum.

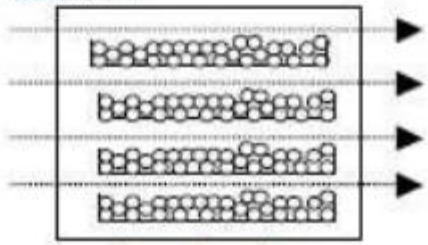
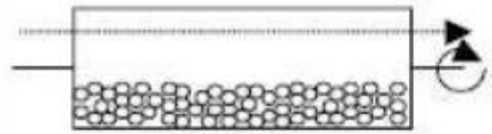
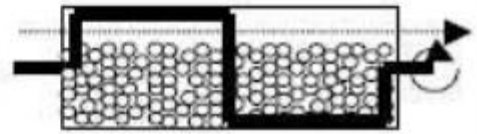
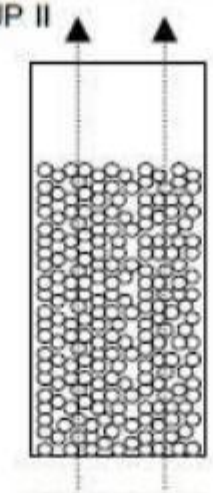
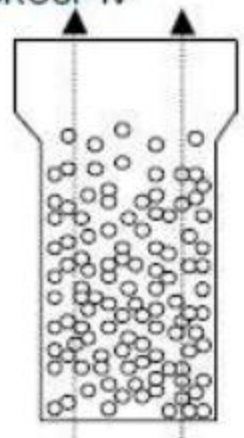
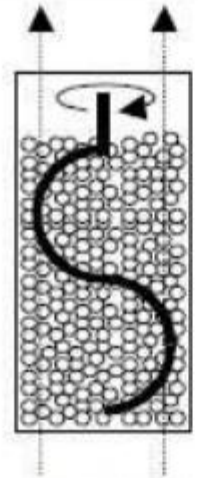
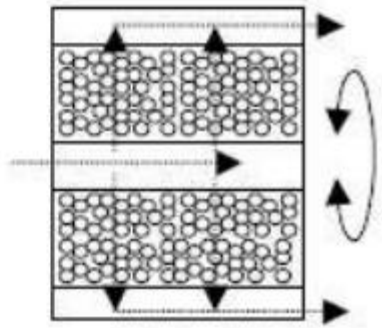
- **•Bioreactor preparation**
- The bioreactor must be cleaned after the previous fermentation, and may need to be sterilized before addition of the substrate, although, as noted above, in some cases it might be appropriate to sterilize the substrate inside the bioreactor.
- **•Inoculation and loading**
- The inoculation step may occur either prior to loading or after loading.
- If the substrate bed cannot be mixed within the bioreactor, inoculation must be done outside the bioreactor.
- If the bed can be mixed, then the best method of inoculation might be to spray the inoculum as a mist over the bed as it is being mixed.
- If the substrate is pasteurized or sterilized and inoculated outside the bioreactor, it may be necessary to undertake the loading step quite carefully in order to prevent or at least minimize the entry of contaminants.
- At large scale, loading will need to be mechanically assisted.

- **•Bioreactor operation**
- The details will depend on the specific bioreactor design, however, the general task is to manipulate various operating variables, such as the flow rate and temperature of the inlet air, the bed mixing speed, and the cooling water temperature, in order to control key fermentation parameters, such as bed temperature and water activity, at the optimum values for growth and product formation.
- **•Unloading**
- In some cases a leaching or drying step is undertaken within the bioreactor, in other cases the product recovery steps are undertaken outside of the bioreactor.
- In any case, solids must eventually be removed from the bioreactor.
- At large scale, unloading will need to be mechanically assisted.

- **Downstream processing**
- Depending on the process, either the whole of the fermented solids represents the product or a specific product is recovered from the solids and then purified.
- In the latter case, the extraction of the product from the solids represents a step in SSF processes that is not necessary in SLF processes.
- However, after extraction, the general principles of downstream processing are similar for both SSF and SLF.
- **Waste disposal**
- SSF is often suggested as a means of minimizing the impact of waste solid organic materials by preventing their being dumped in the environment.
- In some cases the whole solid is used as the product, for example, as a food or animal feed, but in others there will be a solid residue that must be disposed of adequately.

Types of Bioreactors

- SSF bioreactors can be divided into four groups on the basis of how they are mixed and aerated
  - **Group I:** Bioreactors in which the bed is static, or mixed only very infrequently (i.e., once or twice per day) and air is circulated around the bed, but not blown forcefully through it. These are often referred to as “tray bioreactors”.
  - **Group II:** Bioreactors in which the bed is static or mixed only very infrequently (i.e., once per day) and air is blown forcefully though the bed.(packed bed)
  - **Group III:** Bioreactors in which the bed is continuously mixed or mixed intermittently with a frequency of minutes to hours, and air is circulated around the bed, but not blown forcefully through it.
  - Two bioreactors that have this mode of operation, using different mechanisms to achieve the agitation, are “stirred drum bioreactors” and “rotating drum bioreactors”.
  - **Group IV:** Bioreactors in which the bed is agitated and air is blown forcefully through the bed. This type of bioreactor can typically be operated in either of two modes, so it is useful to identify two subgroups.
    - Group IVa bioreactors are mixed continuously
    - Group IVb bioreactors are mixed intermittently with intervals of minutes to hours between mixing events.
  - Various designs fulfill these criteria, such as “gas-solid fluidized beds”, the “rocking drum”, and various “stirred-aerated bioreactors”.

Mixing → / ↓ Aeration	No mixing (or very infrequent)	Continuous mixing, or frequent intermittent mixing
No forced aeration (air passes around bed)	GROUP I  Tray chamber	GROUP III  Rotating drum  Stirred drum
Forced aeration (air blown forcefully through the bed)	GROUP II  Packed bed	GROUP IV  Gas-solid fluidized bed  Stirred bed  Rocking drum

Applications of SSF

- SSF technology has been used for many centuries. Some examples of traditional SSF processes are:
- 1.Tempe involves the cultivation of the fungus Rhizopus oligosporus on cooked soybeans. The fungal mycelium binds the soybeans into a compact cake, which is then fried and eaten as a meat substitute. This fermented food is quite popular in Indonesia;
- 2.The koji step of soy sauce manufacture, which involves the cultivation of the fungus Aspergillus oryzae on cooked soybeans. During the initial SSF process of 2 to 3 days, the fungal mycelium not only covers the beans but also secretes a mixture of enzymes into them. The fermented beans are then transferred into brine, in which, over a period of several months, the enzymes slowly degrade the soybeans, leaving a dark brown sauce.
- Ang-kak, or “red rice”, which involves the cultivation of the fungus Monascus purpureus on cooked rice. The fungus produces a dark red pigment. At the end of the fermentation the red fermented rice is dried and ground, with the powder being used as a coloring agent in cooking.

- Interest in SSF technology over past decades has led to the production of a myriad of different products like:
  - enzymes such as amylases, proteases, lipases, pectinases, tannases, cellulases, and rennet
  - pigments
  - aromas and flavor compounds
  - “small organics” such as ethanol, oxalic acid, citric acid, and lactic acid
  - gibberellic acid (a plant growth hormone)
  - protein-enriched agricultural residues for use as animal feeds
  - animal feeds with reduced levels of toxins or with improved digestibility
  - antibiotics, such as penicillin and oxytetracycline
  - biological control agents, including bioinsecticides and bioherbicides
  - spore inocula (such as spore inoculum of Penicillium roqueforti for blue cheese production).
- There is also research into the use of microorganisms growing in SSF conditions to mediate processes such as:
  - decolorization of dyes;
  - biobleaching;
  - biopulping;
  - bioremediation

Submerged Fermentation

- In Submerged Liquid Fermentation (SLF) it is relatively easy to control the conditions to which the process organism is exposed:
- The fungal hyphae / bacterial cells are bathed in a liquid medium and do not run the risk of desiccation(drying)
- Temperature control is typically not overly difficult, such that the organism is exposed to a constant temperature throughout its growth cycle
- The availability of O2 to the biomass can be controlled reasonably well at a particular level of saturation of the medium (although this can become very challenging in high density cultures);
- The availability of the nutrients to the organism can be controlled within relatively narrow limits if desired, through the feeding of nutrient solutions (at least in those processes in which soluble carbon and energy sources are provided);
- Although shear forces do occur within mechanically stirred bioreactors, the nature and magnitude of these forces are well understood and it is possible to use bioreactors that provide a low-shear environment, if the organism is highly susceptible to shear damage, such as bubble columns or air lift bioreactors;
- pH control is relatively easy to provide.

Principle of Submerged Fermentation

- Submerged fermentation involves the growth of the microorganism as a suspension in a liquid medium in which various nutrients are either dissolved or suspended as particulate solids in many commercial media.
- 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.
- The process involves taking a specific microorganism such as fungi and placing it in a small closed flask containing the rich nutrient broth.
- A high volume of oxygen is also required for the process. The production of enzymes then occurs when the microorganisms interact with the nutrients on the broth resulting in them being broken down.
- The bioactive compounds are secreted into the fermentation broth.

Advantages of Submerged Fermentation

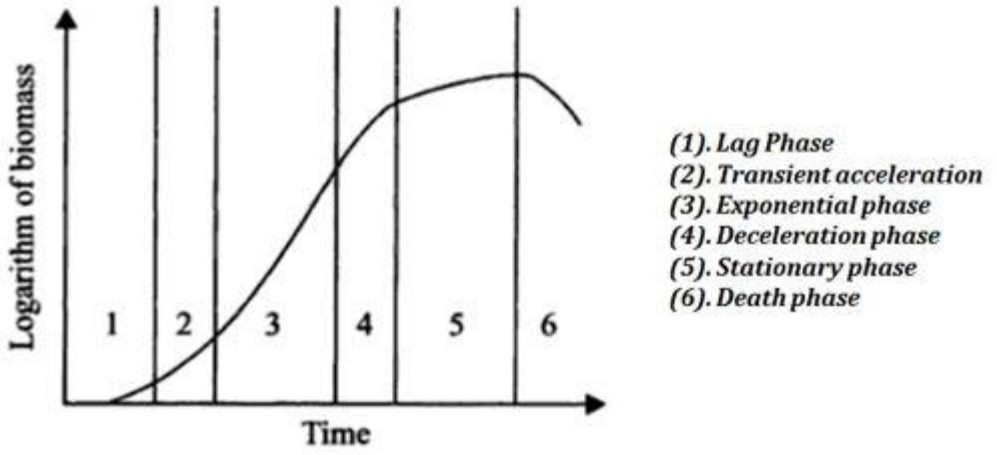
- Submerged fermentation technology has the advantages of short period, low cost and high yield.
- Purification of products is easier.
- In liquid culture the control of the fermentation is simpler and consequently significant reductions in fermentation times can be achieved.
- In the same way, the use of submerged culture can benefit the production of many secondary metabolites and decrease production costs by reducing the labour involved in solid-state methods.

Limitations of Submerged Fermentation

- In recent years, many researchers have demonstrated that SSF has a large impact on productivity, leading to higher yields and improved product characteristics compared to SmF
- Low volumetric productivity
- Relatively lower concentration of the products
- More effluent generation
- Complex fermentation equipments

Methods of Carrying Out Submerged Fermentation

- There are three different process of fermentation viz.:
  - Batch fermentation
  - Feb-batch fermentation and
  - Continuous culture
- Batch fermentation



Characteristic Growth Curve of Microbes in Batch Culture

...Batch fermentation

- This term is attributed to that type of fermentation wherein there is change in culture medium, number of microorganisms and the amount of the product produced (i.e. the metabolite or target protein). In batch fermentation six phases of the microbial growth are seen.

(a) Lag phase: Immediately after inoculation, there is no increase in the numbers of the microbial cells for some time and this period is called lag phase.

- This is in order that the organisms adjust to the new environment they are inoculated into.

(b) Acceleration phase: The period when the cells just start increasing in numbers is known as acceleration phase.

(c) Log phase: This is the time period when the cell numbers steadily increase.

(d) Deceleration phase: The duration when the steady growth declines.

(e) Stationary phase: The period where there is no change in the microbial cell number is the stationary phase.

- This phase is attained due to depletion of carbon source or accumulation of the end products.

(f) Death phase: The period in which the cell numbers decrease steadily is the death phase.

- This is due to death of the cells because of cessation of metabolic activity and depletion of energy re-sources.

- Depending upon the product required the different phases of the cell growth are maintained. For microbial mass the log phase is preferred.
- For production of secondary metabolites i.e. antibiotics, the stationary phase is preferred.

Fed-batch fermentation

- Fed- batch process is the enhancement of the closed batch process.
- In this process, substrate is added in increments as the fermentation progresses, which increases the fermenter volume.
- The formation of many secondary metabolites is subject to catabolite repression by high concentrations of glucose, other carbohydrates, or nitrogen compounds.
- Hence, the critical elements of the nutrient solution are added in small concentrations at the beginning of the fermentation & these substances continue to be added in small doses during the production phase.
- Fed batch mode is useful where a substrate causes viscosity problems or is toxic at high concentrations.
- Fed-batch with recycle of cells (biomass) can also be used for some ethanol fermentations & waste-water treatment processes.
- Some examples of fed-batch use in industry : Bakers yeast, Hepatitis B surface antigen (HbsAg) expressed in recombinant yeast, Penicillin, cellulase by *Trichoderma reesei*

…Fed-batch fermentation

- Two basic approaches to the fed-batch fermentation can be used: the constant volume fedbatch culture - Fixed Volume Fed-Batch - and the Variable Volume Fed-Batch.
- Fixed volume fed-batch
  - In this type of fed-batch, the limiting substrate is fed without diluting the culture.
  - The culture volume can also be maintained practically constant by feeding the growth limiting substrate in undiluted form, for example, as a very concentrated liquid or gas (ex. oxygen).
  - Alternatively, the substrate can be added by dialysis or, in a photosynthetic culture, radiation can be the growth limiting factor without affecting the culture volume.
- Variable volume fed-batch
  - As the name implies, a variable volume fed-batch is one in which the volume changes with the fermentation time due to the substrate feed.
  - The feed can be provided according to one of the following options:
  - (i) the same medium used in the batch mode is added;
  - (ii) a solution of the limiting substrate at the same concentration as that in the initial medium is added; and
  - (iii) a very concentrated solution of the limiting substrate is added at a rate less than (i), (ii) and (iii) .

Advantages of the fed-batch reactors

- 1. The nutritional environment can be maintained approximately constant during the course of the batch.
- 2. The production of by-products that are generally related to the presence of high concentrations of substrate can also be avoided.
- 3. Since this method usually permits the extension of the operating time, high cell concentrations can be achieved and thereby, improved productivity [mass of product/(volume.time)]. This aspect is greatly favored in the production of growthassociated products.
- 4. This method allows the replacement of water loss by evaporation and decrease of the viscosity of the broth.
- 5. Fed-batch might be the only option for fermentations dealing with toxic or low solubility substrates.
- 6. In a fed-batch fermentation, not many special piece of equipment is required in addition to that one required by a batch fermentation.

Disadvantages:

- it requires previous analysis of the microorganism, its requirements and the understanding of its physiology with the productivity it requires a substantial amount of operator skill for the set-up, definition and development of the process
- in a cyclic fed-batch culture, care should be taken in the design of the process to ensure that toxins do not accumulate to inhibitory levels and that nutrients other than those incorporated into the feed medium become limiting. Also, if many cycles are run, the accumulation of non-producing or low-producing variants may result.
- the quantities of the components to control must be above the detection limits of the available measuring equipment.

Continuous fermentation

- Continuous culture is an open system where fresh medium is continuously added & culture is simultaneously removed at the same rate , resulting in a constant working volume.
- Cells grow exponentially for extended periods at a specified predetermined growth rate.
- The system can reach a steady state in which the concentration of limiting nutrient & the cell number do not vary with time.
- Rate of growth also depends on the Dilution Rate (D). D = F/ V; where F = Flow (L/hr), V = reactor volume (L), D = dilution rate (per hr).
- Addition of fresh medium can be controlled at a fixed value, therefore the addition of the rate-limiting nutrient is constant.
- This rate of medium input determines the growth rate & rate of loss of cells from reactor (within certain limits).

…Continuous fermentation

- Under steady state conditions, the net biomass balance can be described as:
- dx/dt = rate of growth in reactor – rate of loss from reactor (wash-out).
- $dx/dt = \mu x - Dx$
- Under steady state, rate of growth = rate of loss,
- hence, $dx/dt = 0$
- Therefore, $\mu x = Dx$ $\mu = D$ (till μmax).
- Residual Substrate concentration can be calculated by substituting D in place of μ in the Monod equation.
- Continuous culture can be carried out in chemostat & turbidostat (in lab scale).

…Continuous fermentation

- Microbial activity can be controlled by either turbidostat or chemostat approaches.
- ***Turbidostat:*** *total cell population is held constant by employing a device that measures the culture turbidity so as to regulate both nutrient feed rate to the fermentor and the culture withdrawl rate from fermentor.*
  - *Growth rate should always be maximum as no limiting nutrient consciously imported .*
  - *But greater residual of unused nutrient to be lost from fermentation (The turbidostat operates best at high dilution rates).*
- ***Chemostat:*** *maintains the nutrient feed and harvest culture withdrawal rates at constant values, but always less than that which allows maximum growth rate.*
- *Chemostat is most stable and effective at lower dilution rates. If the dilution rate rises too high, the microorganisms can actually be washed out of the culture vessel before reproducing because the dilution rate is greater than the maximum growth rate. The limiting nutrient concentration rises at higher dilution rates because fewer microorganisms are present to use it.*
- This type of fermentation is used for the production of single cell protein (S.C.P), antibiotics and organic solvents.