AN INTRODUCTION TO FERMENTATION

FERMENTATION BASICS

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Fermentation Basics

Fermentation is the term used by microbiologists to describe any process for the production of a **product** by means of the mass culture of a **microorganism**.

The **product** can either be:

1. The cell itself: referred to as biomass production.
2. A microorganisms own metabolite: referred to as a product from a natural or genetically improved strain. See Table 1.
3. A microorganisms foreign product: referred to as a product from recombinant DNA technology or genetically engineered strain, i.e. recombinant strain. See Table 2.

Amino acids	Lipids
Antibacterial agents	Nucleotides and precursors
Antifungal agents	Organic synthesis intermediates
Antiprotozoal agents	Pharmaceutical significant compounds
Carbohydrates	Plant growth factors
Dyes and cosmetics	Steroids
Enzymes	Toxins
Foods	Vitamins and coenzymes

Table 1. Products produced by microbial activity

Human therapeutics
Enzymes
Amino acids

Table 2. Products being addressed by recombinant technology

2.1. Classification of microorganisms

The kingdom Protista comprises unicellular organisms capable of self duplication or of directing their own replication. Prokaryotes do not possess a true nucleus or a nuclear membrane, whereas eukaryotes have a nucleus enclosed within a distinct nuclear membrane. The non-cellular protists do not undergo self-replication, instead they direct their reproduction within another cell termed the host.

Cyanobacteria (blue-green algae) in Fig.1 have been classified as a separate group of microorganisms, although they are frequently considered to be included with other bacteria. Fungi may be subdivided into lower fungi as well as slime moulds and higher fungi which comprises yeast's. Yeast's are free-living, single cells, unlike fungi, which they closely resemble. Protozoa are free-living, minute organisms, which although not generally employed for biotechnological processes have been included for completeness. Myxomycota, commonly known as slime moulds (or slime fungi) are widely used as research organisms. Viruses can be regarded as intracellular parasites.

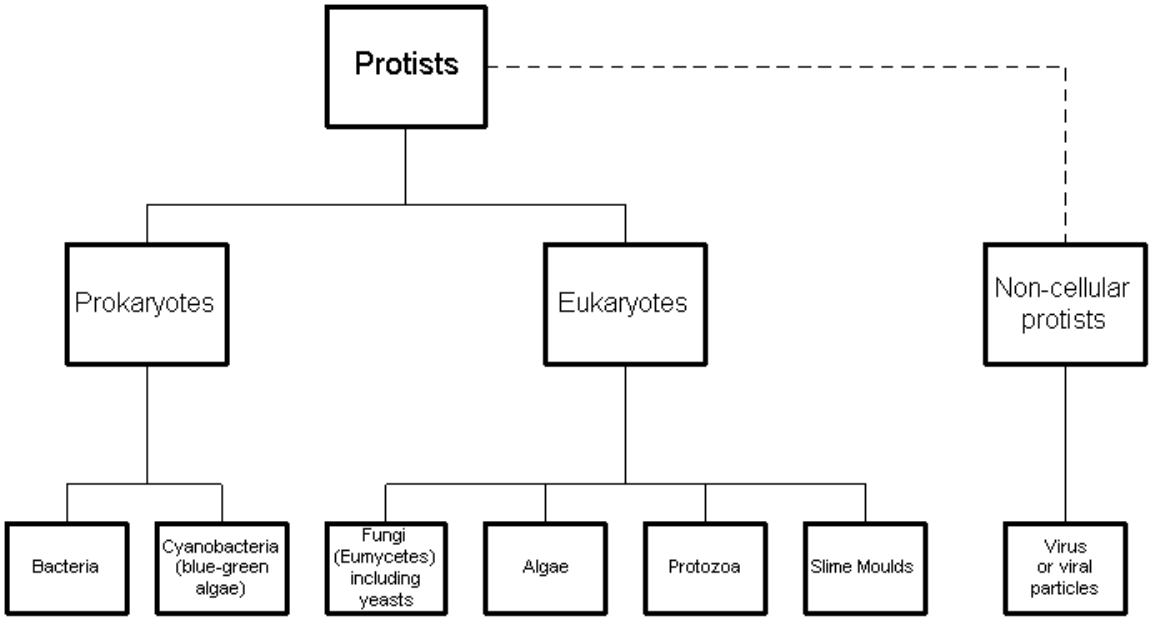

Fig.1. General classification of microorganisms

The criteria used for the classification of microorganisms include morphology, reproductive mechanisms, pigment presence, means of motility, physiology and structural features.

2.2. Microbial activity

Microorganisms in the process of self replication, produce numerous complex macromolecules from about 100 different monomer units. In the biochemical pathways to achieve this a bacterial cell uses well over 1000 different enzymes and a eukaryotic cell may employ twice as many.

The biochemical metabolism can be divided into two broad classes: the **anabolic** pathways (anabolism) synthesize the complex molecules and their intermediate precursors, and the **catabolic** pathways (catabolism) supply the energy needed for the anabolic processes. These two divergent activities are closely linked.

Microorganisms that carry out their metabolism using oxygen are referred to as **aerobic** microorganisms. Some microorganisms can substitute nitrate, others sulfate or ferric ion, for oxygen and thus grow in the absence of oxygen. These microorganisms are referred to as **anaerobic**.

Microorganisms can be classified according to the lowest temperatures at which significant growth occurs. See Table 3

Classification	**Minimum growth temperatures (°C)**
Psychrophiles	<20
Mesophiles	20-45
Thermophiles	45-60

Table 3: Thermal characterization of microorganisms

Growth of yeast is optimal in the region of 20-30°C for mesophiles species.

In general a shift in the incubation temperature from the optimum to a lower temperature results in a temperature-dependent reduction of metabolic activity. An increase in the incubation temperature can cause a reduction in both the biomass concentration and cell viability due to a temperature- and exposure-dependent decrease in enzyme activity.

Many microorganisms display an optimum pH for growth at around 7, with the majority favoring the pH range 5-8. However, there are exceptions including acetic acid bacteria, thiobacilli and urea decomposing bacteria. In addition numerous algae live in natural waters above pH 10.

2.3. Microbial kinetics

BATCH FERMENTATION

A **batch fermentation** can be considered to be a closed system. At time t=0 the sterilized nutrient solution in the fermentor is **inoculated** with microorganisms and incubation is allowed to proceed. In the course of the entire fermentation, nothing is added, except oxygen (in case of aerobic microorganisms), an antifoam agent, and acid or base to control the pH. The composition of the culture medium, the biomass concentration, and the metabolite concentration generally change constantly as a result of the metabolism of the cells.

After the inoculation of a sterile nutrient solution with microorganisms and cultivation under physiological conditions, four typical phases of growth are observed as indicated in Fig.2.

Lag phase

Physicochemical equilibration between microorganism and the environment following inoculation with very little growth.

Log phase

By the end of the lag phase cells have adapted to the new conditions of growth. Growth of the cell mass can now be described quantitatively as a doubling of cell number per unit time for bacteria and yeast's, or a doubling of biomass per unit time for filamentous organisms as fungi. By plotting the number of cells or biomass against time on a semilogarithmic graph, a straight line results, hence the term log phase. Although the cells alter the medium through uptake of
substrates and excretion of metabolic products, the growth rate remains constant during the log phase. Growth rate is independent of substrate concentration as long as excess substrate is present.

Stationary phase

As soon as the substrate is metabolized or toxic substances have been formed, growth slows down or is completely stopped. The biomass increases only gradually or remains constant du-
ring this stationary phase, although the composition of the cells may change. Due to lysis, new substrates are released which then may serve as energy sources for the slow growth of survivors. The various metabolites formed in the stationary phase are often of great biotechnological interest.

Death phase

In this phase the energy reserves of the cells are exhausted. A straight line may be obtained when a semilogarithmic plot is made of survivors versus time, indicating that the cells are dying at an exponential rate. The length of time between the stationary phase and the death phase is dependent on the microorganism and the process used. The fermentation is usually interrupted at the end of the log phase or before the death phase begins.

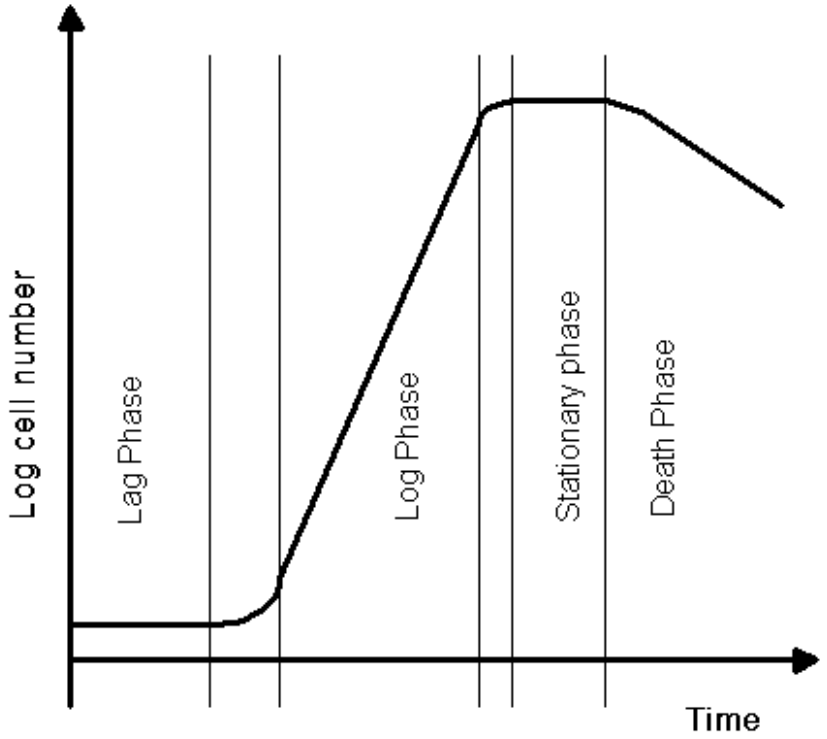

Fig. 2. Growth curve of a bacterial culture.

FED BATCH FERMENTATION

In the conventional batch process just described, all of the substrate is added at the beginning of the fermentation. An enhancement of the closed batch process is the **fed-batch** fermentation. In the fed-batch process, substrate is added in increments as the fermentation progresses. In the fed-batch method the critical elements of the nutrient solution are added in small concentrations at the beginning of the fermentation and these substances continue to be added in small doses during the production phase.

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 the case of a homogeneously mixed bioreactor we refer to a **chemostat** or a **turbidistat**. In the chemostat in the steady state, cell growth is controlled by adjusting the concentration of one substrate. In the turbidistat, cell growth is kept constant by using turbidity to monitor the biomass concentration and the rate of feed of nutrient solution is appropriately adjusted.

2.4. Nutrient requirements

All microorganisms need for their microbial activity the presence of several nutrients.

Carbohydrates

Carbohydrates are capable of being used by all microorganisms, although in no case is there an absolute requirement for this group of organic compounds. Glucose is the most readily metabolized sugar. Most fungi can use disaccharide's.

Lipides

Microbial requirements for steroids, and long-chain fatty acids can be summarized as follows. Long-chain fatty acids like linoleic acid and oleic acid are required for bacteria and fungi. Generally, steroids, other than cholesterol, are not required or utilized by microorganisms. In all fungi, and including yeast, ergosterol is a nutritional requirement.

Purines and pyrimidines

It is generally only in bacteria that cases of purine and pyrimidine metabolism have been reported. Algae do not utilize these compounds at all.

Vitamins and growth factors

There is considerable species variation in the requirements of vitamins and related factors by other microorganisms. Generally, vitamins A, C, D, and K are not necessary for growth.

Amino acids

Amino acids are not generally required by algae, although several algae species are capable of utilizing them. Species of other microorganisms are capable of utilizing all amino acids, except for yeast's, where there is no evidence of critrulline being used. It is usually the L-form of the acids that are biologically active but, unlike higher animals, some bacteria can also utilize the D-amino acids.

Nitrogen sources

It should be stressed that not all species require or utilize these compounds but rather that some species have been identified that are able to utilize these compounds. Fungi require ammonia, nitrate and nitrite.

Sulfur sources

Some species of yeast's can utilize elemental sulfur and sulfate. Generally yeast's do not require or utilize sulfur containing organic compounds. Bacteria require glutathione and

thio-acetic acid while yeast's require sulphonic acid amides, thioacetate, thiocarbonate, thioglycolate and glutathione.

Chemical elements and inorganic ions

Mineral nutrients required by microorganisms are species dependent but consists generally of Fe, K, Mg, Mn. Sometimes S, N, Ca, Co, Cu, P, Zn is required.

2.5. Fermentor systems

A microbial fermentation can be viewed as a **three-phase system**, involving liquid-solid, gas-solid, and gas-liquid reactions.

The **liquid phase** contains dissolved nutrients, dissolved substrates and dissolved metabolites.

The **solid phase** consists of individual cells, pellets, insoluble substrates, or precipitated metabolic products.

The **gaseous phase** provides a reservoir for oxygen supply and for CO_2 removal.

2.5.1. Stirring and mixing

The transfer of energy, nutrients, substrate and metabolite within the bioreactor must be brought about by a suitable mixing device. The efficiency of any one nutrient may be crucial to the efficiency of the whole fermentation.

For the three phases, the stirring of a bioreactor brings about the following:

- Dispersion of air in the nutrient solution
- Homogenization to equalize the temperature and the concentration of nutrients throughout the fermentor
- Suspension of microorganisms and solid nutrients
- Dispersion of immiscible liquids

The relative velocity between the nutrient solution and the individual cell should be about 0.5 m/sec. Nutrient solutions can be subdivided into two groups according to the way they behave when stirred: **viscous** solutions with Newtonian and non-Newtonian properties; and **viscoelastic** solutions, in which normal liquid-state properties are not observed in stirred vessels. The **viscosity**, the ability of a material to resist deformation, is the most significant property affecting the flow behavior of a fluid. Such behavior has a market effect on pumping, mixing, heat transfer, mass transfer and aeration.

There are only a few examples which fall into the second group, e.g. polysaccharides and certain antibiotic fermentation's. Most fermentation solutions fall into the first category. Uninoculated solutions and bacterial cultures often behave as simple Newtonian liquids.

With many mycelial organisms, changes occur during the fermentation not only in the amount of mycelium, but in the characteristics of the nutrient solution. Substrates are taken up during metabolism and the proportion of undissolved substrates is reduced. At the same time, metabolites are excreted, thus affecting the viscosity of the solution.

2.5.2. Gas exchange and mass transfer

One of the most critical factors in the operation of a fermentor is the provision of adequate gas exchange. **Oxygen** is the most important gaseous substrate for microbial metabolism, and **carbon dioxide** is the most important gaseous metabolic product.

When oxygen is required as a microbial substrate, it is frequently a limiting factor in fermentation. Because of its low solubility, only 0.3 mM O_2, equivalent to 9 mg/l, dissolves in one liter of water at 20 degrees Celsius in an air/water mixture. This amount of oxygen will be depleted in a few seconds by an active and concentrated microbial population unless oxygen is supplied continuously. In contrast, during the same period the amount of other nutrients used is negligible compared to the bulk of concentrations. Therefor most aerobic microbial processes are oxygen limited. This is the reason why the concept of gas-liquid mass transfer in bioprocesses is centered on oxygen transfer even if other gasses such as carbon dioxide, hydrogen, methane and ammonia can also be involved.

Due to the influence of the culture nutrients, the maximal oxygen content is actually lower than it would be in pure water.

The solubility of gasses follows Henry's law in the gas pressure range over which fermentors are operated. This means that if the oxygen concentration in the gas phase increases, the O_2 proportion of the nutrient solution increases. Consequently the highest O_2 partial pressure are attained during aeration with pure oxygen. Compared to the value in air (9 mg O_2/l), 43 mg O_2/l dissolves in water when pure oxygen is considered.

As temperature rises, the O_2 solubility decreases. For example the solubility at 33 degrees Celcius is 7,2 mg O_2/l.

For oxygen to be transferred from a gas bubble to an individual cell, several independent partial resistance's must be overcome
(Fig.3).

* 1 resistance within the gas film to the phase boundary
* 2 penetration of the phase boundary between gas bubble and liquid
* 3 transfer from the phase boundary to the liquid
* 4 movement within the nutrient solution
* 5 transfer to the surface of the cell

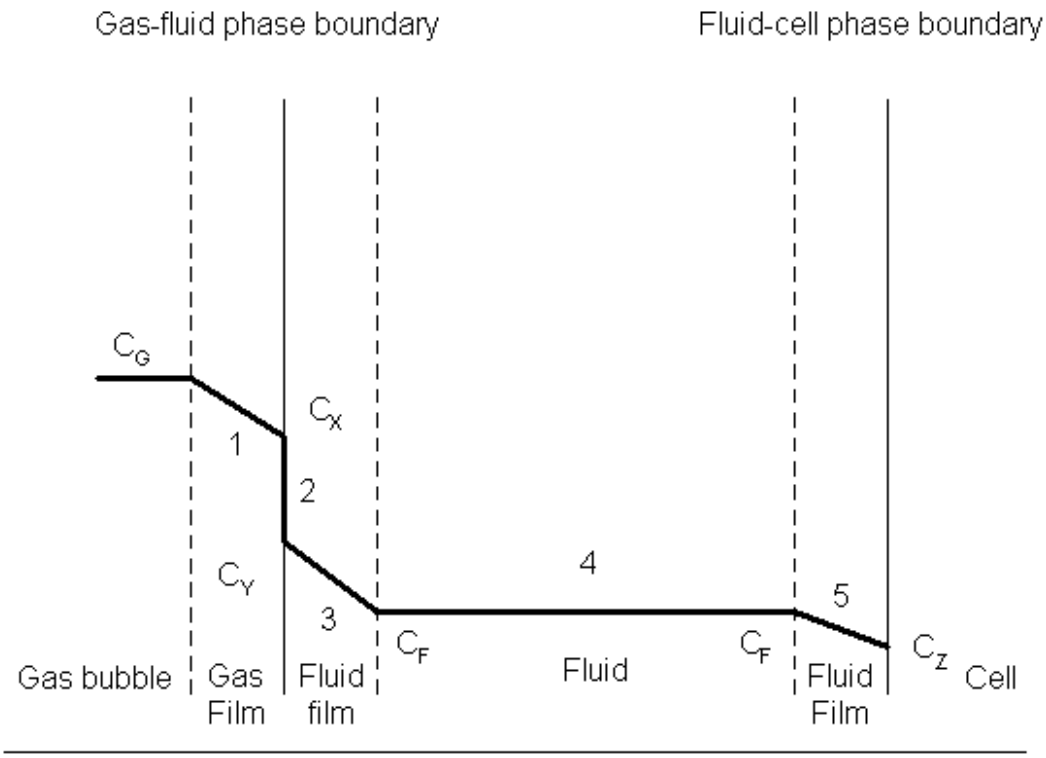

Fig.3. Resistance's for oxygen transfer from air bubble to the microbial cell

For fermentation's carried out with single celled organisms such as bacteria and yeast's, the resistance in the phase boundary between the gas bubble and the liquid is the most important factor controlling the rate of transfer.

Microbial cells near gass bubbles may absorb oxygen directly through the phase boundary and the rate of gas transfer to such cells is increased.

In cell agglomerates or pellets, the O_2 transfer within the agglomerate can become the limiting factor.

The mass transfer of oxygen into liquid can be characterized by the **oxygen tranfer rate (OTR)** or by the **volumetric oxygen transfer coefficient (k_La)**. These values have been thoroughly examined as a critical parameter for bioreactor function. The oxygen transfer rate and the volumetric oxygen transfer coefficient are dependent on the following parameters:

* the vessel geometry: diameter, capacity

* mixing properties: power, impeller configuration and size, baffles
* aeration system: sparger rate, geometry, location
* the nutrient solution: composition, density, viscosity
* the microorganism: morphology, concentration
* the antifoam agent used
* the temperature

The baffles serve to disrupt the vortex pattern that develops around a single-shaft impeller rotating in an unconstrained fluid. The baffles produce a large planar liquid surface and a uniform flow pattern as well as increase the liquid hold-up for a given fermentor volume.

Surface-active substances such as **antifoam agents** reduce the oxygen transfer rate. In pure water, the bubble surface is constantly renewed through vibration and oscillation. As soon as surface-active substances are added, the renewal of the bubble surface by bubble movement ceases.

Microorganisms themselves have an effect on the oxygen transfer rate by acting as a barrier, thus inhibiting the O_2 transfer. With filamentous microorganisms, there are variations depending on whether the mycelium is in loose form or in pellets. While the oxygen transfer rate decreases gradually as the pellets increases in size, there is a much steeper decline with loose forms.

The gas bubbles are replenished in locations of the bioreactor where there is negative pressure, such as behind the agitator blades. As the aeration rate increases, various conditions can be characterized. At low aeration rates, large gas bubbles form behind individual turbine blades and smaller bubbles are spun off centrifugally into the nutrient solution. As the aeration rate is increased, gass bubbles collect behind all turbine blades and continue to accumulate. The energy input is one-third less than that used in unaerated systems. In this intermediate stirring range, gas dispersion is the best. At very high aeration rates, many large gass bubbles adhere to each other and the impeller is flooded with gas, resulting in sharply lowered gas dispersion.

The **critical oxygen concentration** is the term used to indicate the value of the **oxygen uptake rate** or **oxygen absorption rate** which permits respiration without hindrance. Generally the critical oxygen concentrations are 5-25% of the oxygen saturation value in cultures. At oxygen absorption rates which are lower than the critical concentrations, respiration rate is correlated with the O_2 concentration in the solution. Above this value, no dependence between respiration rate and dissolved oxygen has been observed. In Newtonian fluids, such as those occurring in yeast's and bacterial fermentation's, the critical oxygen concentration is constant and is not affected by fermentation conditions. In non-Newtonian solutions, such as those occurring with filamentous microorganisms, the critical oxygen concentration has been shown to be dependent on fermentation conditions.

2.5.3. Heat production

In order to obtain optimal yields, fermentation's must be carried out at **constant temperature**. We now discuss the parameters affecting the heat balance of a fermentation process.

The rate of heat production due to stirring, gassing/aeration and due to the metabolic activity of the microorganisms must be balanced by the heat loss resulting from evaporation and radiation plus heat removal by the cooling system.

During metabolism, heat evolution is a consequence of the thermodynamics of the overall microbial activity. Apart from anaerobic digestion and some other thermophilic microbial activity, the amount of heat produced is usually so high that if it is not removed it raises the temperature of the contents of the fermentor to a level beyond the optimum range for the system.

The evolution of heat during metabolic activity is related to the utilization of the carbon and the energy source. When the carbon source is being actively incorporated into biomass trough anabolism during growth, about 40-50 % of the available enthalpy in the substrate is conserved in the biomass, the rest being given off as heat. When the carbon source is being catabolized to provide energy for cell maintenance, all the enthalpy associated with the oxidation of the substrate is released as heat. If a biochemical product is formed, the heat evolved lies between the heat released during maintenance and that evolved during active growth. The amount of heat evolved is related to the stoichiometry for growth and product formation, whereas the rate of heat evolution is related to the rate of microbial activity.

2.6. Sterilization

In virtually all fermentation processes, it is mandatory to have contamination free seed cultures at all stages, from the preliminary culture to the fermentor. A fermentor can be sterilized either by destroying the microorganisms with some lethal agent such as heat, radiation, or a chemical, or by removing the viable microorganisms by a physical procedure such as filtration.

During fermentation the following points must be observed to ensure sterility:

- sterility of the culture media
- sterility of incoming and outgoing air
- appropriate construction of the bioreactor for sterilization and for prevention of contamination during fermentation

STERILIZATION OF THE CULTURE MEDIA

Nutrient media as initially prepared contain a variety of different vegetative cells and spores, derived from the constituents of the culture medium, the water and the vessel. These must be eliminated by a suitable means before inoculation. A number of means are available for sterilization, but in practice heat is the most often used mechanism.

A number of factors influence the success of heat sterilization: the number and type of microorganisms present, the composition of the culture medium, the pH value, and the size of the suspended particles. Vegetative cells are rapidly eliminated at relatively low temperatures such as 60 degrees Celcius for 5-10 minutes, but for destruction of spores, temperatures of 121 degrees Celcius are needed during 15 minutes.

During heat sterilization there is always the possibility of destroying ingredients in the medium. Apart from the degradation of heat-labile components, also contributes to the loss of nutrient quality during sterilization. A common phenomenon is the occurance of the Maillard-type browing reactions which cause discoloration of the medium as well as loss of nutrient quality. These reactions are normally caused by carbonyl groups, usually from reducing sugars, interacting with amino groups from amino acids and proteins. Separate sterilization of the carbohydrate component of the medium may be necessary to prevent such reactions.

Filter sterilization is often used for all components of nutrient solutions which are heat sensitive. Sugars, vitamins, antibiotics or blood components are examples of heat-labile components which must be sterilized by filtration.

Most nutrient media are presently sterilized in batch volumes in the bioreactor at 121 degrees Celcius. Approximate sterilization times can be calculated from the nature of the medium and the size of the fermentor. Not only the nutrient media, but also the fittings, valves and electrodes of the fermentor itself must be sterilized. Therefor, actual sterilization

times are significantly longer than calculated ones and must be empirically determined for the specific nutrient solutions in the fermentor. Smaller fermentors are sterilized in an autoclave while larger fermentors are sterilized by indirect or direct steam injection.

STERILIZATION OF FERMENTATION AIR

Most fermentation's are operated under high aeration and the air supplied to the fermentor must be sterilized. The number of particels and microorganisms in air varies greatly depending on the location, air movement, and previous treatment of the air. On the average, outdoor air has 10-100,000 particles per m^3 and 5-2,000 microorganisms per m^3. Of these, 50% are fungus spores and 40% are Gram-negative bacteria.

Fermentors generally work with aeration rates of 0.5-2 vvm (air volume/liquid volume per minute). The methods available for sterilizing gases include filtration, gas injection (ozone), gas scrubbing, radiation (UV) and heat. Of these, only filtration and heat are practical.

APPROPRIATE CONSTRUCTION OF THE FERMENTOR

There should be a minimum number of openings in the fermentor to favor maintenance of sterility. Small openings must be made leakproof with O-rings, larger openings with flat gaskets. Whenever a movable shaft penetrates the fermentor wall, special problems of sterility maintenance should be solved.

2.7. Fermentation processes

An overall scheme of a fermentation process can be described as follows:

* Stage 1: inoculum preservation
* Stage 2: inoculum build-up
* Stage 3: fermentor culture

STAGE 1: INOCULUM PRESERVATION

The objective of preservation is to maintain strains as long as possible without cell division. The optimal method of preservation must be worked out for each strain. The following three techniques are most commonly used:

* Storage at low temperatures (2-6 degrees Celcius)
* Frozen storage (-18, -80 or -196 degrees Celcius)
* Lyophilization

Storage at 2-6 degrees Celcius is the least secure, there is a relatively high risk of contamination and reverse mutation through frequent transfer. The frozen storage is the most common and frozen cultures may be kept for several years. The proportion of survivors is critical because upto 95% of the microorganisms are generally killed during freezing and subsequent thawing. The best method of strain preservation is Lyophilization (freeze-drying).

STAGE 2: GROWTH OF INOCULUM

The preserved culture is initially revived by growth in a erlenmeyer flask on a biological shaker or on a solid medium (if spore formation is needed). In order to obtain sufficient inoculum for small fermentors, a second series of shake cultures is usually made in more flasks. Out from lyophilized strains the growth of inoculum takes around 4-10 days, out from frozen cultures the growth of inoculum takes 4-48 hours for bacteria and 1-7 days for fungi. Finally out of refrigerated cultures the growth of inoculum takes 4-24 hours for bacteria and 1-5 days for fungi.

STAGE 3: FERMENTOR CULTURE

The nutrient media for production must be optimized not only in the ingredients used but also how the medium is prepared and sterilized, pH value before and after sterilization. The most important parameters during the fermentation are:

* Temperature
* Aeration
* Stirring

2.8. Process management

The process management is concerned with:

* Setting up the initial process conditions
* Monitoring to ascertain whether the process is following the required course
* Facilitating manual adjustments to the process variables
* Deciding when to terminate the process and/or to transfer or harvest the product
* Calculating the mass and thermal balances, rates of reaction, kinetics and yields (see Table 4)
* Providing information for statistical records on consistency and for archival purposes
* Monitoring contamination and process hygiene

Measurement	Derived process data
pH	Acid product formation
Dissolved oxygen	Oxygen transfer rate
Oxygen in exhaust gas Gas flow rate	Oxygen uptake rate
Carbon dioxide in exhaust gas Gas flow rate	Carbon dioxide evolution rate
Oxygen uptake rate Carbon dioxide evolution rate	Respiratory coefficient
Sugar level Sugar feed rate Carbon dioxide evolution rate	Yield, cell density

Table 4: Examples of process data derived from direct measurement

Since many bioprocesses, particularly those involving fermentation, are operated batch wise they represent initial-value problems. Consequently **process measurement** is concerned with setting up the operation accurately and reproducibly. **Process control** is concerned with making adjustments to the process consequent upon the measurement of one or more of the variables that change as a result of the action of the process. This concerns measuring dependent variables as indicated in Table 5.

Measurement	Control function
Agitation speed rpm	Change agitation rpm
Agitation power	
Temperature ^{o}C	Heat/cool
Flow rate l/min	Change flow rate l/min
Dissolved oxygen %	Change agitation rpm Change flow rate l/min Change gas composition % Change pressure bar
pH	Acid or alkaline addition Carbon source feed rate
Foam	Anti-Foam agent addition
Exhaust gas analysis	Change feed rate
Pressure	Change pressure bar
Weight	Change flow rate l/min Change feed rate
Redox	Additives to change redox potential
Turbidity	Change feed rate

Table 5: Examples of control functions

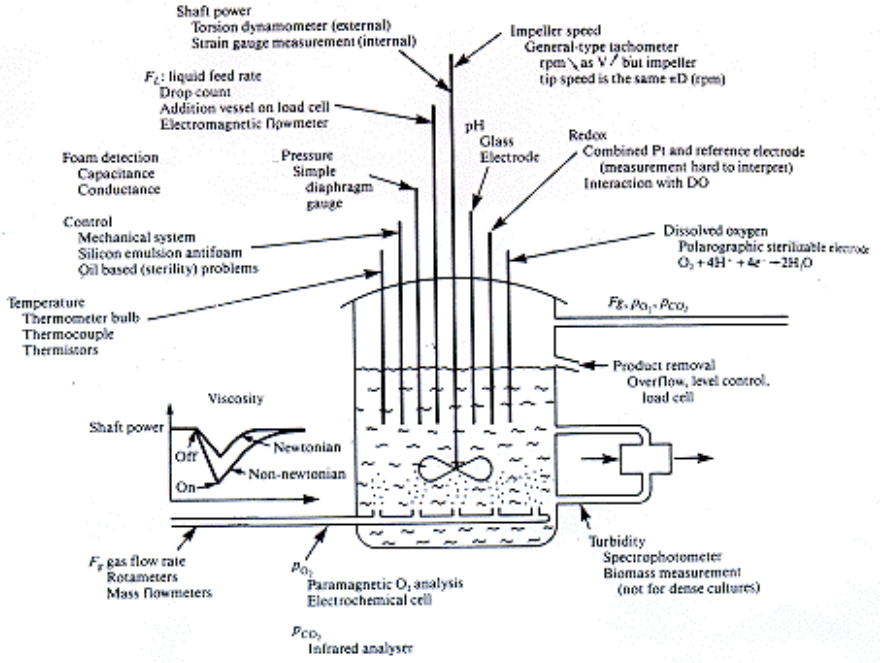

Fig.4. Illustrates both the application of these measurements to a fermentor and common methods used in their determination.

Measurement	Method	Comments
Agitator speed	Frequency counter	More precise
	Tachogenerator	Less reliable
Agitator power	Torque sensor	Difficult
	Electrical power	Recommended
Temperature	Resistance	Probably best
	Thermometer	Fragile
	Thermistor	Satisfactory
	Thermocouple	Not recommended
Flow rate	Rotameter	Satisfactory
	Orifice meter	Less accuracy
	Thermal mass flow meter	Setpoint control
Dissolved oxygen	Galvanic probe	Widely used
	Polarographic probe	Widely used
pH	pH electrode	Widely used
Foam	Conductivity probe	Satisfactory
Liquid feed rates	Burette	Large scale only
	Coriolis effect meter	Large scale only
	Magnet flowmeter	Large scale only
	Peristaltic pumps	Widely used
	Syringe pumps	Limit capacity
	Turbine flow meter	Delicate
	Weighed reservoir	Satisfactory
Exit gas analysis	CO_2 infrared analyzer	Complicated
	O_2 paramagnetic analyzer	Complicated
	Mass spectrometer	Expensive
Pressure	Pressure transducer	Satisfactory
Weight	Dp cell	Limited use
	Load cell	Expensive
	Balance	Small scale
Redox	Redox electrode	Empirical value

Turbidity	Turbidity sensor	Complex

Table 6. Physical and chemical measurements and their methods of measurement.

Other chemical parameters of interest are **substrate concentration** and **product concentration**. These parameters have to be analyzed off-line in the laboratory.

The biological parameters of interest like **doubling time**, **total cell count**, **cell viability**, **cell vitality** and **cell mass dry weight** must all be measured outside the fermentor.