1. Overview of Fermentation Technology

The word fermentation comes from the **LATIN** term '**ferver**' which means '**to boil**'. It is actually referred as the physical state of boiling process. The bubbling appearance in fermentation is the indication of production of gases during fermentation process that is particularly CO_2 which is produced by anaerobic catabolism of sugars present in the extract by the action of microorganisms.

Definition of Fermentation:
Fermentation applies to the growth of microorganisms in media under either condition (aerobic and anaerobic) to produce economically useful products.

Fermentation in Terms of Biochemist:
Biochemists consider fermentation as 'an energy-generating process in which organic compounds act both as electron donors and acceptors'; hence fermentation is 'an anaerobic process where energy is produced without the participation of oxygen or other inorganic electron acceptors'**.**

Fermentation in Terms of Microbiologist:
Microbiologists consider fermentation as 'any process for the production of a product by means of mass culture of micro-organisms'.

Fermentation in Terms of Biotechnologist:
In biotechnology, the microbiological concept is widely used. Fermentation in biotechnology can be referred to the "Use of Biological system for the sake of development / improvement / facilitation in a process by mass culture of cells".

As the Fermentation is a process of mass culturing of the cells for the sake of desired product so according to this definition fermentation involve following:

i. *Mass culturing techniques*

Classical techniques for mass culture of microorganisms require the use of liquid culture in large vessels with the accompanying requirements for aeration and agitation etc. Automated fermenters, which are bulky and expensive, can be used in this technique.

ii. *Types of cells and organisms*

A wide range of cells and organisms can be culture depending upon the type of process and product that is being produce.

iii. *Fermentation Products*

A range of capacity of fermentation products can be produced depend upon the requirement and type of process as well as microorganisms used.

Fermentation products include:

- **Food products:** from milk (yogurt, kefir, fresh and ripened cheeses), fruits (wine, vinegar), Vegetables (pickles, sauerkraut, soy sauce), meat (fermented sausages: salami)
- **Industrial chemicals:** (solvents: acetone, butanol, ethanol; enzymes; amino acids)
- **Specialty chemicals** (Vitamins, Pharmaceuticals)

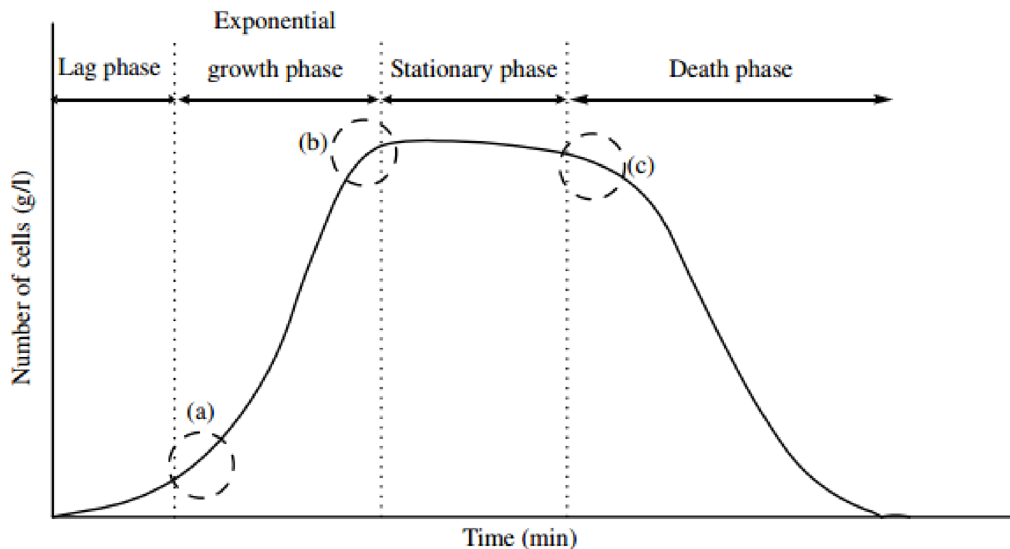
Important Products of Fermentation Process:

- ➢ Acids
- ➢ Enzymes
- ➢ Gasses
- ➢ Alcohols
- ➢ Hormones
- ➢ Single Cell Protein
- ➢ Yogurt
- ➢ Cheese
- ➢ Wines
- ➢ Bread
- ➢ Pickles

2. Microbial Growth Kinetics

Batch Culture:

Batch culture requires enough nutrients to maintain the growth



This figure shows an increase of cell at the start of the cultivation (fermentation) process. Due to the presence of enough nutrients for the cell to grow the amount of nutrient decreases as it being consumed by the cell. Other side products such as carbon dioxide or ethanol are also formed simultaneously. In batch cultures, the cell properties such as:

- Size of cells
- Internal nutrient
- Metabolic function varies considerably during the above growth phases.
- No apparent increase of the amount of cell at the start of cultivation, this is termed as the lag phase.

After this period (can be between 10 to 15mins) the number of cells increases exponentially thus, this stage is called the exponential growth phase; the cell properties tend to be constant. The next stage is the stationary phase where the population of cell achieves it maximum number. This is because: all nutrients in the closed system have been used up by the cell. Lack of nutrient will eventually stop the cell from multiplying.

The rate of cell growth is given by; r X = μx where r X is the volumetric rate of biomass production with units such as $kg.m^{-3}.s^{-1}$, x is the viable cell concentration with units of $kg.m^{-1}$ and μ is the specific growth rate with units of s^{-1}. By following the above equation, the growth is said to follow the first-order autocatalytic reaction.

Similarly, the rate can be written as:
rX = d/ dt and thus; dx/dt = μx

Which upon rearrangement gives; μ = 1/x. dx/dt

Since the equation is only valid if μ is unchanging, therefore the plot can be used to asses whether the specific growth rate is constant for a particular cell growth.

Cell growth rates are normally expressed in terms of **doubling time**, t_d.

When $x = 2x_0$ at $t = t_d$ thus the equation becomes;

$$2x_0 = x_0 e^{\mu t_d}$$

$$e^{\mu t_d} = 2$$

Taking natural log on both sides;

$$\mu t_d = \ln 2$$

or

$$t_d = \frac{\ln 2}{\mu}$$

The final stage of cell cultivation is the **death phase**. The decrease of the number of cell occurs exponentially which happens when the cell breaks open (lysed).

The rate of death normally follows the first-order kinetics given by;

$$\frac{dN}{dt} = -k_d^{'}N$$

The relationship of cell growth and substrate/nutrient concentration can be expressed in terms of Monod Model given by;

$$\mu = \frac{\mu_{max}[S]}{K_S + [S]}$$

The function gives a hyperbolic curve upon plotting the data point.

upon integration leads to

$$N = N_s e^{-k_d^{'}t}$$

where N_s is the concentration of cells at the end of the stationary phase and at the beginning of the death phase and $k_d^{'}$ is the first order death rate constant.

Both stationary and death phase, it is important to recognise that there is a distribution of properties among the cells in a population. A summary of the different phases of cell growth is given in the table overleaf.

Activate V

Growth phase	Rate of growth	Comments
Lag	Zero	Inoculum adapting with the changing condition (temperature, pH)
Acceleration	Increasing	Trivial
Exponential	Constant	Population growth changes the environment of the cells
Retardation	Decreasing	The effect of changing conditions appear
Stationary	Zero	One or more nutrients are exhausted to the threshold level of the cell

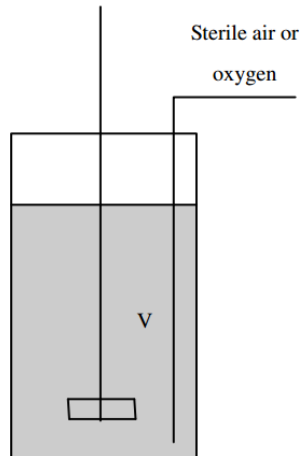
Growth phase	Rate of growth	Comments
Decline	Negative	The duration of stationary phase and the rate of decline are strongly dependent on the kind of organisms
Death phase	Negative	Cells lyse due to lack of nutrient

The balance of a batch reactor is given by the rate of accumulation of product equals to the rate of formation of the product due to chemical reaction or can be simply written as;

$$d/dt\ (VR \cdot c) = VR \cdot r$$

$$dc/dt = r$$

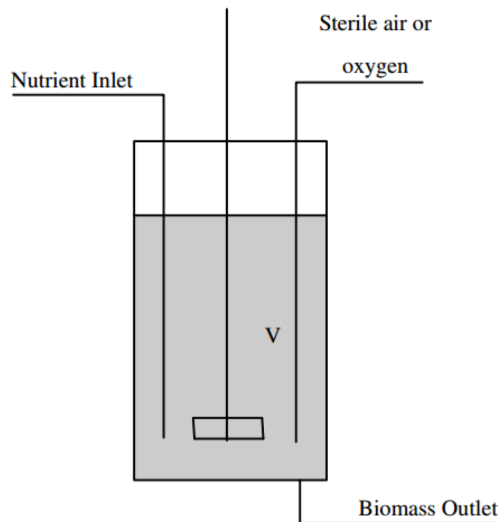
Where c is the amount of the component and r is the reaction rate. VR in the first line of the equation is the total volume of the culture in the reactor.



The balance of a batch reactor is given by the rate of accumulation of product equals to the rate of formation of the product due to chemical reaction or can be simply written as; d/dt (VR · c) = VR · r dc/dt = r (10) where c is the amount of the component and r is the reaction rate. VR in the first line of the equation is the total volume of the culture in the reactor.

Continuous Culture:

In a continuous culture system, nutrients are supplied to the cell at a constant rate. To maintain a constant volume of biomass in the reactor, an equal volume of cell culture is removed. This will allow the cell population to reach a steady-state condition. The reactor configuration of a continuous process is given overleaf.



The air is pumped into the culture vessel through a sterile filter. Bubbling of air provides: supplying air for the growth of aerobic culture. It also circulate and agitate the culture and

pressurize the head space of the culture vessel such that to provide a force during the removal of the media (and cells) from the vessel for analysis (OD, cell viability etc.). It is highly difficult to control the delivery of the nutrient and the removal of the cell so that equal amounts of medium are maintain in the vessel.

This can be tackled by changing the configuration of the reactor into a semi-continuous or fed-batch type reactor. The rate of flow of medium into a system of continuous culture is known as the dilution rate. When the number of cells in the culture vessel remains constant over time, the dilution rate is said to equal the rate of cell division in the culture, since the cells are being removed by the outflow of medium are being replaces by an equal number through cell division in the culture.

Similar to that of the batch cultivation, the material balance for a continuous culture can be written as;
$d/dt\,(V_R \cdot c) = F_o c_o - F_i c_i - V_R \cdot r$ (11) in order to maintain the volume within the vessel; $F_i = F_o = F$

Thus,

$$\frac{d}{dt}(V_R \cdot c) = F(c_o - c_i) - V_R \cdot r$$

$$\Rightarrow \quad \frac{dc}{dt} = \frac{F}{V_R}(c_o - c_i) - r - c\frac{dV_R}{dt}$$

For a reactor without a recycle system,

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

therefore,

$$\frac{dc}{dt} = \frac{F}{V_R}(c_o - c_i) - r \qquad (13)$$

let the term $\frac{F}{V_R}$ denote as D, the final equation leads to,

$$\frac{dc}{dt} = D(c_o - c_i) - r \qquad (14)$$

where D is the **dilution rate** of a CSTR cultivation system.

Activate Win
Go to Settings to

Batch Growth:

Material balance:

- consider the **cell** concentration in the fermenter;

$$\frac{d}{dt}(V_R x) = V_R r_x$$

$$\Rightarrow V_R \frac{dx}{dt} = V_R r_x$$

since $r = \frac{\mu_{max}[S]}{K_S+[S]}$, thus;

$$\Rightarrow \frac{dx}{dt} = \left(\frac{\mu_{max}[S]}{K_S + [S]}\right)$$

Activate Wi

At steady-state, $dx/dt = 0$ thus, $\Rightarrow \mu max[S] / KS + [S] = 0$

This clearly shows that when there is no growth in the bioreactor, there will be no cell to divide, thus no new cells to be produced.

Consider the substrate/nutrient concentration in the fermenter; $d/dt\,(VR[S]) = -\,VR\ r\ x$

At steady-state condition, $d[S]/dt = 0$ which leads to $[S] = 0$ as well.

Continuous Growth:

The balance on the continuous stirred-tank fermenter/reactor is similar to that of the batch, provided that there are inlet and outlet to and from the fermenter respectively. Consider the cell concentration, x; $d/dt\,(VR\ x) = Fixi - Fo\ x\ o + VR\ rx$ but $rx = \mu x$

thus;

$$\frac{d}{dt}(V_R x) = F_i x_i - F_o x_o + V_R \mu x$$

upon expansion and let $\mu = \frac{\mu_{max}[S]}{K_S+[S]}$ gives,

$$V_R \frac{dx}{dt} = F_i x_i - F_o x_o + V_R \left(\frac{\mu_{max}[S]}{K_S + [S]}\right) x$$

upon rearranging;

$$\frac{dx}{dt} = \frac{F_i}{V_R} x_i - \frac{F_o}{V_R} x_o + \left(\frac{\mu_{max}[S]}{K_S + [S]}\right) x$$

for a constant volume in a bioreactor,

$$F_i = F_o = F$$

thus,

$$\frac{dx}{dt} = \frac{F}{V_R}x_i - \frac{F}{V_R}x_o + \left(\frac{\mu_{max}[S]}{K_S + [S]}\right)x$$

let $\frac{F}{V_R} = D$, which represents the **dilution rate** of the fermenter, hence,

$$\frac{dx}{dt} = Dx_i - Dx_o + \left(\frac{\mu_{max}[S]}{K_S + [S]}\right)x$$

at a steady-state condition, $\frac{dx}{dt} = 0$;

$$\Rightarrow Dx_i - Dx_o + \left(\frac{\mu_{max}[S]}{K_S + [S]}\right)x = 0$$

since the cells must be grown in a sterile environment, therefore;

$$x_i = 0$$

and

$$x_o = x$$

then the equation becomes;

$$Dx = \left(\frac{\mu_{max}[S]}{K_S + [S]}\right)x$$

$$\Rightarrow D = \mu_{max}\left(\frac{[S]}{K_S + [S]}\right)$$

where;

$$D_{max} = \mu$$

At a washout steady-state, as the dilution rate, D of the continuous fermenter increases, the concentration of substrate, [S] also increases, where $D > D_{max}$ at $x = 0$.

The feed substrate, [S] will be such that [S] ≫ KS and Dmax becomes approximately equals to the maximum specific growth rate, μmax, $D_{max} \approx \mu_{max}$
Solving for the substrate concentration leads to; $[S] = K_S D / \mu_{max} - D$

consider the **substrate** concentration, $[S]$;

$$\frac{d}{dt}(V_R[S]) = F_i[S]_i - F_o[S]_o - \frac{1}{Y_{X/S}}V_R r_x$$

where $Y_{X/S}$ is defined as;

$$Y_{X/S} = \frac{mass\ of\ biomass/cells\ produced}{mass\ of\ substrate\ used}$$

expanding and rearranging the terms gives;

$$\frac{d[S]}{dt} = D([S]_i - [S]_o) - \frac{1}{Y_{X/S}}\left(\frac{\mu_{max}[S]}{K_S + [S]}\right)x$$

at steady-state condition, $\frac{d[S]}{dt} = 0$, therefore,

$$\Rightarrow D([S]_i - [S]_o) - \frac{1}{Y_{X/S}}\left(\frac{\mu_{max}[S]}{K_S + [S]}\right)x = 0$$

let the outlet substrate concentration, $[S]_o = [S]$
therefore,

$$\Rightarrow D([S]_i - [S]) - \frac{1}{Y_{X/S}}\left(\frac{\mu_{max}[S]}{K_S + [S]}\right)x = 0$$

and upon substitution of the term $[S]$ into the above equation gives;

$$x = Y_{X/S}\left([S]_i - \frac{K_S D}{\mu_{max} - D}\right)$$

3. Fermenter Design

Fermenter Design with Multi-impeller

Agitators achieve the following objectives; (a) bulk fluid and gas-phase mixing, (b) air dispersion, (c) oxygen transfer, (d) heat transfer, (e) suspension of solid particles, and (f) maintenance of a uniform environment throughout the vessel. These objectives are achieved by a suitable combination of the most appropriate agitator, air sparger and baffles, and the best positions for nutrient feeds, acid or alkali for pH control and antifoam addition. Agitators are of several different types, e.g., (i) disc turbines, (ii) vaned discs, (iii) open turbines of variable pitch and (iv) propellers.

Multi-impeller stirred vessels utilize two, three, or more impellers in a single shaft configuration. The number of studies reporting work on dual- and triple-impeller configurations are relatively few even though they are common in the industry, and fewer still focus on systems with more than three impellers because increasing the number of impellers resulted in increased flow complexity. Additionally, the tank height required for systems with more than three impellers is not practical for industrial applications. Stirred vessels using multiple impellers are widely used in chemical, biotechnology, pharmaceutical, food processing, and many other industries for mixing processes. Researchers have focused on multiple-phase systems such as gas-liquid, gas-liquid-solid, and solid-liquid systems in view of the wide industrial applications of multiple-impeller stirred vessels.

Disc turbine consists of a disc with a series of rectangular vanes set in a vertical plane around its perphery. The vaned disc turbine has a series of rectangular vanes attached vertically to the underside of the disc. In case of variable pitch open turbine, the vanes are attached directly to a boss on the agitator shaft.

The marine propeller is similar to variable pitch open turbine, except that it has blades in the place of vanes. In case of disc and vaned disc turbines, the air bubbles from the sparger first-hit the underside of disc before being broken into smaller bubbles and dispersed by the vanes. But in the case of the latter two types of agitators, air bubbles contact the vanes/blades directly and arc broken up and dispersed by them. These basic agitation devices have been variously modified. For example, the variable pitch open turbine scheme has been modified to develop four modern agitator types, viz., Scaba 6SRGT, Prochem Maxflo T, Lightning A315 and the Ekato Intcrmig.

The Rushton disc turbine, having a diameter of one-third the fermenter diameter, has been long considered optimum for many fermentation processes. The disc turbine was considered optimum because it was shown to be able to break up a fast air stream without itself becoming flooded in air bubbles; the latter situation seriously hampers oxygen dispersal in the broth.

In contrast, the impeller and open turbine were found to have the tendency to be flooded in air at higher aeration rates. In subsequent studies, it was found that in low viscosity broths, all the four agitator types can achieve good gas dispersion provided the agitator speed is high enough.

In such broths, agitator type does not appear to be a significant factor affecting oxygen transfer efficiencies. In high viscosity broths, however, gas dispersal presents problems and is greatly reduced. In view of this, a number of agitators have been developed for high viscosity broths, e.g., Scaba 6SRGT, Prochem Maxflow T, Lightning A315 and Ekato Intermig.

These agitators are larger, require lower power input (they do not lose as much power as the Rushton turbines when aerated), are able to handle higher air volumes without flooding, and give better bulk blending and heat transfer in more viscous media. But they can cause mechanical problems mostly of vibrational nature. Good mixing and aeration in high viscosity broths may also be achieved by a dual impeller combination in which the lower impeller primarily dispenses the air, while the upper impeller primarily enhances mixing of the broth.

Fermenter Design: Construction Materials

The function of the fermenter or bioreactor is to provide a suitable environment in which an organism can efficiently produce a target product - the target product might be cell biomass, metabolite and bioconversion Product. It must be so designed that it is able to provide the optimum environments or conditions that will allow supporting the growth of the microorganisms. The design and mode of operation of a fermenter mainly depends on the production organism, the optimal operating condition required for target product formation, product value and scale of production. The choice of microorganisms is diverse to be used in the fermentation studies. Bacteria, Unicellular fungi, Virus, Algal cells have all been cultivated in fermenters. Now more and more attempts are tried to cultivate single plant and animal cells in fermenters. It is very important for us to know the physical and physiological characteristics of the type of cells which we use in the fermentation. Before designing the vessel, the fermentation vessel must fulfill certain requirements that are needed that will ensure the fermentation process will occur efficiently. Some of the actuated parameters are: the agitation speed, the aeration rate, the heating intensity or cooling rate, and the nutrients feeding rate, acid or base valve. Precise environmental control is of considerable interest in fermentations since oscillations may lower the system efficiency, increase the plasmid instability and produce undesirable end products.

Bioreactors are commonly cylindrical vessels with hemispherical top and/or bottom, ranging in size from some liter to cube meters, and are often made of stainless steel and glass. The difference between a bioreactor and a typical composting system is that more parameters of the composting process can be measured and controlled in bioreactors. The sizes of the bioreactor can vary over several orders of magnitudes. The microbial cell (few mm^3), shake flask (100-1000 ml), laboratory fermenter (1 – 50 L), pilot scale (0.3 – $10m^3$) to plant scale (2 – 500 m^3) are all examples of bioreactors. The design and mode of operation of a fermenter mainly depends on the production organism, the optimal operating condition required for target product formation, product value and scale of production. The design also takes into consideration the capital investment and running cost.

• Large volume and low value products like alcoholic beverages need simple fermenter and do not need aseptic condition.

• High value and low volume products require more elaborate system of operation and aseptic condition.

Requirements of Bioreactors Due to above mentioned demands made by biological systems on their environment, there is no universal bioreactor. However, the general requirements of the bioreactor are as follows:

1. The vessel should be robust and strong enough to withstand the various treatments required such as exposure to high heat, pressure and strong chemicals and washings and cleanings.
2. The vessel should be able to be sterilized and to maintain stringent aseptic conditions over long periods of the actual fermentation process.
3. The vessel should be equipped with stirrers or mixers to ensure mass transfer processes occur efficiently.
4. It should have sensors to monitor and control the fermentation process.
5. It should be provided with inoculation point for aseptic transfer in inoculum.
6. Sampling valve for withdrawing a sample for different tests.
7. Baffles should be provided in case of stirred fermenter to prevent vertex formation.
8. It should be provided with facility for intermittent addition of an antifoam agent.
9. In case of aerobic submerged fermentation, the tank should be equipped with the aerating device.
10. Provision for controlling temperature and pH of fermentation medium. Main hole should be provided at the top for access inside the fermenter for different purposes. It is obvious that the design of the fermenter will involve co-operation between experts in microbiology, biochemistry, chemical engineering, mechanical engineering and costing.

Basic points of consideration while designing a fermenter:

- Productivity and yield
- Fermenter operability and reliability
- Product purification
- Water management
- Energy requirements
- Waste treatment

Fermenter Design: Pilot Scale & Industrial Scale Fermenters

Laboratory scale bioreactor:

In fermentation with strict aseptic requirements it is important to select materials that can withstand repeated sterilization cycles. On a small scale, it is possible to use glass and/or stainless steel.

Glass is useful because it gives smooth surfaces, is non-toxic, corrosion proof and it is usually easy to examine the interior of vessel. The glass should be 100% borosilicate, e.g. Pyrex® and Kimax®. The following variants of the laboratory bioreactor can be made:

1. Glass bioreactor (without the jacket) with an upper stainless steel lid.
2. Glass bioreactor (with the jacket) with an upper stainless steel lid.
3. Glass bioreactor (without the jacket) with the upper and lower stainless steel lids.

4. Two part bioreactor - glass/stainless steel. The stainless steel part has a jacket and ports for electrodes installation.
5. Stainless steel bioreactor with peepholes. Vessels with two stainless steel plates cost approximately 50% more than those with just a top plate.

Pilot scale and large scale bioreactors:

When all bioreactors are sterilized in situ, any materials use will have to assess on their ability to withstand pressure sterilization and corrosion and their potential toxicity and cost. Pilot scale and large scale vessels are normally constructed of stainless steel or at least have a stainless steel cladding to limit corrosion. The American Iron and Steel Institute (AISI) stated that steels containing less than 4% chromium are classified as steel alloys and those containing more than 4% are classified as stainless steel. Mild steel coated with glass or phenolic epoxy materials has occasionally been used. Wood, concrete and plastic have been used when contamination was not a problem in a process. Although stainless steel is often quoted as the only satisfactory material, it has been reported that mild-steel vessels were very satisfactory after 12 years use for penicillin fermentations and mild steel clad with stainless steel has been used for at least 25 years for acetone-butanol production. The corrosion resistance of stainless steel is thought to depend on the existence of a thin hydrous oxide film on the surface of metal. The composition of this film varies with different steel alloys and different manufacturing process treatment. The film is stabilized by chromium and is considered to be continuous, non-porous, insoluble and self healing. If damaged, the film will repair itself when exposed to air or an oxidizing agent.

The minimum amount of chromium needed to resist corrosion will depend on the corroding agent in a particular environment, such as acid, alkalis, gases, soil, salt or fresh water. Increasing the chromium concentration enhances the resistance to corrosion, but only grades of steel containing at least 10 to 13% chromium develop the effective film. The inclusion of nickel in high percent chromium steels enhances resistance and improves their engineering properties. The presence of molybdenum improves the resistance of stainless steels to solution of halogens salts and pitting by chloride ions in brine or sea water. Corrosion resistance can also be improved by tungsten, silicon and other elements.

Fermenter Design: Grading quality of Construction Material

Construction quality is a central concept in the approaches used to value commercial and industrial improvements. The quality of the material and workmanship used in constructing an improvement, together with its design elements, will influence its cost new. Construction quality, and the resultant quality grade assigned, is a composite characteristic. It describes the cumulative effects of workmanship, the costliness of materials, and the individuality of design used in constructing an improvement. Although the construction quality of individual components of an improvement may vary, the overall construction quality tends to be consistent for the entire structure. Workmanship quality can easily be observed in an inspection of the property. Good quality workmanship is evidenced by plumb vertical surfaces; level horizontal surfaces, properly located and installed mechanical systems, and an overall pride in workmanship. Material quality is also easily observable during an inspection of the property. Primary indicators of material

quality are type and spacing of framing members, type and grade of interior and exterior finishing materials, type and grade of plumbing and electrical fixtures, and type and grade of mechanical systems. Design is also an indicator of quality of construction. Although most commercial and industrial structures are designed primarily for utility and not for looks, in some occupancies (e.g. office buildings) the importance of appearance and amenities is equal to the importance of pure utility. The fenestration and ornamentation plus the overall layout and design of the building should be considered in determining quality grade.

AISI grade 316 steels which contains 18% chromium, 10% nickel, 2-2.5% molybdenum are now commonly use for fermenter or bioreactor construction. In citric acid fermentation where pH may be 1 to 2, it will be necessary to use a stainless steel with 3-4% molybdenum (AISI grade 317) to prevent leaching of heavy metals from the steel which would interfere with the fermentation. AISI grade 304, which contains 18.5% chromium and 10% nickel, is used extensively for brewing equipment. Now also Stainless steels (e.g..: 1.4435, 1.4539, etc.), Hastelloy, Incolloy, Inconel, Monel, Titanium grades 1, 2, 7, 11 are used in construction of bioreactor. With plant and animal cell tissue culture, a low-carbon version (type 316L) is often used. The thickness of the construction material will increase with scale. At 300,000 to 400,000 dm3 capacity, 7-mm plate may be used for the side of the vessel and 10-mm plate for the top and bottom, which should be hemispherical to withstand pressure.

It is also important to consider the ways in which a reliable aseptic seal is made between glass and glass, glass and metal, metal and metal joints such as between a fermenter vessel and a detachable top or base plate. With glass and metal a seal can be made with a compressible gasket, a lip seal or an ‘O’ ring. With metal to metal joints only ‘O’ ring is suitable. A single ‘O’ seal is adequate for GILSP and levels 1 and B2; a double ‘O’ ring seal is required for levels 2 and b3; a double ‘O’ ring seal with steam between the seals (steam tracing) is necessary for levels 3 and B4.

Fermenter Design: Seals & O Rings

One of the most stringent requirements in the design of a fermenter is the ability of the fermenter to maintain strict aseptic integrity throughout the fermentation process. Of course this is applicable in monoseptic fermentation involving single pure culture fermentation. For such requirements, theoretically there should be complete isolation or barrier between the fermenter content and the surrounding environment. This would literally mean that the fermenter is completely closed by wall or structure to the environment with no openings or holes that expose the content of the fermenter to the environment. In reality this is difficult to achieve as fermenters need holes or ports that allow important connections for the insertion of impellers, electrodes, inlet gas and outlet gas and even sampling ports. If this is so then the design of the fermenter must reach a compromise where while on one hand it maintains its aseptic integrity and on the other hand it allows port holes for the necessary components to enter or leave the fermenter but by not compromising its aseptic integrity.

The solution to this dilemma is by:
1. Providing seals
2. Maintaining the seals continually aseptic.

The seal is especially crucial at the stirrer or agitator entrance to the fermenter. The stirrer is the essential component in any fermenter as it is involves in the mixing and homogenization of the contents of the fermenter. Any seal that is used in the agitator system will have to fulfil various requirements such as:

1 It should be able to maintain the state of aseptic integrity of the fermenter while functioning.
2 It should be able to withstand the stress of repeated sterilizations of the fermenter such as SIPs.
3 It should be able to withstand the various CIP procedures.
4 It should be able to sustain the pressure developed within the fermenter.
5 If the fermenter is involved in food and pharmaceutical fermentations, it should be built of sanitary materials
6 The seal components will not contaminate the fermentation process
7 It should be able to isolate the contents inside the fermenter from the external environment. In any seal there is usually a need for: Packing structure within the seal, Lubricant to smoothen the rotation of the shaft within the seal, Steam sterilization of the seal to ensure no contaminations occurring through the seal

There are other seals within the fermenter which is made up of the O - rings that help in the close air tight sealing between two surfaces. Sealing assembly provides most satisfactory sealing to the stirrer shaft, which is required to provide agitation. This assembly can be operated aseptically for long periods. Three types seal assembly are used:

Packed gland seal: The shaft is sealed by several layers of packing rings of asbestos, pressed against the shaft by a gland. The packing rings are regularly replaced and checked to prevent unsatisfactory heat penetration.

Mechanical seal: The seal is consists of two parts, the stationary part in the bearing and the other rotating on the shaft. The two components are pressed together by springs. Stem condensate are used to lubricate and cool seals during operation.

Magnetic drives: It consists of two magnets, one driving and another driven. The driving magnet is held in bearing on the outside of head plate and connected to the drive shaft. The internal driven magnet is placed on one end of the impeller shaft and held in bearings on the inner surface of the head plate.

Sealing between top plate and vessel is an important criteria to maintain airtight condition, aseptic and containment. Sealing have to be done between three types of surfaces viz. between glass-glass, glass- metal and metal-metal. There are three types of sealing. They are gasket, lip seal and ‘O’ ring. This sealing ensures tight joint in spite of expansion of vessel material during fermentation. The materials used for sealing may be fabric-nitryl or butyl rubbers. The seals should be changed after finite time. There are two way of sealing in O ring type simple sealing and double sealing with steam between two seals.

O-rings are in constant use in a fermentation laboratory. They are used as the compressible material when a seal is made between glass and metal or between metal and metal. O-Rings are usually composed of nitrile or butyl rubber, sometimes of silicone. Remember that items such O-

rings have a finite life span dependent upon how often they are autoclaved, and whether or not they are deformed by over-compression; they will thus have to be carried out as part of the regular maintenance programs.

Fermenter Design: Basic Features of a Stirred Tank Bioreactor: Agitation System

The agitator is required to achieve a number of mixing objective.

- Bulk fluid and gas-phase mixing
- Air dispersion
- Oxygen transfer
- Heat transfer
- Suspension of solid particles and maintain a uniform environment throughout the vessel contents
- Enhancement of mass transfer between dispersed phases.

Bulk mixing and micro mixing both are influenced strongly by impeller type, broth rheology, and tank geometry and internals.

Impellers used bioreactors are: Rushton disc turbines, vaned discs, open turbines of variable pitch and propeller. The disc turbine consists of a disc with a series of rectangular vanes set in vertical plane around the circumstances and vaned disc has a series of rectangular vanes attached vertically to the underside. Air from the sparger hits the underside of the disc and is displaced towards the vanes where the air bubbles are broken up into smaller bubbles. The vanes of variable pitch open turbine and the blade of marine impellers are attached directly to a boss on the agitator shaft. In this case air bubbles do not initially hit any surface before dispersion by the vanes or blades.

The axial flow hydrofoil impellers have become increasingly popular. These axial flow systems can pump liquid either down or up. They have been shown to give superior performance (compare to Rushton radial flow impellers) with respect to lower energy demands for the same level oxygen transfer. Further, they show reduced maximum shear rates, making them usable with sensitive cultures such as animal cell culture, while still being capable of giving excellent performance with viscous mycelial fermentation.

Combination of axial flow and radial flow impeller systems are sometimes used. One way to improve bulk mixing while maintaining good oxygen transfer rate is to use Rushton turbine at lower position in tank and an axial flow impeller at the top. Marine propellers and A-215 work quite well in this scheme for many applications over a wide range of scales. The A-315 (hydrofoil design) will work even better because it has a higher power number than the others; hence it should contribute more to oxygen transfer than the others. The designer can help to minimize top-to-bottom mixing problem by keeping the ratio of liquid height to tank diameter under 2, and by spacing the impeller properly. If the impellers are placed too closely, they interfere with each other, thereby decreasing OTR and mixing quality. If they are spaced too far apart, overall homogeneity suffers. Researchers have found that spacing between 1 and 1.5

impeller diameters gives good mixing in most practical cases, and oxygen transfer is not affected significantly by spacing within this range.

Agitation impellers: The vessel, 130 mm in diameter, had a working volume of 1.8 L in a 3.0 L nominal volume. Figure shows the agitator configuration used. Sizes and positions of the impeller and ring sparger are listed in Table. The vessel was fitted with a thermocouple and a pH electrode.

The temperature of the test fluid was maintained at 30°C in each experiment by circulating temperature-controlled water in a jacket. The impellers were driven by a variable-speed dc motor and the agitation speeds were measured by a tachometer generator indicating system.
Good mixing and aeration in high viscosity broths may also achieved by a dual impeller combination, where the lower impeller acts as the gas dispenser and upper impeller acts primarily as a device for aiding circulation of vessel contents.

Approximately 66% less power requirement even when viscous and oxygen transfer efficiency improved. Intermig agitator has two units. Unlike the earlier design agitator/ vessel diameter ratio is 0.6-0.7. For this agitator larger air sparger is used and top to bottom mixing not efficient. New turbine designs with dual impeller have been introduced. One for gas dispersion and other for aiding circulation, with multirod mixing.

Agitator design and Operation-1: Technically speaking, for a liquid mixed in a tank with a rotating agitator, the shear rate is greatest in the immediate vicinity of the agitator. The shear rate decreases exponentially with distance from the agitator. Thus the shear stresses and strain rates vary greatly throughout an agitated liquid in a tank. As the viscosity of the fluids to be mixed increases, the physics fluid mechanics change from that of turbulent flow (like in liquid agitators) to that in which viscous drag forces dominate. Additionally, some fluids exhibit Non-Newtonian behavior – their viscosity cannot be designated by a single coefficient. Mixing of such fluids requires special heavy duty agitators. As the dynamic viscosity of a Newtonian liquid is independent of shear at a given temperature, its viscosity will be the same at all points in the tank. In contrast the apparent viscosity of a non-Newtonian liquid varies throughout the tank. This in turn significantly influences the mixing process.

For shear thinning liquids, the apparent viscosity is at a minimum in the immediate vicinity of the agitator. The progressive increase in the apparent viscosity of a shear thinning liquid with distance away from the agitator tends to dampen eddy currents in the mixing tank. In contrast, for shear thickening liquids, the apparent viscosity is at a maximum in the immediate vicinity of the agitator. In general shear thinning and shear thickening liquids should be mixed using high and low speed agitators respectively. Wherever possible, the movement of the entire contents of the agitator vessel should be avoided as rapid movement tends to segregate the components due to centrifugal forces.

The mixing time is short if the components to be mixed undergo a large number of changes of location. This can take the form of movement of the agitator itself or of material flows generated

by the agitator. They can be achieved by impact, flow around obstacles, crossing directions of flow and speed differentials at the interfaces of parallel flows.

Baffles are metal strips roughly one-tenth of the vessel diameter and attached radially to the fermenter wall. They are normally used in fermenters having agitators to prevent vortex formation and to improve aeration efficiency.

Usually, four baffles are used, but larger fermenters may have 6 or 8 baffles. Extra cooling coils may be attached to baffles to improve cooling. Further, the baffles may be installed in such a way that a gap exists between the baffles and the fermenter wall. This would lead to a scouring action around and behind the baffles, which would minimize microbial growth on the baffles and the fermenter wall.

During agitation of a low-viscosity liquid, the rotating impeller imparts tangential motion to the liquid. Without baffling, this swirling motion approximates solid-body rotation in which little mixing actually occurs. Think about stirring a cup of coffee or a bowl of soup: The majority of the mixing occurs when the spoon is stopped or the direction of stirring is reversed. The primary purpose of baffling is to convert swirling motion into a preferred flow pattern to accomplish process objectives. The most common flow patterns are axial flow, typically used for blending and solids suspension, and radial flow, used for dispersion. However, baffling also has some other effects, such as suppressing vortex formation, increasing the power input and improving mechanical stability.

Agitator design and Operation-2

The most common flow patterns in mixing are axial (down and up) and radial (side to side) flow. These flow patterns also describe the generic classes of impellers: axial and radial.

1. Axial flow impeller:
Axial (down and up) pumping is an important flow pattern because it addresses two of the most common challenges in mixing; solid suspension and stratification. In this process both the superficial and annular velocities can be calculated to determine and control the level of mixing.

2. Radial flow impeller:
Unlike axial impellers, radial impellers are commonly selected for low level mixing (known as a tickler blade) or elongated tanks. They typically give high shear rates because of their angle of attack. They also have a relatively low pumping number, making them the most sensitive to viscosity. Radial impellers do not have a high tank turnover flow like axial flow impellers.
Agitators are of several different types, e.g., (i) disc turbines, (ii) vaned discs, (iii) open turbines of variable pitch and (iv) propellers.

Disc turbine consists of a disc with a series of rectangular vanes set in a vertical plane around its periphery. The vaned disc turbine has a series of rectangular vanes attached vertically to the underside of the disc. In case of variable pitch open turbine, the vanes are attached directly to a boss on the agitator shaft. The marine propeller is similar to variable pitch open turbine, except

that it has blades in the place of vanes. In case of disc and vaned disc turbines, the air bubbles from the sparger first-hit the underside of disc before being broken into smaller bubbles and dispersed by the vanes. But in the case of the latter two types of agitators, air bubbles contact the vanes/blades directly and arc broken up and dispersed by them. These basic agitation devices have been variously modified. For example, the variable pitch open turbine scheme has been modified to develop four modern agitator types, viz., Scaba 6SRGT, Prochem Maxflo T, Lightning A315 and the Ekato Intcrmig.

Rushton disc turbines, vaned discs, open turbines of variable pitch and propeller. The disc turbine consists of a disc with a series of rectangular vanes set in vertical plane around the circumstances and vaned disc has a series of rectangular vanes attached vertically to the underside. Air from the sparger hits the underside of the disc and is displaced towards the vanes where the air bubbles are broken up into smaller bubbles. The vanes of variable pitch open turbine and the blade of marine impellers are attached directly to a boss on the agitator shaft. In this case air bubbles do not initially hit any surface before dispersion by the vanes or blades. The The axial flow hydrofoil impellers have become increasingly popular. These axial flow systems can pump liquid either down or up. They have been shown to give superior performance (compare to Rushton radial flow impellers) with respect to lower energy demands for the same level oxygen transfer. Further, they show reduced maximum shear rates, making them usable with sensitive cultures such as animal cell culture, while still being capable of giving excellent performance with viscous mycelial fermentation. Combination of axial flow and radial flow impeller systems are sometimes used. One way to improve bulk mixing while maintaining good oxygen transfer rate is to use Rushton turbine at lower position in tank and an axial flow impeller at the top. Marine propellers and A-215 work quite well in this scheme for many applications over a wide range of scales. The A-315 (hydrofoil design) will work even better because it has a higher power number than the others; hence it should contribute more to oxygen transfer than the others.

Agitator design and Operation-3: Mixing is very crucial for the maximum productivity in microbial fermentation and it could be achieved by means of aeration and agitation. But agitation at higher stirring speeds may cause disruption of free cells in the reactor by forces and formation of vortex which may result in poor mass transfer (oxygen/substrate).

However, if the rotational speed of the impeller is sufficiently high, superimposed on the distribution process is turbulence. Turbulence flow occurs when fluid no longer travels along streamlines but moves erratically in the form of cross-currents. The kinetic energy of turbulent fluid is directed into regions of rotational flow called eddies; masses of eddies of various size coexist during turbulent flow. Large eddies are continuously formed by action of the stirrer; these break down into small eddies which produce even smaller eddies. Eddies, like spinning tops, possess kinetic energy. When eddies become so small that they can no longer sustain rotational motion, their kinetic energy is dissipated as heat.

The process of breaking up bulk flow into smaller and smaller eddies is called dispersion; dispersion facilitates rapid transfer of material throughout the vessel. The degree of homogeneity

as a result of dispersion is limited by the size of the smallest eddies which may be formed in a particular fluid.

Shear rates are very difficult to determine, and are thus often neglected. Since shear is the gradient of velocity in respect to space, very finely spaced velocity measurements must be made in order to determine the effect of shear on gas and solid particles of similar dimensions. In other words, a shear gradient determined over a path length of 1 mm is irrelevant to a particle with a 10-µm size. Likewise, the shear gradient over a 10-µm path will have no effect on 1 mm particles. The integration of all the velocity profiles over the immediate discharge area of the impeller results in the determination of the primary flow. Integration along a plane in the discharge zone of the impeller until the point of flow reversal, results in the determination of total impeller flow of an impeller. The total flow generated by an impeller is a result of the primary flow and the impeller's entrained flow. The ratio of entrained to direct flow is a function of the impeller's diameter to tank diameter ratio. The laser is thus a very useful tool to determine both the flow and shear characteristics of an impeller. Positioning the laser at one point and tracing the flow fluctuations with time, results in the determination of local energy fluctuations or time related shear gradients. High shear impellers also produce high-energy fluctuations.

Radial impellers are typically available in 4 or 6 blade designs. They are known to provide more shear and less flow per unit of applied horsepower than axial flow designs, and in comparison, radial flow impellers do not have a high tank turnover flow. They are sensitive to viscosity, which makes them an excellent impeller in dispersion applications like pigment pastes or caulking compounds.

Axial flow impellers are very useful in mixing solid-liquid suspensions because they prevent the solid particles from settling at the bottom of the tank. The type and geometry of impeller used will vary from process to process and the determination of the best geometry for a specific process requires a great deal of experimentation. Radial flow impellers should be used in situations where high shear rates are needed, such as in dispersion processes.

Agitator design and Operation-4: The Rushton turbine is a radial flow impeller used for many mixing applications (commonly for gas dispersion applications). The design is based on a flat disk with vertical flat blades vertically mounted. Recent innovations include the use of concave or semi-circular blades.

The Rushton disc turbine, having a diameter of one-third the fermenter diameter, has been long considered optimum for many fermentation processes. The disc turbine was considered optimum because it was shown to be able to break up a fast air stream without itself becoming flooded in air bubbles; the latter situation seriously hampers oxygen dispersal in the broth.

The Rushton disc turbine is the one used most often for highly aerobic fermentations, because it has among the highest power draws of any the commercially available impellers, and it is better characterized than others; hence, its behavior is easier to predict. Rushton disc turbine of one third of the fermenter diameter has been considered the optimum design for use in fermentation processes. Disc turbine is most suitable in a fermenter since it can break up a fast air stream

without itself becoming flooded in air bubbles. A marine propeller is an axial flow impeller which provides good top-to-bottom mixing. It is low power device does not provide large oxygen-transfer rates.

One way to improve bulk mixing while maintaining good oxygen transfer rate is to use Rushton turbine at lower position in tank and an axial flow impeller at the top. Marine propellers and A-215 work quite well in this scheme for many applications over a wide range of scales. The A-315 (hydrofoil design) will work even better because it has a higher power number than the others; hence it should contribute more to oxygen transfer than the others. The designer can help to minimize top-to-bottom mixing problem by keeping the ratio of liquid height to tank diameter under 2, and by spacing the impeller properly. If the impellers are placed too closely, they interfere with each other, thereby decreasing OTR and mixing quality. If they are spaced too far apart, overall homogeneity suffers. Researchers have found that spacing between 1 and 1.5 impeller diameters gives good mixing in most practical cases, and oxygen transfer is not affected significantly by spacing within this range. The effect of broth rheology on mixing are most pronounced for non-Newtonian broths. If Rushton turbine used in a pseudoplastic broth, the shear rate drops off rapidly and the viscosity increases rapidly with distance from the turbine tip; therefore, there is good mixing only in the immediate vicinity of the impeller, and air tends to channel around the impeller and rise up to shaft. One way to overcome this problem is to use very large diameter turbines.

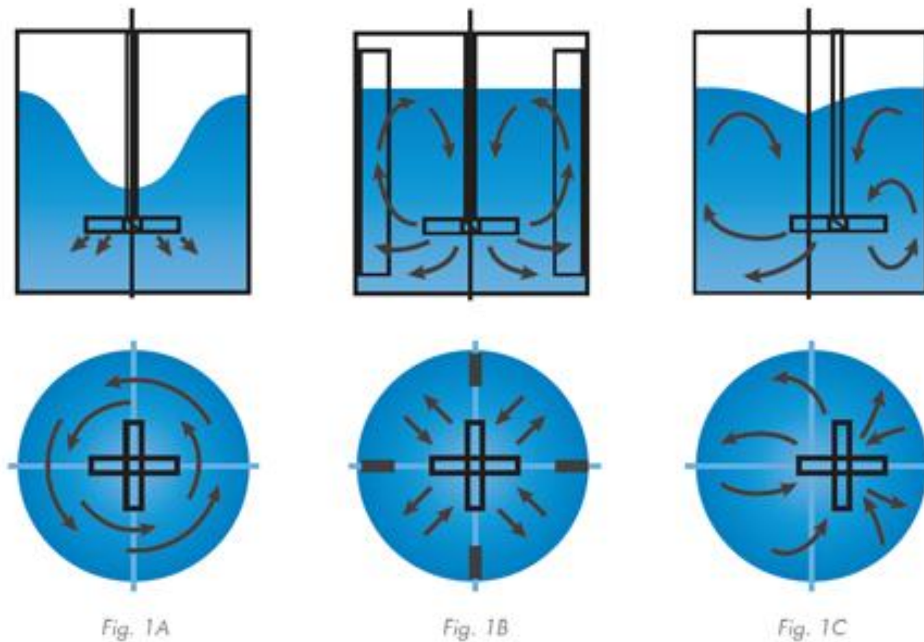
There are some new impellers that have been designed to give adequate axial flow and very good OTR, particularly for non-newtonian broths. Also to overcome problems associated with efficient bulk blending in high-viscosity fermentation. Indeed, the Prochem Hydrofoil and the Mixco A-315 have been shown to do all this, and to require less power than Rushtons for the same OTR at commercial scale. The Scaba 6SRGT agitator is one which at a given power input can handle a high air flow rate before flooding. This radial-flow agitator is also better for bulk blending than a Rushton turbine, but does not give top to bottom blending in a large fermenter which leads to lower concentrations of oxygen in broth away from the agitators and higher concentration of nutrients, acid or alkali or antifoam near to the feed points.

Agitator design and Operation: Axial Flow Impellers-1

Axial flow impellers are very useful in mixing solid-liquid suspensions because they prevent the solid particles from settling at the bottom of the tank. The type and geometry of impeller used will vary from process to process and the determination of the best geometry for a specific process requires a great deal of experimentation

The axial flow hydrofoil impellers have become increasingly popular. These axial flow systems can pump liquid either down or up. They have been shown to give superior performance (compare to Rushton radial flow impellers) with respect to lower energy demands for the same level oxygen transfer. Further, they show reduced maximum shear rates, making them usable with sensitive cultures such as animal cell culture, while still being capable of giving excellent performance with viscous mycelial fermentation.

Axial flow impellers have an up and down flow pattern, ideal for applications where solids suspension or stratification is a challenge. The flow pattern produced by typical axial flow impeller produces an excellent top to bottom motion when the agitator is center mounted, and the vessel is fully baffled (see Fig 1B). If the baffles are removed, the fluid in the vessel will swirl and vortex (Fig 1A), resulting in a rather poor mix.



Propeller: The propeller is typically used in mixers that are small and portable. This type of impeller tends to be heavy and quite expensive in larger sizes.

Pitched Blade: A pitched blade impeller is used when a balance of flow and shear is required. It is especially useful in applications where 2 or more liquids are blended, and is well suited for situations with low bottom clearance or low liquid submergence.

Hydrofoil: The hydrofoil impeller offers the best high flow design. It is known for its low turbulence, and is excellent for shear sensitive applications.

Agitator design and Operation: Axial Flow Impellers-2

These impellers have blades which make an angle of less than 90° to the plane of rotation and promote axial top-to-bottom motion such as propellers – pinched blade turbine Fluid leaving the impeller is driven downwards until it is} deflected from the floor of the vessel Then, it spreads out over the floor and flows up along the} wall before being drawn back to the impeller The impellers are useful when strong vertical} currents are required i.e if the fluid contain solids, strong axial flow of liquid leaving will discourage settling at the bottom of the tank.

Intermig is an interference multistage counter flow impeller that has an inner pitched blade and an outer double blade arranged in a staggered position with an opposing blade angle. Due to the

staggering of the outer blades, interference is produced causing a distinct axial deviation of the flow. As a result, these low-shear axial flow agitators used at large diameter ratios (0.5T-0.95T) are highly suitable for blending, suspending, dispersing, as well as heat transfer applications in both the laminar and transitional flow regimes. Typically, following manufacturers' instructions (EKATO, 1991), Intermigs are installed in pairs, each being rotated 90° respect to one another and are separated vertically by a distance of 0.5T to effectively mix at Reynolds numbers (Re) > 100.

Due to their versatility and effective performance for a number of applications in varying flow regimes, it is not surprising that Intermig impellers are readily used in the process industries. However, it appears that there have been very few studies published in the literature that concern the flow and performance of these agitators. Nienow (1990) found that when the inner blades of the Intermig pump downwards in gas-dispersion applications, flooding occurs much more easily than when they are up-pumping. Ibrahim and Nienow (1995) used streak photography to examine the flow patterns produced by a pair of Intermigs and concluded that the flow patterns produced are complex and very different from the schematic diagram given by the manufacturers. They also compared the power curves of the impellers with down-pumping and up-pumping inner blades for laminar through to turbulent flow regimes.

Knowing that the pumping direction does not improve the fluid exchange between the impellers at low Re, the impellers are rotated by 45° clockwise or anti-clockwise so that the impeller configuration resembles a spiral staircase. For these configurations, the circulation loops are well established and the vectors (not shown) suggest that there is good fluid exchange between the Intermig impellers.

Overall it appears that the direction in which the Intermigs are rotated relative to one another (clockwise or anticlockwise) does not influence the interference behaviour. The power consumption, however, is slightly lower for the anti-clockwise rotation, being equivalent to the typical 90° configuration. For Re = 60, there is fluid exchange between the four impeller stages, with a slightly weaker connection between the two lower Intermigs. The performance is similar to that of the typical configuration. At Re = 37, there is still adequate connection between the four upper circulation regions, which suggests better axial mixing than for the configuration with 90° rotation. When the Re is lowered to 27, the 45° configurations still ensure fluid exchange between the upper three Intermig impellers, with compartmentalisation of only the lowest Intermig.

4. Media for Industrial Fermentations

Growth Factors

Specific nutritional requirements of microorganisms used in industrial fermentation processes are as complex and varied as the microorganisms in question. Not only are the types of microorganisms diverse (bacteria, molds and yeast, normally), but the species and strains become very specific as to their requirements. Microorganisms obtain energy for support of biosynthesis and growth from their environment in a variety of ways. Besides a source of energy, organisms require a source of materials for biosynthesis of cellular matter and products in cell operation, maintenance and reproduction. These materials must supply all the elements necessary to accomplish this. Some microorganisms utilize elements in the form of simple compounds; others require more complex compounds, usually related to the form in which they ultimately will be incorporated in the cellular material. The four predominant types of polymeric cell compounds are the lipids (fats), the polysaccharides (starch, cellulose, etc.), the information-encoded poly-deoxyribonucleic acid and poly-ribonucleic acids (DNA and RNA), and proteins.

Lipids are essentially insoluble in water and can thus be found in the non-aqueous biological phases, especially the plasma and organelle membranes. Lipids also constitute portions of more complex molecules, such as lipoproteins and liposaccharides. Lipids also serve as the polymeric biological fuel storage. Natural membranes are normally impermeable to highly charged chemical species such as phosphorylated compounds. This allows the cell to contain a reservoir of charged nutrients and metabolic intermediates, as well as maintaining a considerable difference between the internal and external concentrations of small cations, such as H', Kf and Na'. Vitamins A, E, K and D are fat-soluble and water-insoluble. Sometimes they are also classified as lipids.

Typically 30-70% of the cell's dry weight is protein. All proteins contain C, H, N, and 0. Sulfur contributes to the three-dimensional stabilization of almost all proteins. Proteins show great diversity of biological functions. The building blocks of proteins are the amino acids. The predominant chemical elements in living matter are: C, H, O, and N, and they constitute approximately 99% of the atoms in most organisms. Carbon, an element of prehistoric discovery, is widely distributed in nature. Carbon is unique among the elements in the vast number and variety of compounds it can form. It has been estimated that hydrogen makes up more than 90% of all the atoms or three quarters of the mass of the universe. Oxygen makes up 21% and nitrogen 78% volume percent of the air. These elements are the smallest ones in the periodic system that can achieve stable electronic configurations by adding one, two, three or four electrons respectively. This ability to add electrons, by sharing them with other atoms, is the first step in forming chemical bonds, and thus, molecules.

Some microorganisms need preformed compounds popularly known as "growth factors", as every microorganism cannot synthesize all most all the basic requirements to build up the cell components The commonly required growth factors are vitamins, selected amino acids, fatty acids, sterols etc. The need of some or many required growth factors can be satisfied by natural carbon and nitrogen sourced added in the media formulation. The vitamin deficiency can often be satisfied by careful blending of materials added in the medium. But, in case when there is a

need of only one type of vitamin, it is very costly to add in pure form. Calcium pantothenate can be used in one medium formulation for vinegar production.

Growth factors and trace nutrients are included in the fermentation broth for organisms incapable of producing all of the vitamins they require. Yeast extract is a common source of micronutrients and vitamins for fermentation media. Inorganic nutrients, including trace elements such as iron, zinc, copper, manganese, molybdenum and cobalt are typically present in unrefined carbon and nitrogen sources, but may have to be added when purified carbon and nitrogen sources are used. Fermentations which produce large amounts of gas (or which require the addition of gas) will tend to form a layer of foam, since fermentation broth typically contains a variety of foam-reinforcing proteins, peptides or starches. To prevent this foam from occurring or accumulating, antifoaming agents may be added. Mineral buffering salts, such as carbonates and phosphates, may be used to stabilize pH near optimum. When metal ions are present in high concentrations, use of a chelating agent may be necessary.

Buffers

Microbial growth is pH specific and thus the control of pH is very important if optimal yield is to be achieved. Certain compounds can be added to the medium to serve exclusively as a buffer. Many media are buffered by the incorporation of calcium carbonate at about pH 7.0 and during the process, if the pH decreases the carbonate is decomposed. Phosphate is an important part of several media. Many time, phosphate play an important role in buffering during production of secondary metabolites. The pH of the medium can also be controlled by balance use of the carbon and nitrogen sources, because buffering capacity can be provided by the amino acid, peptides and protein present in corn steep liquor. Addition of sulphuric acid, ammonia and sodium hydroxide is also an alternative way to control the pH externally.

It is advantageous to add various buffering agents to the medium in order to insure favorable pH conditions or by-products which may inhibit the growth of the organism or the elaboration of the desired products do not accumulate during the course of the fermentation. Various combinations of salts of weak acids and strong alkalies or strong acids and weak cations may be used in order to buffer the medium in cases where the buffering is on the basis of the acidity or alkalinity of the medium is desired. In cases where the medium tends to become acidic as the fermentation proceeds and where such acidity may inhibit the growth of the organism, it is advantageous to add buffering salts such as calcium carbonate to the medium. Other buffers may also be used to maintain the proper pH during the fermentation. Precipitants may be added to remove undesirable metabolites. Enzymes may be added to assist the organism in the utilization of the medium.

Buffers are also added to prevent drastic changes in pH, and antifoam would often be needed when complex media are used. For many fermentations e.g., antibiotic production, medium suited for rapid cell growth is unsuitable for product formation. In such cases, specialized media for production have to be devised.

Precursors

Precursors are defined as “substances added prior to or simultaneously with the fermentation which are incorporated without any major change into the molecule ofthe fermentation product and which generally serve to increase the yield or improve the quality of the product”. They are required in certain industrial fermentations and are provided through crude nutritive constituents, e.g., corn steep liquor or by direct addition of more pure compounds. Some fermentations must be supplemented with specific precursors, notably for secondary metabolite production. When required, they are often added in controlled quantities and in a relatively pure form, examples include, D-threonine is used as a precursor in L-isoleucine production by Serratia marcesans, and anthranillic acid additions are made to fermentations of the yeast Hansenula anomola during L-tryptophan production. The use of corn steep liquor as side-chain precursors in penicillin fermentations results in six different penicillins as opposed to the use of phenylacetic acid which results in mainly Penicillin G formation.

Instead of rather supporting the growth of the microorganism, a number of components of a fermentation medium assist to regulate the production of the product. These additives are called precursors, inhibitors and inducers. These compounds can be exploit to influence and manage the progress of the fermentation. When we add certain compounds in some selected fermentation processes, these compounds are directly incorporated into the desired product. For instance, for improving penicillin yields, precursors are added. A variety of diverse side chains can be incorporated into the side chain of penicillin molecule. Corn steep liquor, which was found to contain phenylethylamine, when added in the medium, improved the yield of penicillin from 20 unit cm-3 to 100 unit cm-3. It was found that phenylethylamine present in the corn steep liquor preferentially incorporated into the penicillin molecule to yield benzyl penicillin. As we know that the activity of penicillin rest in the side chain. The limiting factor to synthesize active penicillin was the synthesis of its side chain. Now, it has become a normal practice to add the side chain precursors to the medium, in particular phenylaceticacid. As reported by Smith and Bide (1948), the production of penicillin can be increased three time when phenyl acetic acids and its derivatives are added in the medium As on this date, the phenylacetic acid is the most widely used precursor in the penicillin production.

Inhibitors

Inhibitors are used to redirect metabolism towards the target product and reduce formation of other metabolic intermediates; others halt a pathway at a certain point to prevent further metabolism of the target product. An example of an inhibitor specifically employed to redirect metabolism is sodium bisulphite, which is used in the production of glycerol by *S. cerevisiae*. Some GMMs contain plasmids bearing an antibiotic resistance gene, as well as the heterologous gene(s). The incorporation of this antibiotic into the medium used for the production of the heterologous product selectively inhibits any plasmid-free cells that may arise.

When certain inhibitors are added to fermentations, more of a specific product may be produced, or a metabolic intermediate which is normally metabolized is accumulated. One of the earliest examples is the microbial production of glycerol. Glycerol production depends on modifying the ethanol fermentation by removing acetaldehyde. The addition of sodium bisulphite to the broth leads to the formation of the acetaldehyde bisulphite addition compound (sodium hydroxy ethyl

sulphite). Since acetaldehyde is no longer available for re-oxidation of $NADH_2$, its place as hydrogen acceptor is taken by dihydroacetone phosphate, produced during glycolysis. The product of this reaction is glycerol-3-phosphate, which is converted to glycerolInhibitors have also been used to affect cell-wall structure and increase the permeability for release of metabolites. The best example is the use of peniclin and surfactants in glutamic acid production.
In most cases the inhibitor is effective in increasing the yield of the desired product and reducing the yield of undesirable related products. A number of studies have been made with potential chlorination inhibitors, e.g. bromide, to minimize chlortetracycline production during tetracycline fermentation.

Inducers

Enzymes are biocatalysts produced by living organisms to bring about specific biochemical reactions which form a part of the cellular metabolism. Enzymes are exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries. Today, more than 3000 different enzymes have been identified of which several of them find application in biotechnology and industries. Even small improvements in biotechnological enzyme production processes are considered significant for commercial production and process. Microbial proteases are extracellular enzymes that catalyse proteolysis by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. Proteases are the most valuable commercial enzymes and account for 60% of the total enzyme market
The majority of the enzymes used in industrial fermentation are inducible and are synthesized in response of inducers: e.g. starch for amylases, maltose for pollulanase, pectin for pectinase,olive oil and tween are also used at times.

Most inducers which are included in microbial enzyme media are substrates or substrate analogues, but intermediates and products may sometimes be used as inducers. For example, maltodextrins will induce amylase and fatty acids induce lipase. However, the cost may prohibit their use as inducers in a commercial process.
One unusual application of an inducer is the use of yeast mannan in streptomycin production. During the fermentation varying amounts of streptomycin and mannosidostreptomycin are produced. Since mannosidostreptomycin has only 20% of the biological activity of streptomycin, the former is an undesirable product.

It has been reported that phenylacetic acid and its salts induces the production of penicillin G acylase in *E.coli* and other bacteria. A very little data has been published on the type and mode of addition of inducer for the biosynthesis of penicillin V acylase.

Most inducers which are included in microbial enzyme media are substrates or substrate analogues, but intermediates and products may sometimes be used as inducers. For example, maltodextrins will induce amylase and fatty acids induce lipase.

It is now possible to produce a number of heterologous proteins in yeasts, fungi and bacteria. These include proteins of viral, human, animal, plant and microbial origin. However, heterologous proteins may show some degree of toxicity to the host and have a major influence

on the stability of heterologous protein expression. As well as restricting cell growth as biomass the toxicity will provide selective conditions for segregant cells which no longer synthesize the protein at such a high level.

The majority of the enzymes used in industrial fermentation are inducible and are synthesized in response of inducers: e.g. starch for amylases, maltose for pollulanase, pectin for pectinase,olive oil and tween are also used at times.

Therefore, optimum growth conditions may be achieved by not synthesizing a heterologous protein continuously and only inducing it after the host culture has grown up in a vessel to produce sufficient biomass. In cells of *Saccharomyces cerevisiae* where the *Gal*1 promoter is part of the gene expression system, product formation may be induced by galactose addition to the growth medium which contains glycerol or low non-repressing levels of glucose as a carbon source. During growth on methanol, which also acts as an inducer, the promoter is induced to produce about 30% of the cell protein.

<u>Oxygen Requirements, Trace Elements, pH and Osmolality</u>

The main purpose of a stirring system is the oxygen supply to keep the cell respiratory activity. Thus, the point is to transfer the oxygen from the gas phase to the liquid phase and let the dissolved oxygen reach the suspended cells, penetrating into the cells and, finally, being consumed in the reaction. The mechanism of oxygen transfer from the gas phase to the liquid phase is controlled by the liquid phase mass transfer resistance. The rate of mass transfer or the rate of oxygen absorption can be stated mathematically. Therefore, for studying both the oxygen transfer and the uptake kinetics of the cells, it is essential to know about the values of saturation oxygen concentration in the liquid phase and concentration of oxygen in the liquid phase

It is sometimes forgotten that oxygen, although not added to an initial medium as such, is nevertheless a very important component of the medium in many processes, and its availability can be extremely important in controlling growth rate and metabolite production.

The culture may become *oxygen* limited because sufficient oxygen cannot be made available in the fermenter if certain substrates, such as rapidly metabolized sugars which lead to a high oxygen demand, are available in high concentrations. The individual components of the medium can influence the viscosity of the final medium and its subsequent behaviour with respect to aeration and agitation. Many of the antifoams in use will as surface active agents and reduce the transfer rate.

The equilibrium oxygen concentration (C_0) was determined in a complex fermentation media containing sucrose, lysine, molasses, corn steep liquor, antifoam agents and biomass. In simple systems, with all the components being dissolved, C_0 represents the oxygen solubility and linearly decreases with increasing solute concentrations. In complex solutions with multi-phase structure an increase in C_0 can be detected. It suggests that C_0 consists of two components — one being oxygen solubility, the other being determined by the amount of oxygen adsorbed on the interphase and bound by macromolecules.

The role of trace elements in medium formulation can be significant. Cultured cells normally require Fe, Zn, Cu, Se, Mn, Mo and V. These are often present as impurities in other media components. Citric acid yields of 98.7% (sugar consumption basis) were reached in shaker flasks with mutant Aspergillus niger in a resin-treated sucrose medium, yields of 75% were obtained in medium with resin-treated clarified syrup and 68% with ferrocyanide-treated blackstrap molasses. Optimal conditions included selection of appropriate pellets as inoculum at 3%, pH of 4.5, temperature at 30 C, agitation at 250 rev/min, and fermentation time of 8 days. The mutant tolerated high concentrations of trace elements.

Experimental data revealed pyruvate overflow inducing conditions, which typically occur in oxygen limited zones of large-scale fermentations as a major reason leading to norvaline and norleucine synthesis during *E. coli* cultivation. Previous approaches to suppress misincorporation of norleucine and norvaline considered growth media supplementation with the relevant canonical isostructural compounds, but no research was performed on the impact of the overflow metabolism related trace elements molybdenum, nickel and selenium. These elements form essential parts of the formate hydrogen lyase (FHL) metalloprotein complex, which is a key enzyme of anaerobic pyruvate metabolism in *E. coli* and could therefore represent a crucial connection to the pyruvate accumulation associated biosynthesis of rare amino acids.

The normal buffer system in tissue culture media is the CO_2-bicarbonate system. This is a weak buffering system and can be improved by the use of a zwitterinic buffer such as HEPES, either in addition to or instead of the CO_2-bicarbonate buffer. Continuous pH control is achieved by the addition of sodium bicarbonate or sodium hydroxide (with fast mixing) when too acid. The pH does not normally become too Alkaline so acid additions are not required but provision may be made for CO_2 additions. In order to prevent poor fermentation yields and to improve the quality and reliability of the products, it is important to maintain proper control starter production. This control may be achieved by studying the effects of process parameters on the growth kinetics of the bacteria and on their acidification activity and physiological state in growing conditions. Among all process parameters, pH and harvesting time are key factors that strongly influence the physiological state of lactic acid bacteria after fermentation and stabilization.

The optimum range of osmotic pressure for growth is often quite narrow and varies with the type of cell and the species from which it was isolated. It may be necessary to adjust the concentration of NaCI when major additions are made to a medium.

Escherichia coli is able to grow at increased NaCl concentrations that provides an increase in medium osmolarity and cellular Na+ content. The addition of 0.5 M NaCl to the growth medium led to a substantial decrease in growth rate during anaerobic fermentation on glucose at pH of 7.3 or 9.0. This inhibitory effect of 0.5 M NaCl was at least threefold stronger than that seen under aerobic conditions, and stronger than equivalent concentrations of sucrose, KCl, or potassium glutamate under anaerobic conditions. Further, proline was found to stimulate the growth rate at high NaCl concentration under anaerobic and to a lesser extent, under aerobic conditions.

Growth of *Zymomonas mobilis* strain 113 S and its ethanol and levan production under the conditions of increasing sucrose medium osmolality caused by NaCl, KCl, sorbitol or maltose.

The increase in medium osmolality (700–1,500 mosml/kg) was accompanied by the inhibition of growth (growth rate, biomass yield) and ethanol production (specific productivity and yield) In contrast, levan synthesis was less affected or even stimulated and, as a consequence, levan specific productivity was increased significantly. A decrease in the anabolic growth parameters correlated with a parallel inhibition of glucose-6-P dehydrogenase and alcohol dehydrogenase (isoenzyme ADH II) activities.

Antifoams

Antifoams are necessary to reduce foam formation during fermentation. Foaming is largely due to media proteins that become attached to the air-broth interface where they denature to form a stable foam "skin" that is not easily disrupted. If uncontrolled the foam may block air filters, resulting in the loss of aseptic conditions; the fermenter becomes contaminated and microorganisms are released into the environment. Of possibly the most importance is the need to allow "freeboard" in fermenters to provide space for the foam generated. If foaming is minimized, then throughputs can be increased. There are three possible approaches to controlling foam production: the use of a defined medium and a modification of some of the physical parameters, e.g. pH, temperature, aeration and agitation (if the foam is due to media components), use of chemical foam breakers and addition of chemical antifoams. Antifoams are surface active agents that reduce the surface tension in the foams and destabilize the protein films by (i) hydrophobic bridges between two surfaces; (ii) displacement of the adsorbed protein; and (iii) rapid spreading on the surface of the film.

The ideal antifoam should have the following properties:

1 readily and rapidly dispersed with rapid action
2 high activity at low concentrations
3 prolonged action
4 non-toxic to fermentation microorganisms, humans or animals
5 low cost
6 thermostability
7 compatibility with other media components and the process, i.e., having no effect on oxygen transfer rates or downstream processing operations
8 be heat sterilizable

- The most common cause of foaming is due to proteins in the medium, such as corn-steep liquor, meal, soybean meal, yeast extract or meat extract.
- These proteins may denature at the air-broth interface and form a skin which does not rupture readily.

The foaming can cause removal of cells from the medium which will lead to autolysis and the further release of microbial cell proteins will probably increase the stability of the foam.

If uncontrolled, then numerous changes may occur and physical and biological problems may be created. These include reduction in the working volume of the fermenter due to oxygen

exhausted gas bubbles circulating in the system changes in bubble size, lower mass and heat transfer rates, invalid process data due to interference at sensing electrodes and incorrect monitoring and control.

Natural antifoams include plant oils (e.g., from soya, sunflower and rapeseed), deodorized fish oil, mineral oils and tallow. The synthetic antifoams are mostly silicon oils, poly alcohols and alkylated glycols. Since antifoams are of low solubility, they need a carrier, e.g., lard oil, liquid paraffin or castor oil, which may be metabolised and therefore affect the fermentation process. Many of the surface-active agents, particularly the oils, are added as emulsions of suspended oil droplets which can destabilise the foams by acting as hydrophobic bridges between the two film surfaces or by displacing the stabilising adsorbed material, e.g. protein, at the bubble–liquid interface. However, those conditions, which cause collapse of the foam structure, can also favour the coalescence of bubbles in the body of the liquid. This results in an increase in the mean bubble diameter and a reduction in gas hold-up. Both of these effects will tend to reduce the specific interfacial area available for mass transfer. The concentrations of many antifoams which are necessary to control foaming may reduce the oxygen transfer rate by as much as 50%. Thus, antifoam addition should be kept to an absolute minimum. Some antifoams may reduce the oxygen transfer rate as well as adversely affect downstream processing steps, especially membrane filtration. If the oxygen transfer rate is too severely affected mechanical foam breakers may have to be considered.

Antifoams are surface active agents, reducing the surface tension in the foams and destabilizing protein films by:

(a) hydrophobic bridges between two surfaces,
(b) displacement of the absorbed protein, and
(c) rapid spreading on the surface of the film.

Unfortunately, the concentrations of many antifoams which are necessary to control fermentations will reduce the oxygen-transfer rate by as much as 50%; therefore antifoam additions must be kept to an absolute minimum. There are also other antifoams which will increase the oxygen-transfer rate. If the oxygen-transfer rate is severely affected by antifoam addition then mechanical foam breakers may have to be considered as a possible alternative. Various studies concluded that foam control in industry is still an empirical art. The best method for a particular process in one factory is not necessarily the best for the same process on another site. The design and operating parameters of a fermenter may affect the properties and quantity of foam formed.

Many of the surface-active agents, particularly the oils, are added as emulsions of suspended oil droplets which can destabilize the foams by acting as hydrophobic bridges between the two film surfaces or by displacing the stabilizing adsorbed material, e.g. protein, at the bubble–liquid interface. However, those conditions, which cause collapse of the foam structure, can also favor the coalescence of bubbles in the body of the liquid. This results in an increase in the mean bubble diameter and a reduction in gas hold-up.

Antifoams are necessary to reduce foam formation during fermentation. Foaming is largely due to media proteins that become attached to the air-broth interface where they denature to form a stable foam “skin” that is not easily disrupted. If uncontrolled the foam may block air filters, resulting in the loss of aseptic conditions; the fermenter becomes contaminated and microorganisms are released into the environment. Of possibly the most importance is the need to allow “freeboard” in fermenters to provide space for the foam generated. If foaming is minimized, then throughputs can be increased.

Medium optimization

When considering the biomass growth phase in isolation it must be recognized that efficiently grown biomass produced by an 'optimized' high productivity growth phase is not necessarily best suited for its ultimate purpose, such as synthesizing the desired product.

In order to get high yield and best physiological state of desire product a number of conditions are investigated to set the growth conditions. There may be a sequence of phases each with a specific set of optimal conditions. Classical method of media optimization is to change one independent variable (nutrient, antifoam, pH, temperature, etc.) while fixing all the others at a certain level can be extremely time consuming and expensive for a large number of variables.
To make a full factorial search which would examine each possible combination of independent variable at appropriate levels could require a large number of x^n experiments, where as

x = number of levels
n = number of variables

This may be quite appropriate for three nutrients at two concentrations (2^3 =8 trials) but not for six nutrients at three concentrations (3^6 =729). Industrially the aim is to perform the minimum number of experiments to determine optimal conditions. There are alternative strategies which allow more than one variable to change at a time.

Medium optimization is an integral part of biopharmaceutical process development. This commonly involves the addition of various supplements to an existing basal medium formulation. There is an on-going debate within the industry as to the various advantages and disadvantages of both defined and undefined media and media components. The choice of which type of system to employ is often motivated by risk mitigation with respect to consistency of performance, as weighed against the underlying goal of achieving the highest possible product titers for any given system. Process development scientists must make these assessments within the context of their own targets and timelines, as well as the tools available to achieve these goals.

As is evidenced by the data generated and presented by Kerry, defined and undefined supplementation solutions may not be mutually exclusive. Not all supplements perform equally in different biopharmaceutical production systems. The interaction among components of the basal medium and those of the supplement (batch or fed-batch) can have significant impacts on overall system performance. While both culture media and supplement systems can share a

number of common components, the unique composition of select supplement systems may elicit distinct beneficial responses in cultured cells.
Different combinations and sequences of process conditions need to be investigated to determine the growth conditions which produce the biomass with the physiological state best constituted for product formation. There may be a sequence of phases each with a specific set of optimal conditions.

MEDIUM OPTIMIZATION-Plackett-Burman Design

In 1946, R.L. Plackett and J.P. Burman published their now famous paper "The Design of Optimal Multifactorial Experiments" in Biometrika (vol. 33). This paper described the construction of very economical designs with the run number a multiple of four (rather than a power of 2). Plackett-Burman designs are very efficient screening designs when only main effects are of interest.

Plackett-Burman (PB) designs are used for screening experiments because, in a PB design, main effects are, in general, heavily confounded with two-factor interactions. The PB design in 12 runs, for example, may be used for an experiment containing up to 11 factors. Any factors not assigned to a variable can be designated as a dummy variable.

Alternatively, factors known to not have any effect may be included and designated as dummy variables. Table in the next slide shows a Plackett-Burman design for seven variables (A -G) at high and low levels in which two factors, E and G, are designated as 'dummy' variables.
Consider the variable A; for the trials in which A is high, B is high in two of the trials and low in the other two. Similarly, C will be high in two trials and low in two, as will all the remaining variables.

For those trials in which A is low, B will be high two times and low two times. This will also apply to all the other variables. Thus, the effects of changing the other variables cancel out when determining the effect of A. The same logic then applies to each variable. However, no changes are made to the high and low values for the E and G columns. The effects of the dummy variables are calculated in the same way as the effects of the experimental variables. If there are no interactions and no errors in measuring the response, the effect shown by a dummy variable should be 0. If the effect is not equal to 0, it is assumed to be a measure of the lack of experimental precision plus any analytical error in measuring the response. This procedure will identify the important variables and allow them to be ranked in order of importance to decide which to investigate in a more detailed study to determine the optimum values to use.

1. Determine the difference between the average of the H (high) and L (low) responses for each independent and dummy variable. Therefore the difference = $\Sigma A\,(H) - \Sigma A\,(L)$

The effect of an independent variable on the response is the difference between the average response for the four experiments at the high level and the average value for four experiments at the low level. Thus the effect of

$$A = \frac{\Sigma A\,(H)}{4} - \frac{\Sigma A\,(L)}{4}$$
$$= \frac{2(\Sigma A\,(H) - \Sigma A\,(L))}{8}$$

This value should be near zero for the dummy variables.

2. Estimate the mean square of each variable (the variance of effect). For A the mean square will be = $\frac{(\Sigma A\,(H) - \Sigma A\,(L))^2}{8}$
3. The experimental error can be calculated by averaging the mean squares of the dummy effects of *E* and *G*.
4. The final stage is to identify the factors which are showing large effects. In the example this was done using an *F*-test for

$$\frac{\text{Factor mean square.}}{\text{error mean square.}}$$

Media for Industrial Fermentations:

The microbes used for fermentation grow in specially designed growth medium which supplies the nutrients required by the organisms. A variety of media exist, but invariably contain a carbon source, a nitrogen source, water, salts, and micronutrients. In the production of wine, the medium is grape must. In the production of bio-ethanol, the medium may consist mostly of whatever inexpensive carbon source is available.

Carbon sources are typically sugars or other carbohydrates, although in the case of substrate transformations (such as the production of vinegar) the carbon source may be an alcohol or something else altogether. For large scale fermentations, such as those used for the production of ethanol, inexpensive sources of carbohydrates, such as molasses, corn steep liquor,sugar cane juice, or sugar beet juice are used to minimize costs. More sensitive fermentations may instead use purified glucose, sucrose, glycerol or other sugars, which reduces variation and helps ensure the purity of the final product. Organisms meant to produce enzymes such as beta galactosidase, invertase or other amylases may be fed starch to select for organisms that express the enzymes in large quantity.

Fixed nitrogen sources are required for most organisms to synthesize proteins, nucleic acids and other cellular components. Depending on the enzyme capabilities of the organism, nitrogen may be provided as bulk protein, such as soy meal; as pre-digested polypeptides, such as peptone or tryptone; or as ammonia or nitrate salts. Cost is also an important factor in the choice of a nitrogen source. Phosphorus is needed for production of phospholipids in cellular membranes and for the production of nucleic acids. The amount of phosphate which must be added depends upon the composition of the broth and the needs of the organism, as well as the objective of the fermentation. For instance, some cultures will not produce secondary metabolites in the presence of phosphate.

Growth factors and trace nutrients are included in the fermentation broth for organisms incapable of producing all of the vitamins they require. Yeast extract is a common source of micronutrients and vitamins for fermentation media. Inorganic nutrients, including trace elements such as iron, zinc, copper, manganese, molybdenum and cobalt are typically present in

unrefined carbon and nitrogen sources, but may have to be added when purified carbon and nitrogen sources are used. Fermentations which produce large amounts of gas (or which require the addition of gas) will tend to form a layer of foam, since fermentation broth typically contains a variety of foam-reinforcing proteins, peptides or starches. To prevent this foam from occurring or accumulating, antifoaming agents may be added. Mineral buffering salts, such as carbonates and phosphates, may be used to stabilize pH near optimum. When metal ions are present in high concentrations, use of a chelating agent may be necessary.

EXAMPLES OF COMMONLY USED NITROGEN SOURCES:

Most industrially used micro-organisms can utilize inorganic or organic sources of nitrogen. Inorganic nitrogen may be supplied as ammonia gas, ammonium salts or nitrates. Ammonia has been used for pH control and as the major nitrogen source in a defined medium for the commercial production of human serum albumin by *Saccharomyces cerevisiae*. Ammonium salts such as ammonium sulphate will usually produce acid conditions as the ammonium ion is utilized and the free acid will be liberated.

On the other hand nitrates will normally cause an alkaline drift as they are metabolized. Ammonium nitrate will first cause an acid drift as the ammonium ion is utilized, and nitrate assimilation is repressed. When the ammonium ion has been exhausted, there is an alkaline drift as the nitrate is used as an alternative nitrogen source.

One exception to this pattern is the metabolism of *Gibberella fujikuroi* (Borrow *et al*., 1961, 1964). In the presence of nitrate the assimilation of ammonia is inhibited at pH 2.8-3.0. Nitrate assimilation continues until the pH has increased enough to allow the ammonia assimilation mechanism to restart.

Organic nitrogen may be supplied as amino acid, protein or urea. In many instances growth will be faster with a supply of organic nitrogen, and a few microorganisms have an absolute requirement for amino acids. Amino acids are more commonly added as complex organic nitrogen sources which are non-homogeneous, cheaper and readily available. In lysine production, methionine and threonine are obtained from soybean hydrolysate since it would be too expensive to use the pure amino acids.

Table: Best nitrogen sources for some secondary metabolites

Product	Main nitrogen source(s)	Reference
Penicillin	Corn-steep liquor	Moyer and Coghill (1946)
Bacitracin	Peanut granules	Inskeep *et al*. (1951)
Riboflavin	Pancreatic digest of gelatine	Malzahn *et al*. (1959)
Novobiocin	Distillers' solubles	Hoeksema and Smith (1961)
Rifomycin	Pharmamedia Soybean meal, $(NH_4)_2SO_4$	Sensi and Thiemann (1967)
Gibberellins	Ammonium salt and natural plant nitrogen source	Jefferys (1970)
Butirosin	Dried beef blood or haemoglobin with $(NH_4)_2SO_4$	Claridge *et al*. (1974)
Polyenes	Soybean meal	Martin and MacDaniel (1977)

Other proteinaceous nitrogen compounds serving as sources of amino acids include corn-steep liquor, soya meal, peanut meal, cotton-seed meal.

MINERALS-1:

All micro-organisms require certain mineral elements for growth and metabolism. In many media, magnesium, phosphorus, potassium, sulphur, calcium and chlorine are essential components, and because of the concentrations required, they must be added as distinct components. Others such as cobalt, copper, iron, manganese, molybdenum and zinc are also essential but are usually present as impurities in other major ingredients.

When synthetic media are used the minor elements will have to be added deliberately. The form in which the minerals are usually supplied and the concentration ranges are given in Table.

TABLE 4.10. *The range of typical concentrations of mineral components* (g dm^{-3})

Component	Range
*KH_2PO_4	1.0–4.0 (part may be as buffer)
$MgSO_4 \cdot 7H_2O$	0.25–3.0
KCl	0.5–12.0
$CaCO_3$	5.0–17.0
$FeSO_4 \cdot 4H_2O$	0.01–0.1
$ZnSO_4 \cdot 8H_2O$	0.1–1.0
$MnSO_4 \cdot H_2O$	0.01–0.1
$CuSO_4 \cdot 5H_2O$	0.003–0.01
$Na_2MoO_4 \cdot 2H_2O$	0.01–0.1

*Complex media derived from plant and animal materials normally contain a considerable concentration of inorganic phosphate.

The concentration of phosphate in a medium, particularly laboratory media in shake flasks, is often much higher than that of other mineral components. Part of this phosphate is being used as a buffer to minimize pH changes when external control of the pH is not being used.

In specific processes the concentration of certain minerals may be very critical. Some secondary metabolic processes have a lower tolerance range to inorganic phosphate than vegetative growth. This phosphate should be sufficiently low as to be assimilated by the end of trophophase.

The inorganic phosphate concentration also influences production of bacitracins, citric acid (surface culture), ergot, monomycin, novobiocin, oxytetracycline, polyenes, ristomycin, rifamycin Y, streptomycin, vancomycin and viomycin.

MINERALS-2:

Weinberg (1970) has reviewed the nine trace elements of biological interest (Atomic numbers 23-30, 42). Of these nine, the concentrations of manganese, iron and zinc are the most critical in secondary metabolism.

In every secondary metabolic system in which sufficient data has been reported, the yield of the product varies linearly with the logarithmic concentration of the 'key' metal. The linear relationship does not apply at concentrations of the metal which are either insufficient, or toxic, to cell growth. Some of the primary and secondary microbial products whose yields are affected by concentrations of trace metals greater than those required for maximum growth are given in Table.

TABLE *Trace elements influencing primary and secondary metabolism*

Product	Trace element(s)
Bacitracin	Mn
Protease	Mn
Gentamicin	Co
Riboflavin	Fe, Co
	Fe
Mitomycin	Fe
Monensin	Fe
Actinomycin	Fe, Zn
Candicidin	Fe, Zn
Chloramphenicol	Fe, Zn
Neomycin	Fe, Zn
Patulin	Fe, Zn
Streptomycin	Fe, Zn
Citric acid	Fe, Zn, Cu
Penicillin	Fe, Zn, Cu
Griseofulvin	Zn

Chlorine does not appear to play a nutritional role in the metabolism of fungi. It is, however, required by some of the halophilic bacteria. Obviously, in those fermentations where a chlorine-containing metabolite is to be produced the synthesis will have to be directed to ensure that the non-chloro-derivative is not formed. The most important compounds are chlorotetracycline and griseofulvin.

In griseofulvin production, adequate available chloride is provided by the inclusion of at least 0.1% KCI as well as the chloride provided by the complex organic materials included as nitrogen

sources. Some of the other chlorine containing metabolites are caldriomycin, nornidulin and mollisin.

CHELATORS:

Many media cannot be prepared or autoclaved without the formation of a visible precipitate of insoluble metal phosphates. Gaunt *et al.* (1984) demonstrated that when the medium of Mandels and Weber (1969) was autoclaved, a white precipitate of metal ions formed, containing all the iron and most of the calcium, manganese and zinc present in the medium. The problem of insoluble metal phosphate(s) may be eliminated by incorporating low concentrations of chelating agents such as ethylene diamine tetraacetic acid (EDTA), citric acid, polyphosphates, etc., into the medium.

These chelating agents preferentially form complexes with the metal ions in a medium. The metal ions then may be gradually utilized by the microorganism. Gaunt *et al.* were able to show that the precipitate was eliminated from Mandel and Weber's medium by the addition EDTA at 25 mg dm^{-3}. It is important to check that a chelating agent does not cause inhibition of growth the micro-organism which is being cultured. In many media, particularly those commonly used in large scale processes, there may not be a need to add a chelating agent as complex ingredients such as yeast extracts or proteases peptones will complex with metal ions and ensure gradual release of them during growth.

GROWTH FACTORS:

Some micro-organisms cannot synthesize a full complement of cell components and therefore require preformed compounds called growth factors. The growth factors most commonly required are vitamins, but there may also be a need for specific amino acids, fatty acids or sterols. Many of the natural carbon and nitrogen sources used in media formulations contain all or some of the required growth factors. When there is a vitamin deficiency it can often be eliminated by careful blending of materials. It is important to remember that if only one vitamin is required it may be occasionally more economical to add the pure vitamin, instead of using a larger bulk of a cheaper multiple vitamin source.

Calcium pantothenate has been used in one medium formulation for vinegar production. In processes used for the production of glutamic acid, limited concentrations of biotin must be present in the medium. Some production strains may also require thiamine.

BUFFERS:

The control of pH may be extremely important if optimal productivity is to be achieved. A compound may be added to the medium to serve specifically as a buffer, or may also be used as a nutrient source. Many media are buffered at about pH 7.0 by the incorporation of calcium carbonate (as chalk). If the pH decreases the carbonate is decomposed. Obviously, phosphates which are part of many media also play an important role in buffering. However, high phosphate concentrations are critical in the production of many secondary metabolites.

The balanced use of the carbon and nitrogen sources will also form a basis for pH control as buffering capacity can be provided by the proteins, peptides and amino acids, such as in corn-

steep liquor. The pH may also be controlled externally by addition of ammonia or sodium hydroxide and sulphuric acid.

PRECURSORS:

Some components of a fermentation medium help to regulate the production of the product rather than support the growth of the micro-organism. Such additives include precursors, inhibitors and inducers, all of which may be used to manipulate the progress of the fermentation.

Some chemicals, when added to certain fermentations, are directly incorporated into the desired product. Probably the earliest example is that of improving penicillin yields. A range of different side chains can be incorporated into the penicillin molecule. The significance of the different side chains was first appreciated when it was noted that the addition of corn-steep liquor increased the yield of penicillin from 20 units cm^{-3} to 100 units cm^{-3}. Corn-steep liquor was found to contain phenylethylamine which was preferentially incorporated into the penicillin molecule to yield benzyl penicillin (Penicillin G).

Some studies showed that addition of phenylacetic acid and its derivatives to the medium were capable of both increasing penicillin production threefold and to directing biosynthesis towards increasing the proportion of benzyl penicillin from 0 to 93% at the expense of other penicillins. Phenylacetic acid is still the most widely used precursor in penicillin production. Some important examples of precursors are given in Table.

Table: Precursors used in fermentation processes

Precursor	Product	Microorganism
Phenylacetic-acid related compounds	Penicillin G	*Penicillium chrysogenum*
Phenoxy acetic acid	Penicilin V	*Penicillium chrysogenum*
Chloride	Chlortetracycline	Streptomyces aureofaciens
Propionate	Riboflavin	*Lactobacillus bulgaricus*
Cyanides	Vitamin B12	Proprianobacterium, *Streptomyces* spp.
β-Iononones	Carotenoids	*Phycomyces blakesleeanus*
α-Amino butyric acid	L-Isoleucine	*Bacillus subtilis*
D-Threonine	L-Isoleucine	*Serratia marcescens*
Anthranilic acid	L-Tryptophan	*Hansenula anomala*
Nucleosides and bases	Nikkomycins	*Streptomyces tendae*
Dihydronovobionic acid	Dihydronovobiocin	*Streptomyces sp.*

p-Hydroxycinnamate	Organomycin A and B	*Streptomyces organonensis*
DL-α-Amino butyric acid	Cyclosporin A	*Tolypocladium inflatum*
L-Threonine	Cyclosporin C	
Tyrosine or *p*-hydroxy-phenylglycine	Dimethylvancomycin	*Nocardia orientalis*

Having established that the activity of penicillin lay in the side chain, and that the limiting factor was the synthesis of the side chain, it became standard practice to add side-chain precursors to the medium, in particular phenyl acetic acid.

INHIBITORS:

When certain inhibitors are added to fermentations, more of a specific product may be produced, or a metabolic intermediate which is normally metabolized is accumulated. One of the earliest examples is the microbial production of glycerol. Glycerol production depends on modifying the ethanol fermentation by removing acetaldehyde. The addition of sodium bisulphite to the broth leads to the formation of the acetaldehyde bisulphite addition compound (sodium hydroxy ethyl sulphite). Since acetaldehyde is no longer available for re-oxidation of $NADH_2$, its place as hydrogen acceptor is taken by dihydroacetone phosphate, produced during glycolysis. The product of this reaction is glycerol-3-phosphate, which is converted to glycerol.

The application of general and specific inhibitors are illustrated in Table. In most cases the inhibitor is effective in increasing the yield of the desired product and reducing the yield of undesirable related products. A number of studies have been made with potential chlorination inhibitors, e.g. bromide, to minimize chlortetracycline production during a tetracycline fermentation.

Table: Specific and General Inhibitors used in fermentations

Product	**Inhibitor**	**Main effect**	**Micro-organism**
Glycerol	Sodium bisulphate	Acetaldehyde pro-duction repressed	*Saccharomyces cerivisiae*
Tetracycline	Bromide	Chlortetracycline formation repressed	*Streptomyces aureofaciens*
Glutamic acid	Penicillin	Dell wall permeability	*Micrococcus glutamicus*
Citric acid	Alkali metal/ phosphate, pH below 2.0	Oxalic acid repressed	*Aspergillus niger*
Valine	Various inhibitors	Various effects with different inhibitors	*Brevibacterium roseum*
Rifamycin B	Di-ethyl barbiturate	Other rifamycins inhibited	*Nocardia mediterranel*

Inhibitors have also been used to affect cell-wall structure and increase the permeability for release of metabolites. The best example is the use of penicllin and surfactants in glutamic acid production.

INDUCERS-1:

The majority of enzymes which are of industrial interest are inducible. Induced enzymes are synthesized only in response to the presence in the environment of an inducer. Inducers are often substrates such as starch or dextrins for amylases, maltose for pullulanase and pectin for pectinases.

Some inducers are very potent, such as isovaleronitrile inducing nitralase. Substrate analogues that are not attacked by the enzyme may also serve as enzyme inducers. Examples of industrially important enzyme inducers are shown in Table.

Table: Some examples of industrially important enzyme inducers

Enzyme	Inducer	Micro-organism
α-Amylase	Starch Maltose	*Aspergillus spp.* *Bacillus subtilis*
Pullulanase	Maltose	*Aerobacter aerogenes*
α-Mannosidase	Yeast mannans	*Streptomyces griseus*
Penicillin acylase	Phenylacetic acid	*Escherichia coli*
Proteases	Various proteins	*Bacillus spp.* *Streptococcus spp.* *Streptomyces spp.* *Aspergillus spp.*
Cellulase	Cellulose	*Trichoderma viride*
Pectinases	Pectin (beet pulp, apple pomace, citrus peel)	*Aspergillus spp.*

One unusual application of an inducer is the use of yeast mannan in streptomycin production. During the fermentation varying amounts of streptomycin and mannosidostreptomycin are produced. Since mannosidostreptomycin has only 20% of the biological activity of streptomycin, the former is an undesirable product. The production organism *Streptomyces griseus* can be induced by yeast mannan to produce *β*-mannosidase which will convert mannosidostreptomycin to streptomycin.

Most inducers which are included in microbial enzyme media are substrates or substrate analogues, but intermediates and products may sometimes be used as inducers. For example,

maltodextrins will induce amylase and fatty acids induce lipase. However, the cost may prohibit their use as inducers in a commercial process.

INDUCERS-2:

It is now possible to produce a number of heterologous proteins in yeasts, fungi and bacteria. These include proteins of viral, human, animal, plant and microbial origin. However, heterologous proteins may show some degree of toxicity to the host and have a major influence on the stability of heterologous protein expression. As well as restricting cell growth as biomass the toxicity will provide selective conditions for segregant cells which no longer synthesize the protein at such a high level.

Therefore, optimum growth conditions may be achieved by not synthesizing a heterologous protein continuously and only inducing it after the host culture has grown up in a vessel to produce sufficient biomass. In cells of *Saccharomyces cerevisiae* where the *Gal*1 promoter is part of the gene expression system, product formation may be induced by galactose addition to the growth medium which contains glycerol or low non-repressing levels of glucose as a carbon source. Methylotrophic yeasts such as *Hansenula polymorpha* and *Pichia pastoris* may be used as alternative systems because of the presence of an alcohol oxidase promoter. During growth on methanol, which also acts as an inducer, the promoter is induced to produce about 30% of the cell protein. In the presence of glucose or ethanol it is undetectable.