CHAPTER 3

ANTIBIOTICS

1. HISTORICAL DEVELOPMENT OF ANTIBIOTICS

Paul Vuilemin (1889) was the first and foremost scientist who vehemently promulgated the very concep' of **'antibiotic'** activity to introduce the terminology *'influences antibiotiques'* (or *antibiotic influences*) in order to describe the prevailing negative interactions amongst the animals and plants.* Later on, Walksman (1940s) eventually coined the term **'antibiotic'** and also introduced a plausible definition as — *'a chemical substance derived from microorganisms which has the capacity of inhibiting growth, and even destroying, other microorganisms in dilute solutions'*.**

Another school of thought advocates that the **natural product antibiotics** essentially comprise of a specific category of chemical entities invariably termed as the **secondary metabolites.** Besides, on a rather broader perspective such substances may be characterized for possessing chemical structures which are found to be quite unusual when compared with those of the **intermediary metabolites.** Nevertheless, such *natural product antibiotics,* are being generated at an extremely low ebb specific growth rates, and also supported by the fact that these are not absolutely essential the growth of the **'producing organisms'** in a *pure culture medium.* In fact, the **'antibiotics'** are observed to be of highly critical nature with respect to the **producing organisms** in their usual natural environment because their presence is an absolute must not only for the *survival* but also for the *competitive advantage.****

However, the most widely accepted definition of an **'antibiotic'** promulgated by the scientific jargons is — *'a chemical substance produced by a microorgansims, that has the capacity, in low concentration, to inhibit or kill, selectively, other microorganisms'*.

Importantly, the aforesaid definition***** lays particular emphasis on the terminologies like **'selectively'** or **'selective toxicity'** that explicitely suggests that the *substance* either checks the growth of

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* Levy SB : **The Antibiotic Paradox : How Miracle Drugs Are Destroying the Miracle,** Plenium Press, New York, 1992.

** Vandamine EJ, **Antibiotic Search and Production : An Overview,** Vandamine EJ (ed.) **Biotechnology of Antibiotics,** Marcel Dekker, New York, pp. 3–31, 1984.

*** Demain AI : **Functions of Secondary Metabolites :** Hershberger CL *et al.* (eds.) **Genetic and Molecular Biology of Industrial Microorganisms,** American Society for Microbiology, Washington DC, pp. 1–11, 1989.

**** Kar, A : **Pharmacognosy and Pharmacobiotechnology,** New Age International (P) LTD., Publishers, New Delhi, pp. 654–800, 2003.

pathogens or exerts a bactericidal action on the microbes without displaying a similar action on the host organisms i.e., the humans.

Interestingly, one may evidently observe from the above cited definition(s) that it includes the plethora of newer medicinal compounds essentially having the pure synthetic [illegible]. In reality and actual practice, these 'synthetic substances' are virtually treated at par with the [illegible] natural compounds together with their respective derivatives under the terminology '**antimicrobials**' that could be further sub-divided predominantly into two categories namely: **antifungals** and **antibacterials** depending on the specific type(s) of **microbe** undergoing inhibition. Therefore, to circumvent the practical aspects, both the terminologies, viz., '**antibiotic**' and '**antimicrobial**' may be used effectively and interchangeably irrespective of the specific source of the chemical [illegible].

In general, the '**antibiotics**' are produced on a large scale by three well-known and [illegible] methodologies, such as: (a) **fermentation process**; (b) **semi-synthetic process**; and (c) **synthetic process**. A tremendous quantum leap and qualified successful diversification in the specific field of '**biotechnology**' has helped the first two processes (i.e., 'a' and 'b') in accomplishing an enormous enhancement in the rate of production as well as improved upon their yield and purity.

Antibiotic Development: The latest progressive trend in the logistic aspects of **antibiotic development** may be observed vividly by the under mentioned sequence of goals and objectives, such as:

* To screen and evaluate different types of sources of **microorganisms** for the **detection of purposeful** antagonisms.
* To identify and select **modified** versions of microbial mutants, establish optimal environmental and nutritional conditions, and to develop suitable technique(s) for the **recovery of antibiotics from cultures**.
* To induce the production of particular desired metabolites.
* To improve upon and modify the fermentative metabolites either by the aid of biological or chemical manipulations to **accomplish more useful antibiotic substances**.
* To develop an elaborated methods for the '**total synthesis**' of antibiotics from ab initio [illegible] feasible economic advantage, and
* To make use of an 'enhance agent' to distinctly **enhance the impact or availability of an antibiotic**.

2 ANTIMICROBIAL SPECTRUM AND METHODS USED FOR THEIR STANDARDIZATION

Microbiology, in particular clinical medical microbiology, is a scientific discipline chiefly concerned with the **isolation** and subsequent **identification** of causative disease-producing microorganisms (or **pathogens**): **bacteria**, **fungi** (including **yeasts**), **viruses**, **rickettsia**, and **parasites**.

In general, there are well-defined specific as well as non-specific techniques available with regard to the isolation and identification of the '**suspect organisms**' as stated under:

* Propagation on an appropriate primary culture media,
* Selective isolation on special (specific) culture media,
* Application of appropriate living host material e.g., mouse, embryonated egg, tissue culture and the like,

* Determination of morphological features of the organism,
* Determination of staining characteristics of the organism,
* Confirmation by biochemical analysis, and
* Confirmation by immunochemical analysis.

In actual practice, however, appropriate 'animal inoculation', wherever applicable, may be used to establish **pathogenicity**. It is pertinent to mention here that for accomplishing the **final differentiation and confirmation process** one has to take into consideration the prime variables directly associated, namely: site, timing, technique (aseptic), instrumentation, and transportation of clinical specimens.

Specific Tests*: There are three specific tests that may be used to identify the pathogens, namely:

(a) **Enzymatic and Immunological Tests:** The introduction of rapid manual enzymatic and immunological test kits have enormously enabled to identify the presence of '**pathogens**' in the cerebrospinal **fluid (CSF)** analysis.

(b) **Coagglutination Tests:** In this specific tests, the particular antibody is bound to protein A on the surface of a staphylococcal cell, and the very presence of antigen causes agglutination, and

(c) **Latex-Agglutination Tests:** In this particular tests a specific antibody gets coated upon the latex particles and when an antigen is present, the latex particles are visibly distinctly.

The various pathogenic organism(s), its type, occurrence and the identification tests have been duly summarized in Table 1 given below:

Table 1: Identification Test of Various Pathogenic Organism

S.No.	Organism	Type of Organisms	Occurrence	Identification Test
1.	*Staphylococcus aureus (Micrococcus pyogenes var aureus)*	Gram + ve	Normal human skin, mucous membranes, frequently associated with abscesses, septicemia, endocarditis, and osteomyelitis.	It is based on colonial (pigmentation) and microscopic morphology (grape-like clusters), positive catalase production, positive coagulase production (staphylocoagulase plasma clotting factor), and positive mannitol fermentation.
2.	*Streptococcus pyogenes*	Gram + ve	Associated with tonsillitis or pharyngitis, erysipelas, pyoderma, and endocarditis.	Streptococcal groups are identified by **precipitin tests** with group specific antisera for A, B, C, D, F and G.
3.	*Neisseria gonorrhoeae*	Gram – ve	Venereal disease gonorrhoeae	It is based on the primary isolation of the gonococcus from urethral exudates on chocolate agar or Thayer-Martin medium. The microscopic

* Kelsi PJ: Med Lab Observer: 108 (Sept.) 1983

				examination of Gram – ve diplococci resembling the gonococci supports a positive diagnosis of gonorrhoea. The presence of oxidase enzyme activity of the gonococci is usually carried out by a reagent **p-dimethyl aminoaniline**, which oxidally changes reddish – brown colonies black.*
4.	***Enteric bacilli*** *(Enterobacteriaceae)*, such as *Shigella* spp. : *dysentery*, *Salmonella typhi* : *typhoid fever*, *Escherichia coli*, *Proteus* spp., *and Pseudomonas* spp. : urinary tract and tissue infections ; **Klebsiella** spp. : pulmonary infections ;	Gram – ve	Stated under 'organism'	**Enteric bacilli** may be primarily isolated on *selective and differential agar e.g.*, Mac Conkey's medium containing neutral red, bile salts, crystal violet and lactose, besides, *enrichment media e.g.*, **tetrathionate and selenite broth**. **Leifson's desoxycholate citrate agar** (LCD) OR *Salmonella-Shigella* agar (SS) } For primary isolation of *Salmonella* and *Shigella*. **Brilliant green agar BG** and **Bismuth sulphite agar** (BS) } For presumptive diagnosis of *S. typhi*. Identification/confirmation of **enteric bacilli** may be carried out by a host of **serological tests** and **biochemical reactions** *e.g.*,

* 1. A + ve oxidase test by Gram – ve diplococci isolated on TM medium constitutes a presumptive test for *N. gonorrhoeae*.

2. Final identification tests are **typical sugar fermentation** or specific (**fluorescent antibody**) stain

				(ii) H_2S — production (triple – sugar iron agar) ; (iii) Acetylmethyl carbinol production ; (iv) Indole production ; (v) Citrate utilization ; (vi) Activity of various enzymes *e.g.*, urease, lysinase, and arginine decarboxylase ; and phenylalanine deaminase.

Antimicrobial Susceptibility Tests : It may be defined as — *a determination of the least amount of an antimicrobial chemotherapeutic agent that will inhibit the growth of a microorganism in vitro, using a tube dilution method, agar – cup method, or disk diffusion method.*

However, the **antimicrobial susceptibility test** may serve as a vital and critical help essentially needed by the physician in the judicious and precise selection of a chemotherapeutic agent. It is also pertinent to state here that the exact concentration of the **antimicrobial agent** in the **body fluids*** may be estimated by *'biological assay'* with the aid of an *'organism'* having a known **susceptibility** (predetermined) for the specific agent in question.

Laboratory Diagnosis of Viral Infections : In actual practice, the laboratory diagnosis of several **viral infections** is exclusively based upon the following *five* cardinal factors, namely :

1. Examination of the infected tissues for actual **pathognomonic changes** or for the presence of **viral material.**

2. Isolation and identification of the viral agent.

3. Demonstration of an appreciable enhancement in the **'antibody titer value'** to a given virus in the span of the illness.

4. Detection of **viral antigens** present in lesions by employing **fluorescein-labeled antibodies.**

5. **Electron microscopic examination** of either the tissue extracts or the vesicular fluids.

Serological Tests : It is a common practice to use 'blood' for carrying out the serological tests, but quite rarely for virus isolation. However, it is absolutely important and vital that both **acute** and **convalescent-phase blood specimens** should be examined thoroughly in parallel to estimate precisely whether **'antibodies'** have appeared, lowered or enhanced in the **'titer value'** in the span of the disease.

Examples : A few typical examples of **'human viral infections'** are as enumerated under :

- Respiratory infections (*e.g.*, **Adenovirus group**)
- Diseases of the nervous system (*e.g.*, **Polio** and **Coxsackie viruses of the picornavirus group**)
- Small pox (**Poxvirus group**)
- Measles (**Paramyxovirus group**)
- Chicken pox (**Herpesvirus group**)
- Influenza (**Myxovirus group**)

* Blood, sputum, urine, cerebrospinal fluid (CSF), vesicular fluids etc.

Clinical Parasitology : It is indeed a well-defined science that is exclusively concerned with the **parasitic protozoa** (amoeba), the **helminths** (roundworms, tapeworms, flukes, nematodes, threadworms), and the **arthropods**.

Identification of Protozoan Ova : It is based upon the **detailed microscopic examinations studies** (including *nuclei*) by making use of **wet mounts** (*e.g.*, *saline or iodine*) or **stained preparations** (*e.g.*, *iron, hematoxylin*) obtained from *fecal specimens* (fresh or preserved with polyvinyl alcohol) that have been *adequately concentrated* by **sedimentation, centrifugation**, or **flotation techniques**.

Example : Amebic Dysentery : Specifically, in the fecal specimens the presence of **trophozoites** and/or **cystic stages** could be detected along with intestinal protozoa, as in the case of amebic dysentery usually caused by *Entamoeba histolytica*.

Serodiagnosis of Parasitic Diseases : **Serodiagnosis** essentially concerns with the diagnosis by observing the reactions of blood serum. Importantly, the serodiagnosis of parasitic diseases involves the following *critical 'tests'*, namely :

Immunodiagnostic Tests : *Complement fixation* (trichinosis) ; *precipitin test* (schistosomiasis, *hemozoin flocculation* (ascariasis) ; **hemagglutination** (echinococcosis) ; *latex agglutination* (filariasis, etc) ; *cholesterol flocculation* (schistosomiasis) ; *fluorescent antibody* (malaria) ; and *enzyme-linked immunosorbent assay* (toxoplasmosis).

Methods Used for Standardization of Antibiotics

Official compendia invariably make use of the terminology **'antibiotic'** that essentially designates a *'medicinal preparation* ; containing an appreciable quantum of *a chemical entity* which is capable to produce naturally by a microorganism or by a semi-synthetic route artificially, and that possesses the inherent ability to either destroy (bactericidal effect) or *inhibit* (bacteriostatic effect) microorganisms in relatively dilute solution.

Following are some of the standardization certification of various *'antibiotics'* in a chronological order :

Year	Event
1938	: Federal Food, Drug and Cosmetic Act — Introduction in stages of the *'batch certification of antibiotics'* meant for human or veterinary applications.
1945	: Penicillin
1948	: Streptomycin
1949	: Aureomycin, Bacitracin and Chloramphenicol.
1962	: Kefauver-Harris Amendments — as part of these amendments it was mandatory for the **'batch certification'** of **all antibiotics** intended for human use.
1982	: Federal Drug Authority (FDA)-USA issued regulations which totally exempted the **'antibiotics'** from the *batch certification requirements* so long as the article complied with standards ; however, section 507 (*i.e.*, **related to certification of Antibiotics**) remains intact and hence applicable.

***Federal Register* (USA)** : It essentially incorporates the **'Standards of Potency and Purity for Antibiotics'** as established and determined by the FDA in the form of regulations published from time

* A spirillum recorded by its hosts during its growth stage.

to time. It is, however, pertinent to mention here that as all **recognized antibiotics are automatically** subject to the provisions of the regulations, these eventually determine the **official standards**.

The following *three* cardinal points may be taken into consideration with regard to the *'assay' i.e., standardization of Antibiotics*, such as :

(1). FDA regulations governing all aspects of antibiotics testing are extremely detailed and are subject to periodic amendment.

(2). FDA regulations need to be adhered to with regard to the **'prescribed methods'** for the assay of *individual antibiotics and their preparations*, and

(3). While evaluating the potency of **'antibiotics substances'**, the actual and apparent measured effect is the *'degree of inhibition'* of the growth of a suitable strain of microorganisms *i.e.*, the ultimate prevention of the multiplication of the **'test organisms'**.

The procedures usually employed in the **'microbial assay of antibiotics'** may be categorized under *two* heads, namely : (a) Cylinder Plate Method ; and (b) Turbidimetric Method, which shall now be treated briefly as under :

2.1. Cylinder-Plate Method

The **cylinder-plate method of assay** of *antibiotics potency* is solely based upon the measurement of the specific diameter of **'zones of microbial growth inhibition'** immediately surrounding cylinders **containing various dilutions of the 'test compound'** (*i.e.*, the substance under investigation), that are carefully placed on the surface of a solid nutrient agar medium previously inoculated with the **'culture'** of an *appropriate organism*. In actual practice the zone of inhibition caused by the **test compound** is meticulously compared with that produced by a known concentration of a pure **'Reference Compound'**.

2.2. Turbidimetric Method

The **turbidimetric method of assay** of *antibiotics potency* is exclusively based upon the inhibition of microbial growth as indicated by the **corresponding measurement of the turbidity** (*i.e.*, **transmittance**) of suspension of an *appropriate organism* in a fluid medium into **which the graded amounts** of the **'test compound'** have been added duly. Consequently, the changes in the transmittance caused by the **'test compound'** are duly compared with those resulted by known concentrations of the **Reference Standard**.

Note : **(1) Comprehensive account of appropriate microbial assays for specific antibiotic(s) (*viz.*, cylinder-plate or turbidimetric method) has been duly included in the 4th through 6th and 8th supplements of USP 23-NF 18.**

(2) The *'test organism'* recommended for each antibiotic have been duly incorporated in these aforesaid supplements.

3. SCREENING OR SOIL FOR ORGANISMS PRODUCING ANTIOBIOTICS

'Soil' — is nothing but the upper layer of the earth. Ample studies and researches have substantially revealed and established the dictum that — **soil is the best available source** from which one may obtain ultimately a *broad spectrum of viable 'microorganisms'*

Therefore, to ascertain the **'screening approach'** to an effective and justifiable manner, one must have to rigorously explore **'soil'** as a natural microbial source which essentially comprises of various kinds of microorganisms. It is absolutely immaterial whether a relatively large section of these microorganisms may or may not be recognized to exhibit the biosynthetic abilities of genuine interest. It is, however, pertinent to state here that there exists another unexplored possible source which could prove to be a *'Mecklenyle's Gold'* i.e., an unbound source of microorganisms, such as : **ocean water** and **marine mud**. Interestingly, there are several other viable and convenient plausible sources of useful microorganisms, namely : compost, domestic sewage undergoing treatment, manure, rumen contents, and decomposing food stuffs or foodstuffs.

At this juncture, one may raise a crucial and an extremely pivotal question that — *why is soil invariably regarded to be the ideal source from which to obtain diverse types of microorganisms ?* The various **logical explanations** to the above issue may be summarized as given below :

(1) A sizable quantum of the **'debris of the world'** finds its normal passage either onto or into the soil ; and ultimately gets adequately decomposed by one microorganism or the other.

(2) **'Soil'** may be thought of as being of a specified kind of **'huge natural fermentation vat'** wherein a plethora of organisms are actively engaged not only in the actual decomposition and reconversion of simple to complex **organic materials,** but also in carrying out effectively the process of oxidation, reduction and other chemical changes pertaining to **inorganic materials.**

(3) It has been duly demonstrated and established that more than one type, and often many types, of soil microorganisms are invariably capable of performing each of these individual **chemical or biochemical transformation.**

(4) Though a large volume of different types of microorganisms do occur in the soil ; however, it is not yet so clear and evident that actually upto what extent of these organisms, as a whole, have been pinned down and isolated in the form of **purest laboratory culture.**

Explanations : In fact, a host of researchers have more or less determined and established that both **plate counting** and **isolation procedures** as applicable to total numbers and types of the soil microorganisms, even though employing the best recognized media and incubation parameters, perhaps would allow less than 1% of the **soil microorganisms** to be grown in the laboratory logistically. Obviously, these 'microorganisms' urgently need the **'magic tool'** for someone to evolve a *suitable medium* and *cultural parameters* will permit their ample growth in the laboratory environment ultimately.

Evidently, it is of an immense interest and great value to one who wishes to isolate organisms having *predominant newer biosynthetic capabilities*, as it steadily indicates that, at least with **soil,** there exist a plethora of microorganisms not reported or described previously that are just waiting to be isolated and evaluated in the near future.

(5) Importantly, **soil** also admits a certain extent of manipulation in the relative degrees of the various components of its microbial population just prior to the articulated procedures adopted for **screening** and **isolation.**

(6) **Nutrients :** The availability of nutrients in **soil** is invariably found to be relatively at low degree and, therefore, the prevailing **microbial competition** for these nutrients is quite prevalent. In case, a highly desired and specific nutrient is timely incorporated to the **'moistened soil'**, and the treated **soil** is duly incubated then a relatively much appreciable **larger growth**

response takes place amongst the ensuing soil microorganisms that are capable of attacking the **specific nutrient** thereby rendering the isolation of these particular organisms much simpler and convenient. In other words, one may accomplish judiciously the **'enrichment in soil'** for specific microorganisms of our interest.

(7) In the same vein, the resulting **soil** may be adequately incubated in a **particular liquid laboratory culture media** so as to cause enrichment for *specific organisms* before an isolation is commenced. However, a natural phenomenon of enrichment invariably takes place in the **soil** located in the vicinity of plate roots ; and, therefore, the prevailing microorganisms in the specified area may be found out to be quite different from those existing in the **'adjacent soil'** not duly penetrated by roots. In actual practice, this **'rhizosphere effect'** is afforded by root secretions and dead or sloughed debris of root would serve as microbial nutrients.

3.1. Screening

Screening may be defined as — *the application of highly selective, specific and sophisticated sequential procedures so make the detection and isolation of only such microorganisms that are of genuine interest out of a large microbial population.*

Concept of Screening : The various underlying concepts of screening essentially include :

(1) *Segregation of Viable Microorganisms :* It should be highly effective in the sense that either *a few steps of a single step would be able to discard a major portion of the relatively non-useful microorganisms ;* whereas, **simultaneously** allowing the rapid and fast detection of the small percentage of viable and useful microorganisms which are usually present in the populations.

Example : In industrial research programmes an attempt is made from a natural microbial source *e.g.*, **soil** is diluted to obtain a 'cell concentration' in such a **fashion that when** aliquots spread, sprayed, or applied onto the surfaces of sterilized agar plates, in an *aseptic condition*, shall give rise to countable Colonies not essentially touching the neighbouring colonies.

(2) *Detection of Microorganisms by Colour Change :* The various types of microorganisms yielding *organic acids* (attributing acidic characteristics) or *amines* (attributing basic features) generated from various carbon substrates quite often may be detected conveniently by the incorporation of a **pH indicating dye,** for instance : **bromothymol blue** or **neutral red,** into a *slightly buffered agar nutrient medium.* In actual practice, the production of these aforesaid *'chemical entities'* is invariably indicative by exhibiting a definite change in colour of the previously incorporated indicating dye in the periphery of the ensuing colony to a **'colour'** showing either an alkaline or an acidic reaction. However, the usefulness of this *'specific methodology'* may be augmented appreciably if a media having much higher buffer capacity are utilized so that only such microorganisms which are responsible solely for producing significant quantum of either the **'amine'** or the **'acid'** can effectively induce characteristic changes in the colour of the dye.

Drawbacks : The various drawbacks of this technique are as enumerated under :

(a) It fails to give a definite indication about which amine or organic acid has been produced actually. Hence, it should be immediately followed by further testing with the help of certain well known analytical procedures *e.g.*, **paper chromatography, electrophoresis** so as to determine and establish whether the acidic or basic product really is one of interest.

(b) Importantly, in such an event where cultures of microorganisms by virtue of their ... screening procedure, seem to possess "**appreciable fermentative potential**" ... mediately be subjected to *purification* ; and, therefore, subcultured subsequently ... slants of an appropriate agar medium to be maintained subsequently as "*stock cultures*" during further testing devices.

(c) Sometimes, it is indeed quite discouraging to discover a specific organism exhibiting *excellent fermentative potential* only to observe that, via strenuous techniques, ... contamination or for other reasons, the culture in question has been lost ultimately.

(3) **Microorganisms for Producing Antibiotics** : In the recent past, the "**screening approach**" has been exploited both extensively and intensively in the meticulous search for "**naturally occurring specific microorganisms** that are exclusively capable of producing **antibiotics** of ... harmful drug human diseases.

Crowded-plate Technique : It is one of the **simplest screening techniques** invariably employed by the "*antibiotic producers*". In fact, this technique has an added advantage ... exclusively seeking for microorganisms which produce an "**antibiotic**" without any regard ... consideration whatsoever about the types of microorganisms that may be sensitive to ... antibiotic.

Methodology : The various steps involved are as follows :

(1) First of all, the "**soil**" or anyother source of microorganisms is adequately diluted with ... soft concentration in such a manner that the agar plates normally prepared from these ... tions shall be *crowded with individual colonies* on the surface of the agar *i.e.*, approximately 300–400 or even more colonies per plate.

(2) The **colonies that are** solely responsible for producing antibiotic activity are indicated by ... area of agar around the colony which is usually free of growth of other colonies. It is ... common practice to subculture such a colony further in an identical medium, and ... subsequently be streaking, just prior to making "**stock cultures**". The *'purified culture'* ... obtained is now almost *ready for testing to establish precisely the types of microorganisms that are sensitive to the* **antibiotic under investigation**, by, means of the "**minimum inhibition concentration (MIC)**" or the "**microbial inhibition spectrum (MIS)**".

Limitations of Crowded-plate Technique

There are several limitations that are noticeable in the *crowded plate* technique, such as

(1) The crowded-plate technique does not necessarily aid in the precise selection of an antibiotic producing microorganisms by virtue of the fact that the inhibition area (or zone) immediately surrounding the colony may be attributed to other **vital reasons** quite frequently, such as

- marked and pronounced alteration in the pH value of the medium caused due to the metabolism of the colony,
- rapid utilization of essential nutrients in the immediate vicinity of the colony.*

* Requires further testing to ensure that the persisting inhibitory activity intimately associated with a given organism may actually be attributed to the presence of an antibiotic.

(2) It essential has only limited application, because normally one is more inclined in finding a **microorganism producing antibiotic activity** Vs **Specific microorganisms**, and certainly not against the **unknown microorganisms** which were present just as a stroke of luck on the agar plate in the vicinity of an *antibiotic producing microorganisms*.

(3) The "**antibiotic screening profile**" may, however, be expressed by the strategic introduction into the laid-out procedure of a "**test organism**".*

The various "**screening**" aspects discussed under section 3.1 are usually referred to as the "**primary screening**" or the "**preliminary screening**".

3.2. Secondary Screening

Primary screening (or **preliminary screening**) solely enables not only the "*detection*", but also the "*isolation*" of such viable microorganisms that essentially possess potentially interesting and commercially feasible applications. Nevertheless, this screening is invariably followed by a **secondary screening** so as to ascertain more useful information about these organisms, besides their actual inherent capabilities.

It is, however, pertinent to state here that *the primary screening* establishes exclusively the capability of microorganisms that are responsible for producing a compound without giving enough idea either with respect to the yield or production potential for the organisms. On the contrary, the **secondary screening** categorically enables the further "**sorting out**" of those specific microorganisms that essentially possess the "*real industrial value*" for feasible and gainful industrial processes ; and ultimately eliminating those devoid of such a potential.

3.2.1. Methodology

The various steps involved are as follows :

(1) **Secondary screening** is usually carried out on *agar plates* aseptically.

(2) It may also be conducted in flasks or small fermenters containing liquid media, or as a combination of such available **procedures**.

(3) However, one may use "**liquid culture**" as an alternative to "**agar plate**" in the "*secondary screening*" method. Following are some of the important merits and demerits of these two techniques stated briefly

S.No.	Agar Plate Method	Liquid Culture Method
1.	It is not so sensitive.	It is very sensitive.
2.	More information is obtained.	Relatively provides less information.
3.	Usually occupies much lesser space in an incubator ; besides, does not require enough degree of handling and work up effort.	Occupies relatively larger space in an incubator, and also requires sufficient procedural details.

* An organism used as an indicator for the presence of specific antibiotic activity.

4.	It essentially provides only a restricted knowledge with respect to the *actual product yield potentials* amongst the various isolates obtained.	It distinctly provides a much clear idea with regard to the physical, nutritional and productive responses of an organism in comparison to the **actual fermentation production parameters.**

3.2.2. Salient Features of Secondary Screening

The various vital and important **salient features of secondary screening** are enumerated below :

(1) It may be either *qualitative* or *quantitative* in its approach.

(a) **Qualitative Approach :** Provides *valuable information(s)* with regard to the *wide spectrum or ranges of microorganisms* that is significantly sensitive to an altogether **newly discovered** *antibiotic*.

(b) **Quantitative Approach :** Gives authentic information(s) with regard to the *specific yields* of **"antibiotic substance"** that may be expected when the microorganisms are allowed to grow in various **media having** varying composition.

Special Notes :

(i) *There exists no clear cut and necessarily a distinct difference between the qualitative and the quantitative* **secondary screening**.

(ii) *In reality, a* **qualitative screening** *for the ensuing 'microbial inhibition spectra' of an antibiotic eventually gives an ample idea with respect to the* **'test organisms'** *that are found to be sensitive to the antibiotic, whereas it may provide information pertaining to the comparative prevailing sensitivities of these organisms to the* **respective antibiotic**.

(2) *It invariably promulgates a wide spectrum of highly valuable and authentic information(s)* **that are very much** *needed in order to evaluate the precise and actual (true) potential of particular microorganisms for industrial application.*

Example : *It must determine as well as establish the types of microorganisms that are invariably in addition to the fact whether these may be classified at least to various genera or families.*

**Note : The aforesaid information is of immense value, because it broadly makes possible a befitting comparison between the newly isolated organisms with those already reported either in the scientific journals or in the patent* literatures showing a adequate logical evidence to produce fermentation products of commercial value and interest.

(3) **Classification of Organisms :** Interestingly, classification of the organisms due to secondary screening certainly makes a room for the much needed prediction of whether they **organisms** do own any genuine pathogenicity for *humans, animals* or *plants* that would necessarily warrant special precautions in the handling of such organisms. Besides, it gives a reliable prediction of the *growth characteristic features* in the intensive studies of these microorganisms.

(4) **Establishing a More Economically Variable Process :** The secondary screening must provide adequate information(s) with respect to the fact whether the microorganisms isolated

* A newly discovered microorganisms invariably helps in obtaining a **'patent'** as it predominantly adds **'novelty'** or newness to the microbial process.

and identified) are virtually giving rise to **'newer chemical entities'** not reported earlier or, alternatively, for such **fermentation procedures** which are already reported.

Highlights : A few highlights are as given below :

(i) In case, the resulting product happens to be an altogether newly discovered compound then an attempt must be made to establish its real genuine merit.

(ii) In actual practice, the **patents** are usually granted for exclusively new as well as useful products.*

(5) **Real Differences in Product Yield Potentials :** The **secondary screening** must be able to detect the real differences in product yield potentials amongst the various isolates irrespective of the fact whether the outcome of the **'ultimate fermentation product'** is or is not a new (*chemical entity* i.e., compound). Therefore, in order to save on valuable resources, such as : manpower, time, energy and money, it is **almost mandatory** to allow the organisms to grow upon different media in liquid culture for various length of time so as to accomplish quantitative assays effectively. Nevertheless, the aforesaid studies may be carried out only after the **complete** reaction (or elimination) of useless cultures previously ascertained by agar plate procedures.

(6) **Critical Requirements for Specific Microorganisms :** The **secondary screening** should effectively reveal whether the two cardinal phenomena, namely : *(a) growth of the organism* ; and *(b) formation of chemical products*, are dependent on pH, aeration or other critical requirements associated with specific microorganisms. Importantly, it must also detect **'gross genetic instability'** present in various microbial cultures.

Therefore, **secondary screening** essentially expatiates the following relevant facts :

- a microorganisms is of practically little importance if it takes care of to either **mutate** or **change** in some manner thereby drastically losing its innate ability to accumulate high yields of product.
- it must reveal evidently whether some **'medium constituents'** are either missing or perhaps prove to be too toxic to the growth of the organism or as an in built capacity to accumulate fermentation products.
- it should exhibit the **chemical stability profile** of the product ; and consequently, the **product's solubility profile** in different organic solvents.
- it must adequately establish whether the resulting product bears a **simple, complex** or rather a **macro-molecular structure.**
- it should demonstrate explicitely whether the **'ultimate isolated product'** possesses either *typical physical characteristic properties*, for instance : UV-absorption, fluorescence ; or *typical chemical characteristic properties* which may be judiciously exploited to detect the **compound** via various sophisticated analytical techniques or by the aid of elaborated paper chromatographic studies, and which ultimately be of immense value in predicting and assigning the most probable chemical structure of the **'compound'**.
- adequate determination must be made so as to ascertain whether gross *human, animal or plant toxicity* can be attributed to the *fermentation product(s)* obtained specifically during

* It is usually ascertained by the help of paper, thin-layer or high performance thin layer chromatographic procedures so as to compare the **newly discovered compound** with the **known reference compounds.**

the **secondary screening**, in case it is to be utilized (e.g., **antibiotics**) exclusively in the treatment of human ailments. Therefore, the compound must be in its **purest form** so as to obtain both valid and reliable information. Importantly, for studying the detailed toxicity level of an **impure compound**, one should resort to a qualified and educated **'guess'** with respect to the various types of contaminating substances which may be imminently associated with the compound, and ultimately provide *appropriate experimental parameters* for such **identified contaminants** in the ensuing **toxicity testing**.

- it must reveal clearly whether the **'final product'** obtained from a microbial fermentation occurs in the culture broth in a **racemic form** i.e., as a mixture of *optically active d- and l-* forms of which one may turn out to be a biologically active material. Besides, there must also exist two or even more compounds of variant nature that could be obtained from a **'single fermentation'**. Hence, in the *ultimate fermentation broth* one may come across from one to several **intermediate chemical entities** in the prevailing *'metabolic pathways'* leading to **product formation**; besides absolutely **unrelated chemical entities**. Importantly, the presence of additional minor as well as major products are of **distinct interest**, because their subsequent recovery and commercial value as viable by-products may substantially improve upon the economic status of the **'prime fermentation'**.
- it should adequately ascertain the fact whether the ensuing microorganisms are capable of undergoing *changes* under the influences of chemical compounds or even cause destruction of their self-generated fermentation products. Thus the microorganism may, by virtue of its relatively high-level **accumulation** of product present in the culture broth, generate enough **adaptive enzymes** that would greatly destroy the potential value and usefulness of the product.

Examples : (a) A microorganisms may be able to produce a **'racemase enzyme'** which in turn shall alter the *L-configuration* of an amino acid product to an equimolar mixture of its corresponding *D-* and *L-isomers*, with the *D-isomers* exhibiting almost little biological value. (b) A microorganism may strategically respond to the accumulation of an amino acid by adaptively influencing the production of a **'decarboxylase enzyme'** which would specifically remove CO_2 from the resulting molecule, thereby rendering an **organic amine** at the end.

- **secondary** screening thus may give rise to a broad-spectrum of valuable information, such as :
  - (i) it helps in deciding precisely which of the various **microbial isolates** possess probable useful potentialities as a **viable industrial organism**.
  - (ii) it immensely helps most articularly in predicting the approaches to be utilized justifiably in pursuing further productive, aggressive and meaningful research on the selected microorganism and its corresponding fermentation process.

4. FERMENTORS (OR BIOREACTORS)

The most articulate, manipulative and progressive industrial (commercial) usag of microorganisms invariably needs that they be allowed to *grow* in large vessels essentially loaded with considerable quantum of highly nutritive culture media. These specially designed vessels are universally and commonly termed as **fermentors** or **bioreactors**. In reality, these bioreactors (fermentors) could be quite complicated in design by virtue of the fact that most abundantly they should cater for the precise control and meticulous observation of the measurable facets of microbial growth and the biosynthesis

Another school of thought has rightly baptized the above mentioned phenomenon as the **bioprocess** or **fermentation technology**. In a rather broader perspective the *fermentation technology* or, as it is now widely recognized, *bioprocess technology* were conspicuously most derived to put from the use of microorganisms for the generous production of various important and vital products, for instance : *pharmaceutical drugs* [e.g., **antibiotics**, diagnostic agents (enzymes, monoclonal antibodies MABs), enzyme inhibitors, steroids, vaccines] ; *food products* [e.g., cheeses, yoghurts, sauerkraut (chopped pickled cabbage), fermented pickles and sausages, soy sauce, tempeh, miso, mushroom products, starch products, vitamins and amino acids, glucose and high fructose syrups, functional modifications of proteins and peptides] ; *beverages* [e.g., beers, wines, derived spirits] ; *organic chemicals* [e.g., **ethanol**, acetone, acetic acid, citric acid, itaconic acid, n-butanol, perfumeries, enzymes, polymers (mainly polysaccharides)] ; *inorganic chemicals* [e.g., metal beneficiation, bioaccumulation and leaching (Cu, U)] ; *energy* [e.g., ethanol (gasohol), methane (biogas), biomass] ; *agriculture products* [e.g., animal feed stuffs (SCP)*, veterinary vaccines, ensilage and composting processes, **microbial pesticides**, *Rhizobium* and other N-fixing bacterial inoculants, *Mycorrhizal* inoculums, plant-cell and tissue culture (vegetative propagation), embryo production, genetic improvement].**

The aforesaid divergent and highly specific forms of **bioprocessing technology** were nonetheless rightly long regarded as spectacular pieces of *'arts'* or splendid *'crafts'*, but most interestingly these are now attracting wide recognition world wide besides increasingly being subjected to the full array of **modern science** and **magic touch of technological advancements.** Besides, the aforesaid innumerable highly beneficial **product formations** was the legitimate cognizance of the critical and specific roles of the **'microorganism'** essentially utilized in the removal of extremely obnoxious and unhealthy waste products, that has resulted in the world-wide service industries intimately associated with water purification, effluent treatment, and above all the solid waste management efficaciously.

Biofermentors (bioprocessing technology) in its several recognized variants essentially embraces a relatively large multitude of *complex enzyme catalyzed biochemical reactions* within the **specific microorganisms**. Nevertheless, these reactions are exclusively and critically dependent upon the broad spectrum of physical and chemical parameters which predominantly exist in their immediate vicinity. Importantly, the *categorical success* of the bioprocessing phenomenon will take place only when all the vital experimental conditions are duly brought together.

Bioprocessing technology, in the recent time, has spread its tentacles in several *major commercial byproducts solely derived from microbial fermentations*, such as :

- (a) **Primary Metabolites** : i.e., to overproduce certain essential primary metabolites, for instance : citric acid, lactic acid, acetic acid, glycerine, n-butanol, amino acids, vitamins, polysaccharides, perfumeries etc.
- (b) **Secondary Metabolites******* : i.e., to produce most important and life-saving **'antibiotics'** (*pharmaceutical drugs*) *via* well defined *fermentative procedures*******, for instance : Gliocrifins, Penicillin, Cephalosporins, Streptomycin etc.

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* SCP = *single cell protein*.

** Bull AT *et al. Biotechnology International Trends and Perspectives*, OECD, Paris.

*** Metabolites that do not appear to have an obvious role in the metabolism of the producer organism.

**** Most of these procedures are duly protected under the **'Patent Laws'**.

(c) **Enzymes** : *i.e.*, to produce a large variety of industrially viable and useful enzymes *e.g.*, (a) *extracellular enzymes* : invertase, asparaginase, restriction endonucleases etc. (b) *intracellular enzymes* : amylases, pectinases and proteases.

4.1. Salient Features of Bioreactors

The various salient features of '**bioreactors**' or '**biotechnological processes**' are as enumerated under :

(1) In the recent past, biotechnological processes (bioprocess technology) is found to very highly *aggressively and progressively* **specific cells** derived exclusively from higher plants and animals to give rise to several useful and vital products.

Examples :

(a) **Plant Cell Culture** : It is largely aimed at the adequate formation of **secondary plant products solely**, for instance : **drugs** (antibiotics), **flavours**, and **perfumes**.

(b) **Animal Cell Culture (Mammalian Cell Culture)** : It is mainly concerned with the production of extremely potent and life-saving products, such as : (i) **vaccines** ; (ii) **antibody formation** ; and (iii) **protein molecules** *e.g.*, interferons, interleukins etc.

(2) *It has been amply demonstrated, proved and established beyond any reasonable doubt that* the aforesaid '**bioproducts**' cannot be produced economically via other *chemical processes*. Besides, with the advent of latest developments in the specific fields of genetic engineering (or '**organisms**') and technological advances (in **processing modes**) one may accomplish wonderful viable economies in the production of '**bioproducts**'.

Examples :

(a) Huge **quantum** of cells are magnificently *grown under well-defined stringent controlled conditions*, whereby the '**organisms**' may be adequately *cultivated* and *nurtured* to produce the desired products by the help of a **precise physical/technical containment system** (*i.e.*, **bioreactor**) in addition to the *appropriate medium composition* and to *specific environment growth regulating parameters*, for instance : **aeration** and **temperature**.

(b) Articulated optimization of various streams of the '**bioprocess**' spans not only the prevailing *bioprocess* but also the ensuing *technical systems*. In actual practice, the careful and proper exploitation of an **organism's potential** to produce apparently *distinct & stringent products* having *well-defined characteristic features, quality parameters* and *huge quantum* will necessarily require the **in-depth knowledge of the biochemical mechanisms of product generation** daily.

(3) Importantly, the same apparatus, of course with certain modifications, may be used to bring into being several vital products *e.g.*, **antibiotic, enzyme, amino acid** or **single-cell protein**. One may look at the *bioprocess phenomenon* as comprising of several sequential *unit operations*, such as : mixing microorganisms with a nutrient broth, allowing the *components of react* (*e.g.*, yeast cells with a nutrient broth) to give rise to the formation of **ethanol**.

(4) Most *biotechnological procedures* are meticulously carried out very much within *bioreactors* or *containment systems*' whereby large excess of cells that are actually invested in these processes and the **bioreactor** essentially secures their intimate involvement with the suitable correct diets as well as experimental parameters for the *actual growth and product formation*.

(5) *One of the most desired and major functional criterion of a* '**bioreactor**' is to reduce drastically *the cost involved in producing a product or providing a service*.

A few typical examples having the diverse product categories being produced on a commercial scale in **bioreactors** *are summarized as under* :

Category	Examples
Cell mass*	Baker's yeast, single-cell protein
Cell components**	Intracellular proteins
Biosynthetic products**	Antibiotics, vitamins, amino and organic acids
Catabolic products*	Ethanol, lactic acid, methane
Bioconversions*	High fructose corn syrup, 6-amino penicillanic acid (6-APA),
Waste treatment	Activated sludge, anaerobic digestion

4.2. Classifications

Evidences from the literature survey amply justifies that '**bioreactors**' are essentially classified in *two* broad categories, namely :

(a) based on the '**agent used**', and

(b) based on the '**process requirements**'

Bioreactors that are exclusively based on the '**agent used**' may be further sub-divided into two groups, such as :

(i) those based on *living cells*, and

(ii) *those employing enzymes*.

On the contrary, '**bioreactors**' that are solely based upon the '**process requirements**' may be further classified into *four* prominent groups, for instance :

(i) Solid-state fermentation,

(ii) Anaerobic fermentation,

(iii) Aerobic fermentation, and

(iv) **Immobilized** cell bioreactors.

The aforesaid four groups of '**bioreactors**' based on the '*process requirements*' shall now be treated individually in the sections that follows :

4.2.1. Solid State Fermentation

In true sense, such fermentation procedures are usually governed by both *microbial growth* and *product formation* predominantly taking place at the surface of the solid substrates, such as : *mold-ripened cheeses* ; *tissue cultures* ; *mushroom cultivations* etc.

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* *Typical conversion of feedstock into intensive processes.*

** *Typically recovery into intensive processes.*

Importantly, in the recent past, the solid state fermentation approach has been judiciously extended for the production of certain high-value products of interest, namely : enzymes, enzymes, valuable chemical entities, fungal toxins, and fungal spores (commonly employed for **biotransformation processes**).

However, the most traditional substrates essentially comprise of a plethora of "**agro-industrial products**" like rice, maize, wheat, soybean etc. It has been observed duly that the prevailing substrate predominantly caters for a rich and complex source of nutrients that either may or may not require supplementation. Interestingly, 'substrates' belonging to this specific class selectively support the growth of **organisms** that are capable of growing even at an elevated osmotic concentrations, and additionally give rise to variety of **extracellular enzymes**, such as : (a) a huge number of **filamentous fungi** and (b) a relatively small number of bacteria (e.g., **actinomycetes** and one strain of *Bacillus*).

It is, however, pertinent to mention at this point in time that according to the physical characteristic state, the solid state fermentations are invariably categorized into two major heads, namely :

(i) **low moisture solids** fermented either without or with occasional/continuous agitation, or

(ii) **suspended solids** fermented in packed columns through which liquid is circulated.

In actual practice, the fungi which are exclusively employed for carrying out the **solid state fermentations** are normally the **obligate aerobes**. The following table enumerates a few typical examples of the solid state fermentations which are used extensively in Japan for large-scale products like **enzyme** and **organic acidics**.

S.No.	Product	Substrate	Primary Genus	Product Used As	Comments
1.	Amylase*	Rice	*A. Oryzae*	Enzyme	—
2.	Cellulase*	Wheat Bran	**Trichoderma** species [**Synonym** : Viride]	Enzyme	—
3.	Citric acid	Cooked vegetable residues	*Aspergillus niger*	Organic acid	Commonly in Japan
4.	Hamanatto	Soybean ; Wheat	*Aspergillus sp.*	Food	Processed further
5.	Miso	Rice/barley ; Soybean	*A. Oryzae*	Food	— do —
6.	Soy Sauce (Shoyu)	Soybean, Wheat	*Aspergillus Sojae* or *A. Oryzae*	Food	— do —
7.	Sufu	Tofu	*Actinomucor sp.*	Food	— do —
8.	Tempeh	Soybean	*Rhizopus spp.* [*R. Oligosporus*]	Food	Further processing not required

* Enzymes produced commercially ; other enzymes include : **pectinase**, **lipase** and **protease**.

Special Remarks : Following are some of the special remarks with respect of the solid state fermentation procedures, namely :

(1) they make use of either stationary or rotary trays,

(2) invariably both temperature and humidity controlled air is being circulated through the mass packed within,

(3) sporulation rare formations are most rather less frequently,

(4) they usually often possess **unique advantages** besides some vital disadvantages also, and

(5) major commercial application of this phenomenon for the biochemical production is solely confined to Japan.

4.2.2. Anaerobic Fermentation

It is quite evident that in anaerobic fermentation a provision for "aeration" is absolutely not required as shown in Fig. 3.1.

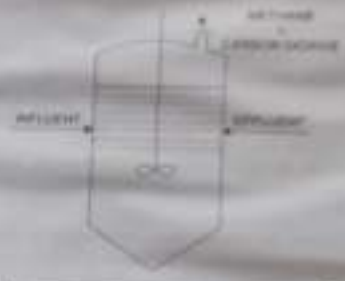


Fig. 3.1. *Anaerobic Digester or Bioreactor*

Salient Features : The salient features of "anaerobic fermentation" are as follows :

(1) Certain specific instances do require aeration or initial oxygen only to build-up inoculum.

(2) Large number of cases do not essentially need a "mixing device", whereas a few of them the initial mixing of the inoculum is an absolute necessity.

(3) Once the fermentation commences the CO_2 generated in the reaction vessel generates sufficient mixing (i.e., causes agitation).

(4) **Air** present in the headspace of the fermentor must be adequately replaced by CO_2, N_2, H_2 or an appropriate mixture of these ; and this specific operation is absolutely vital and important for critical **obligate anaerobes** e.g., *Clostridium*.

(5) Process of "**fermentation**" invariably gives rise to CO_2 and H_2, that are carefully collected in pressurized cylinders and used accordingly in various commercial and production activities, namely :

(c) CO_2 — for making dry ice and methanol,

(ii) CO_2 — for making carbonated beverages *e.g.*, beers, soft-drinks, champagne, soda water, etc., and

(iii) CO_2 — for slowly bubbling into freshly inoculated fermenters.

Note :

(1) Acetogens plus other gas-utilizing organisms it is necessary to bubble through the medium either oxygen free sterile CO_2 or other mixture of gases.

(2) Acetogens may be cultured successfully in 400 L fermentors by carefully bubbling sterile CO_2 ; and thus 2 kg cells could be harvested in every individual operation.

(iii) Recovery of *desired final products* from the anaerobic fermentors does not essentially require anaerobic environments anymore. However, several **'enzymes'** belonging to such cells are **high oxygen-sensitive.** Hence, the sole objective for the recovery of such enzymes may be accomplished by harvesting the cells strictly under **anaerobic conditions.**

4.2.3. Aerobic Fermentation

The *apparent cardinal and most distinct features* of the **'aerobic fermentation'** is the *most critical provision of constant adequate aeration* * It has been observed that in certain specific cases the *actual quantum of air required per hour is almost 60 folds* in comparison to the process medium volume. Hence, **bioreactors** employed invariably for carrying out the **'aerobic fermentation'** have an essential provision for the constant, adequate and compressed (pressurized supply of the **air'** that is usually sparged into the liquid culture medium. Besides, such **'bioreactors'** (fermentors) should possess a befitting device and mechanism for efficient stirring and mixing of the liquid culture medium and the cells.

In actual practice, however, the **'aerobic fermentors'** are of *two* kinds, namely : (a) stirred-tank type fermentors ; and (b) air-lift type fermentors. These two distinct fermentors shall now be discussed separately in the sections that follows :

4.2.3.1. Stirred-tank Type Fermentors (or Stirred Bioreactors)

These are usually made of **'glass'** [*i.e., smaller vessels* having capacity ranging between 1000 L] or **'stainless steel'** [*i.e.*, larger vessels having capacity varying between 2000 to 800 L]. In reality and actual practice, these are closed systems having rather a definite fixed volume and normally agitated with motor-driven stirrers with lots of variation in design specifications, such as curved-bottom for more efficient mixing at low speeds ; water-circulated jacket in place of heating (electrical) temperature control ; mirrored internal finishes to minimize cell-damage drastically as depicted in Fig. 3.2.

* **Aeration** is usually done with absolutely **'sterile air'** dispersed under adequate compression through liquid culture medium.

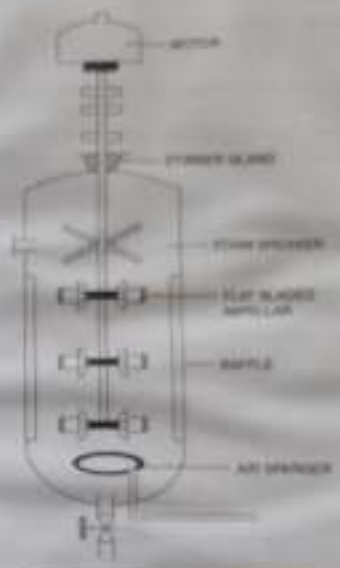


Fig. 3.2. Stirred Tank Type Fermentor

Advantages : The various vital advantages of stirred-tank type fermentors are as stated below :

(1) Several **heterophoid* cell-lines** may be grown successfully in such vessels.

(2) Small scale reactors (cap. 2-50 L) fulfil the need for research biochemicals from cells.

(3) Large scale reactors (cap. 100-5000 L) are largely employed for growing hybridoma cells for the production of *monoclonal antibodies* (MABs) ; whereas, their yields from the 'cultured cells' ranges marginly between 1-2% of those obtained by passing the cells via peritoneal cavity of mice.

Note : Namalva cells grown for 'interferon' ; but in actual practice the maximum size of the 'stirred bioreactor' is 20L only because larger vessels are rather not-so-convenient and difficult to handle, to autoclave, and also to agitate the culture medium effectively.

* Possessing a chromosome number that is not a multiple of the haploid number common of the species.

4.2.3.2. Air-lift Type Fermentors

The cultures in an **air-lift type fermentor** are not only subjected to 'aeration' but also 'mixing' by passing sterilized compressed air bubbles introduced strategically at the bottom of the vessel as shown in Fig. 3.3.

Salient Features of Air-lift Type Fermentors : The various vital salient features of an air-lift type fermentor are as follows :

(1) The fermentor has an inner draft tube into which the air bubbles as well as the aerated medium flow into. Because this effectively gives rise to through mixing of the culture and provides aeration simultaneously.

(2) The air bubbles being lighter lift to the top of the medium and the air subsequently is released through an outlet.

(3) In this process, importantly the cells and the medium which eventually lift out of the draft tube usually move downwards outside the tube and are recirculated duly.

(4) Air-lift type fermentors with a capacity of 2-90L are invariably available for large scale production. However, 2000L fermentors are being employed specifically for the production of monoclonal antibodies (MABs).

Fig. 3.3. Air-lift Type Fermentor

4.2.4. Immobilized Cell Bioreactors

It has been adequately established that the specific cultures based on **immobilized cells** provide several important and vital advantages, namely :

(1) possess relatively *higher cell densities* in the tune of $50 - 200 \times 10^{-6}$ cells ml^{-1},

(2) retain evidently greater *stability* and *longevity* of cultures,

(3) possess *wider applicability* to both suspension and monolayer cultures.

(4) afford a *platform* of systems that essentially protect the cells from shear forces by virtue of the medium flow, and

(5) provide comparatively *less dependence of cells* at higher densities on the external supply of growth factors that eventually saves culture cost significantly.

In actual practice, there exist two basic approaches to cell immobilization, such as : (a) entrapment and (b) entrapment. These two different aspects shall now be treated individually in the sections that follow :

4.2.4.1. Entrapment Cultures

Obviously, in such type of cultures, cells are invariably confined within a medium permeable barrier. In actual practice, one most make use of the classic of '**hollow fibers**' usually packed in an appropriate cartridge (like one such system). In this particular instance, the medium gets circulated through the fiber whereas the cells in suspension are normally present in the cartridge outside the fiber.

Advantages : The various *critical advantages* of this technique are as follows :

(1) an extremely effective technology that retains upto 1 L, and affords cell densities upto $1 - 2 \times 10^8$ cells ml^{-1},

(2) sophisticated units may yield even upto 40g MABs per month,

(3) interestingly membranes permitting medium and gas diffusion medium are also employed to develop **bioreactors** of this particular type, and

(4) commercial availability of both small and large scale versions of **membrane bioreactors.**

4.2.4.2. Entrapment Cultures

In this particular instance, the cells are very much retained within an '**open matrix**' via which the medium flows freely.

Examples :

(1) **Opticell** is the most befitting example wherein the cells are entrapped well within the porous ceramic walls of the unit. In *actual practice*, **opticell units of upto 210 m^2** surface are presently available that may give rise upto 30g MABs per day.

The opticell units can also be connected to **cellulose fibres,** such as : DEAE, TLC, QAE, TEAE. All these fibres are adequately autoclaved (sterilized), and washed subsequently as prescribed. Ultimately these are carefully incorporated into a **stirred/spinner bioreactor** at a concentration of 5g . L^{-1}.

(2) **Porous Microcarriers :** These are rather small beads (having diameter ranging between 170 to 800 μm) made up of gelatin, collagen, glass or cellulose that predominantly possess a network of interconnecting pores.

In fact, these pores afford remarkable advantages, namely :

(i) provide a tremendous enhancement in surface area Vs volume ratio,

(ii) allow adequate and efficient diffusion of medium and product that are absolutely suitable for scaling up, and

(iii) found to be equally beneficial for both monolayer and suspension cultures.

Interestingly, these plus points may be amalgamated and arranged in **different variants of bioreactors** *e.g., fixed bed reactors, fluidized bed reactors,* and *stirred reactors.*

Future Scope : It is, however, earnestly believed that future developments in this direction would probably render the **immobilized cell systems the most prevalent and dominant production systems of the twenty-first century.**

4.3. Design and Bioreactors (Fermentor Variants)

It is, however, pertinent to state here that the various design and types of **bioreactors (fermentors)** presently employed either in small-scale or in large-scale (commercial) utilities and production are essentially of twelve types, namely

(a) Fermcell (laboratory) fermentor,
(b) Loop (recycle) bioreactor,
(c) Activated sludge bioreactor,
(d) Packed bed bioreactor,
(e) Mist **Bioreactor,**
(f) Bubble column bioreactor, and
(g) Bubble-cap fermentor,
(h) Tower bioreactor,
(i) Continuous flow stirred-tank bioreactor,
(j) Trickling film bioreactor,
(k) **Rotating drum** bioreactor
(l) Commercial fermentation plant.

The aforesaid twelve types of **bioreactors** (fermentors) shall now be described in the ensuing text that follows individually :

4.3.1. Fermcell (Laboratory) Fermentor

The **fermcell** or **laboratory fermentor** essentially makes use of the phenomena based on **batch fermentations**. In actual practice, these specific fermentations are practically operated in a continuous mode without emptying the '**fermentor**' at each and every harvest of microbial cells or biosynthetic products. To accomplish this objective, the fresh medium is either added intermittently or continuously to the **fermentor (bioreactor)** so as to replace spent nutrients, and a portion of the broth that invariably comprises of either cells or biosynthetic products, from the '**bioreactor**' is intermittently or continuously withdrawn for recovery of the product.

Nevertheless, '**continuous fermentation**' or **continuous cultivation** predominantly gives rise to near-balanced growth, with almost negligible fluctuation of nutrients, metabolites, and cell mass or biomass. Thus, the ensuing process solely depends upon the fresh medium gaining entrance as a batch system at the particular exponential phase of growth, as shown in Fig. 3.4, having a corresponding **withdrawal of medium** plus cells.

Fig. 3.4. Growth Factor in a Batch Culture of a Microorganism.

The above graphic representation illustrates the six different phases that are encountered in the spirit of '**growth factor**' in a batch culture of a microorganism, such as

1 **(Lag Phase)** : The initial **lag phase** designates a time of no apparent growth, but actual biochemical evolves reveal metabolic intensive density indicative of the fact that the cells are in the process of adaptation to the prevailing environmental conditions, and also suggest that new growth will commence eventually.

2 **(Transient Acceleration Phase)** : In the subsequent **transient acceleration phase,** in fact, the microbes begin to grow.

3 **(Exponential Phase)** : In the **exponential phase** the microbial growth specifically proceeds at the maximum possible attainable rate for that organism by virtue of three vital reasons e.g., (a) absence of growth inhibitors ; (b) excess of nutrients ; and (c) ideal experimental conditions. Nevertheless, particularly in the batch cultivations the exponential growth phase is of very limited duration.

4 **(Deceleration Phase)** : It has been duly observed that when the nutrient parameters start depleting, growth rate decreases first gradually and then drastically thereby paving entry into the **deceleration phase.**

5 **(Stationary Phase)** : The prolongation of the deceleration phase ultimately leads to the **stationary phase** whereby the growth rate virtually comes to a stand still.

6 **(Death Phase)** : The final phase of the growth cycle is termed as the **death phase** when eventually the growth rate has ceased completely.

Caution : A plethora of important biotechnological batch processes are arrested completely before reaching the 'death phase' on account of two vital reasons, namely : (a) cell lysis ; and (b) decreased metabolism.

Fig. 3.5 represents the flow diagram of a self-explained **'continuous laboratory fermentor'** or **'fermcell fermentor'.**

Methodology : In an absolutely **mixed continuous culture system** the sterile medium is made to pass directly into the previously sterilized **'bioreactor'** at a steady flow rate, and the culture broth containing nutrient, waste products and organisms is usually gets released from it at the same rate thereby maintaining the total volume of the culture in the **'bioreactor'** almost constant.

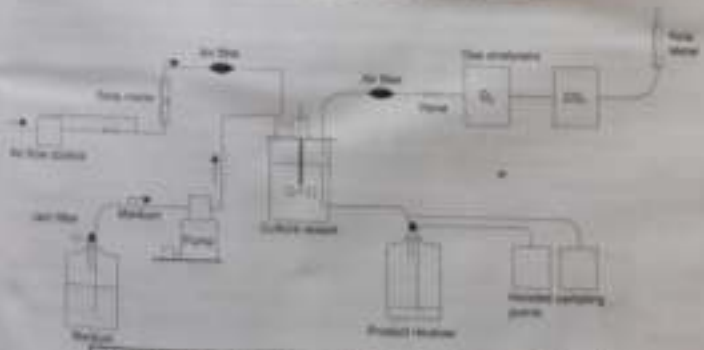


Fig. 3.5. Diagramatic Representation of a Continuous Laboratory Fermentor.

Advantage : The most prominent advantage of it is that various factors, such as : (a) pH of the medium ; and (b) concentration of the nutrients as well as the metabolic products, which invariably undergo some alterations in the course of batch cultivation may be held almost more constant in a continuous cultivation process.

Limitations : In industrial practice, however, the ensuing constantly operated systems are of almost limited application ; and eventually include exclusively such operations as : (a) single-cell protein and ethanol production ; and (b) certain aspects of waste water treatment phenomena.

4.3.2 Bubble-Cap Fermentor

In actual practice, the critical and specific **'gaseous carbon nutrients'** serving as components of the fermentation media invariably pose *special associated problems* with respect to the **'design'** of fermentation equipment.

Examples : Methane and **ethane** (gaseous carbon nutrients) usually represent as two typical and building examples, and to circumvent these types of gaseous carbon, recently the **"Bubble-Cap Fermentor"** has been designed meticulously to allow the proper utilization of such substrates.

Salient Features : The various salient features of a **'bubble-cap fermentor'** are as follows :

(1) It essentially comprises of a tank provided with a series of horizontal plates, as illustrated in Fig. 3.6.

(2) Each plate supports nutrient medium devoid of a carbon source, and subsequently the medium is duly inoculated with the **'required microorganism'**.

(3) Furthermore, each plate is provided with several *short vertical pipes* that are strategically connected to its upper surface, and duly projecting just above the surface of the liquid nutrient medium.

(4) Each *short vertical pipe* has two important provisions, namely : (a) a **hole** in the bottom of this pipe allows legitimate contact with the atmosphere above the medium in the next immediate lower plate ; and (b) the **top** of the lower rim of the inverted cap extends beneath the surface of the nutrient medium.

(5) **Methane and ethane** (i.e., the hydrocarbon gas) are introduced at the bottom of the **bubble-cap fermentor** beneath the above stated plates and eventually rises through the pipes of each plate, thereby getting released from each pipe just below the surface of the liquid medium due to the inverted cap loosely covering the said pipes.

(6) In this manner, the *'quantum of gas'* which fails to get oxidized at a particular plate level of the fermentor usually rises to the next plate to get exposed once again to the prevailing microbial oxidation. However, the **'gas'** rising completely through the *fermentor* may be recycled conveniently to the bottom of the *fermentor* for another passage, and hence used subsequently.

(7) Evidently, an **'alternate procedure'** for carrying out the fermentation of **'gaseous substrates'** is to introduce them along with **'air'** via the **sparger** into a **submerged aerated fermentation device.**

CAUTION. **It is absolutely necessary to adhere for special stringent precautionary measures, otherwise a good proportion of the 'gaseous substrate' (i.e., methane and ethane) shall go as a waste along with the air exhausted from the fermentor simultaneously.**

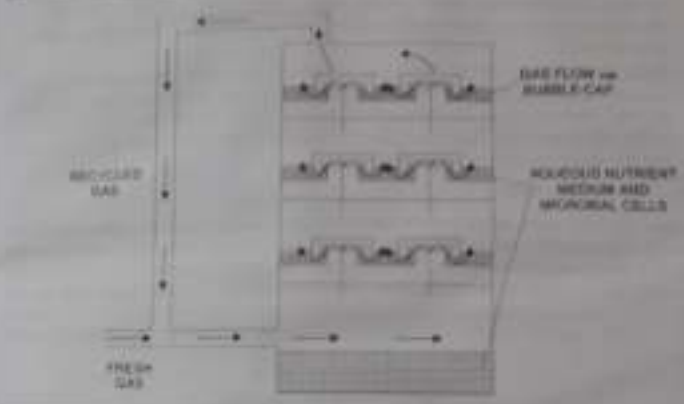


Fig. 3.6 Bubble-Cap Fermentor Illustrating Microbial Attack on Gaseous Substrates e.g., Gaseous Hydrocarbons."
[" Taggart MS Jr. March 19, 1946, US Patent NO : 2, 396, 933]

Fermentations with Liquid Carbon Substrates : In actual manufacturing operational procedures it has been observed that the fermentations employing solely liquid carbon substrates together with water *e.g.*, liquid hydrocarbons which usually float on the surface of the aqueous medium, may be handled conveniently and effectively in one of the following *three methods*, namely :

(a) **Vigorous Impeller Agitation** : In reality, the SS or MS tanks for submerged aerated fermentations are employed quite frequently, accompanied by **vigorous impeller agitation** thereby helping to disperse the *'liquid hydrocarbons'* in the form of small oil droplets throughout the aqueous medium. It is, however, pertinent to state here that an *'emulsifying agent'* may also be incorporated so as augment the phenomenon of **dispersion** adequately.

(b) **Lift (Cyclic) Fermentor** : In this particular process, the required *'liquid hydrocarbon'* substrate is permitted to float upon the surface of the aqueous medium. The latter composing of required/desired microorganism is now withdrawn continuously from the bottom of the fermentor upto a small bore lift pipe strategically located at the side of the fermentor. At this juncture, the column of the liquid medium in this very pipe is raised adequately to two different modes : (a) by introducing sterile air under pressure (*i.e.*, compressed sterile air) ; and (a) by employing mechanical pumps, — right upto the top of the fermentor where it is duly sprayed over the entire surface of the *'liquid hydrocarbon'*.

In fact, the spraying as well as the introduction of the **sterile-air** either into the *lift-pipe* or the *head space* of the fermentor accomplishes the following important aspects, namely : (c) maintains the liquid medium well aerated ; (d) passage of the condensed aqueous spray down the floating liquid hydrocarbon layer renders the *prevailing microorganisms* present in

the sparge zone commence contact with the liquid hydrocarbons ; and (iii) a forceful upward surge tend to break up the **layering effect** at the liquid hydrocarbon/ aqueous phase **'liquid surface'** predominantly.

c.) **Baffle Arrangement :** In this **'fermentor design'**, the immiscible liquid substrates is duly pumped right into a mixture of aqueous culture medium and an immiscible substrate from the main reservoir of the fermentor, which is subsequently forced into a tortuous network of **baffle rearrangement.** Thus, the resulting thoroughly mixed and aerated upflush falls back into the culture reservoir located right below.

4.3.3. Loop (Recycle) Bioreactor

Another vital and major approach to **aerobic bioreactor design** essentially makes use of an *distribution* (having reasonably much lower power consumption) to create both forced and controlled liquid flow in a **loop (recycle) bioreactor.** In this manner, the actual contents of the fermentor are adequately subjected to a **controlled recycle flow**, either involving the *external recycle loop* or very much within the bioreactor. Importantly, the process of stirring has been judiciously replaced by a pumping device, that could be either *pneumatic* or *mechanical*, as may be observed in the instance of **airlift bioreactor** (section 4.2.3.2). Fig. 3.7 depicts a loop bioreactor.

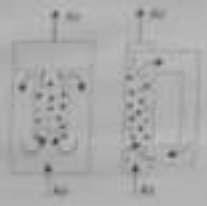


Fig. 3.7. Loop (Recycle) Bioreactor.

4.3.4. Tower Bioreactor

The industrial fermentor *e.g.*, **tower bioreactor** is meticulously designed to provide the best feasible as well as possible growth and biosynthetic conditions meant for industrially vital microbial cultures ; besides, to allow ease of manipulation virtually for almost all operations that are associated with the use of the fermentors. Thus, a tower bioreactor should be strong enough to withstand not only the pressures of large volumes of aqueous medium, but also the material of construction of the fermentor *i.e.*, it should not be either corroded by the fermentation product or even contribute severe toxic ions to the prevailing **growth medium.** Therefore, in an event when the growth of the fermentation microorganism is to take place aerobically, then a subsequent provision should be rendered for rapid introduction of sterile air into the medium in such a fashion that the **oxygen (O_2)** of this air is suitably dissolved in the medium. In short, the oxygen is readily and adequately available to the microorganism, and that the resulting CO_2 obtained from the **'microbial metabolism'** is mostly flushed out from the medium accordingly through a vent provided at the top-end of the **tower bioreactor** as illustrated in Fig. 3.8.

Fig. 3.8. Tower Bioreactor [From : Kristiansen and Chamberlain, 1983*]

The *sample* is introduced into the **tower bioreactor** from the bottom, and the finished fermented product is removed from the top-end outlet as and when required.

4.3.5. Activated Sludge Bioreactor

In actual practice, a huge quantum of **organic waste waters** obtained from either *industrial or domestic* sources, across the globe, are routinely subjected to aerobic and anaerobic systems. In this very context, the **activated sludge bioreactors** are being employed extensively for the specific oxidative treatment of sewage and other liquid wastes. To accomplish such objectives and processes one may utilize effectively either the *batch* or *continuous* **agitated bioreactor systems** to enhance categorically the **'adequate entrainment of air'** to optimize the oxidative breakdown of the organic material. However, these **activated sludge bioreactors** are relatively large in dimensions, and, therefore, to facilitate optimum functioning may have a battery of **'agitator units'** so as to accomplish thorough mixing as well as *oxygen* uptake. They are mostly and abundantly used in **municipal sewage treatment plants.** Fig. 3.9 depicts the diagram of a *typical* **activated sludge bioreactor.**

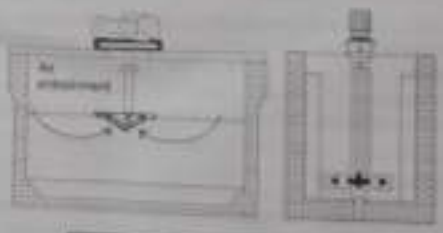


Fig. 3.9. Activated Sludge Bioreactor.

* Kristiansen B. and Chamberlain H. **Fermentor Systems.** In : *The Filamentous Fungi*, Vol. 1, pp. 45-61, Edward Arnold Publishers, London, 1983.

4.3.6. Continuous Flow Stirred Tank Bioreactor

In general, the **continuous flow stirred tank bioreactors** are of large dimensions ; and, therefore, these are obviously less productive and the overall percent conversion of substrate stands at a low level, however, the concentration of the end product is relatively high. Consequently, the high concentration of the product reasonably inhibits the prevailing activity of the 'catalyst' that may drastically reduce productivity. Perhaps this very fact amply explains the ensuing low rate of conversion of the substrate. In actual practice, however, it is absolutely uneconomical for having a conversion factor of more than 90%.

Ingenuously, the *continuous flow bioreactors* are basically of *two* different types, namely : (a) **continuous flow stirred tank bioreactor** having adequate provision of thorough mixing ; and (b) **plug flow bioreactor** having no mixing arrangement. Nevertheless, the various experimental parameters very much within a **continuous flow stirred tank bioreactor** predominantly remain identical to those prevailing in batch.

Fig. 3.10 depicts evidently *two* variants of the **continuous flow stirred tank bioreactors,** such as : (a) provided with a settling tank ; and (b) provided with an ultrafiltration device, as illustrated under :

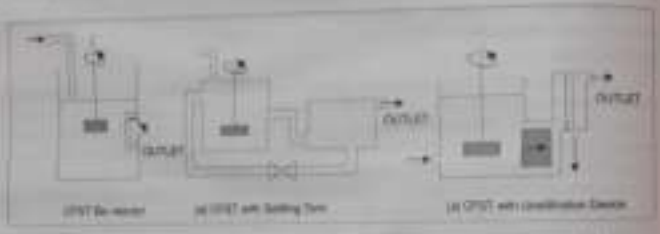


Fig. 3.10. Continuous Flow Stirred Tank (CFST) Bioreactor (a) With Settling Tank (b) With Ultrafiltration Device

The catalyst is duly suspended homogeneously in a big SS tank via which the substrate flows and very much retained within the bioreactor by means of subsequent ultrafiltration followed by filtration or alternatively being attached to the paddles of the stirrer. The reactants present in the bioreactor are mixed thoroughly. Various physical conditions, namely : pH, temperature, replacement of the '*used up catalyst*', — are maintained efficiently. Efforts are also made to hold the **'diffusional limitations'** to a bare minimum level.

4.3.7. Packed Bed Bioreactor

It is indeed worthwhile to state at this juncture that the **packed bed bioreactors** are available in several advantageous designs. Generally, they are of rather small in size and dimension, and on the contrary possess remarkably **high productive output.** This specific **bioreactor** has certain glaring **drawbacks,** namely :

- high viscous substrates do tend to block these bioreactors
- **'diffusional limitations'** may be caused due to poor mixing of the substrate with enzyme.
- compressible nature of the **'catalyst'** may prevent the flow through the packed bed bioreactor.
- flow direction of the **'substrate solution'** in *packed bed bioreactor* must be taken into consideration strictly.

Fig. 3.11. depicts the simple **packed bed bioreactor** wherein the flow of the **'substrate solution'** is indicated upwards.

Fig. 3.11. Packed Bed Bioreactor

Evidently, the downward flow of the ensuing 'substrate solution' invariably gives rise to the compression of the bed of enzyme column. Hence, it is absolutely important and necessary that the said flow of 'substrate solution' is preferably maintained in the upward direction particularly when 'gas' is generated during the enzyme reaction. The **enzymes** may be advantageously incorporated into the **PB-bioreactor** in *two* different forms, for instance : (a) **immobilized enzyme fibre skins ;** and (b) **spirally rolled-up sheets of immobilized enzymes.**

Advantages of these enzymes are as stated below :

- ❖ Ease of handling procedures,
- ❖ Possess 'automatic control' and operational modes',
- ❖ Extremely cost-effective, and
- ❖ Quality control of end-products very easy and convenient

4.3.8. Trickling Film Bioreactor

In the **trickling film bioreactor** the 'culture medium' trickles upon the glass beads. In actual operational mode the culture media is made to recirculate from a reservoir and sprayed carefully on the fixed bed of roots ; and ultimately allowed to follow a downward flow via the root bed. Subsequently, the roots are inoculated on *top of the glass beads* where they usually multiply either on the bed-surface or down the glass beads. It is, therefore, quite necessary to maintain the *actual length of the pipe* between the **air-inlet** (PTFE-filter) and the **air-lift** specifically to the shortest possible dimension so as to reduce the oscillation of the prevailing culture medium very much within the airlift tube. In actual practice, it is vital and important to make a provision of a *screw-clamp arrangement* strategically

positioned in the pipe returning the culture medium to the bottom of the air-lift pipe that would serve essentially as a **throttle-valve** to substantially dampen the oscillations that otherwise may sometimes give rise to the **'reversal of air flow up'** via the base (bottom) of the **trickling film bioreactor** as illustrated explicitly in Fig. 3.12 given below.

Fig. 3.12. Trickling Film Bioreactor

The **trickling film bioreactor configuration** remarkably holds a reasonably high promise with regard to the scale-up operations because the prevailing flow patterns are overwhelmingly under the influence of 'gravity' that predominantly acts more or less uniformly over the bed very much in contrast to the existing **localized power input** due to mechanical agitation. However, the harvesting of the ensuing mats is relatively difficult on account of their intimate adherence to the glass beads.

4.3.9. Mist Bioreactor

In the **mist bioreactor** the culture medium is strategically pumped via the provided **'mist head'** by adequately making use of a *peristaltic pump* fixed at the bottom of the reactor along with a *pump timer*. The base of the bioreactor is inserted with a **glass-wool fibre** to enable the filtration of cells and the other from the root cap or other debris, that might help in clogging the jet-hole (jet) located at the 'mist head'. In fact this kind of **bioreactor** has the added advantages, such as : (a) culture medium may be drained off as and when required ; (b) wet weight of the **'synthetic'** can be determined directly ; and (c) without disturbing the sanity of the sterile cultures one may estimate the wet-weight of the end-product at frequent intervals to know the completion of the on-going fermentative process in the **bioreactor**.

* (PTFE = Polytetrafluoroethylene (Teflon)).

Fig. 3.13 depicts the diagrammatic representation of a **mist bioreactor** showing its various essential parts.

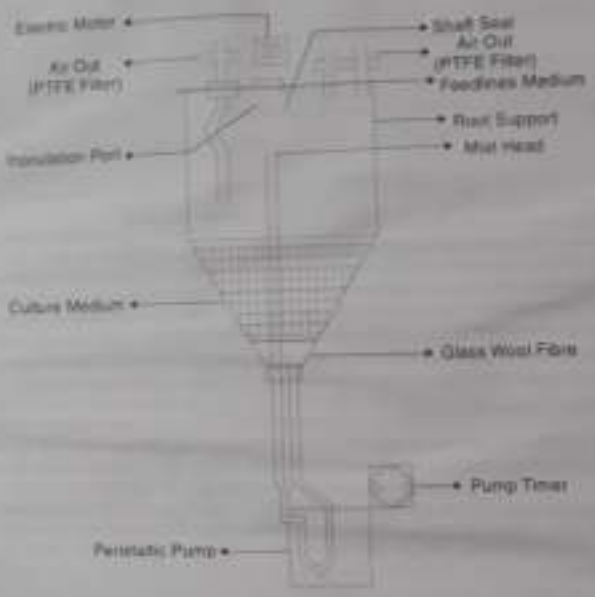


Fig. 3.13. Diagram of Mist Bioreactor.

4.3.10. Rotating Drum Bioreactor

The underlying principle and configuration of a **rotating drum bioreactor** are very much identical to the corresponding **'fill and drain reactor'**, Interestingly, in this particular configuration, the *'filling process'* usually occurs when the tissue specifically remains just below the surface of the 'culture media', whereas, the corresponding *'draining process'* normally takes place when the tissue rotates in the upward direction (*i.e.*, out of the media. It has been duly observed that this configuration substantially lessens the various problems directly or indirectly associated with the scheduled timing of the respective **'fill and drain'** sequence, but may also exhibit certain obvious limitations pertaining to the scale.

Fig. 3.14 illustrates **rotating drum bioreactor** *in a simple and explicit manner.*

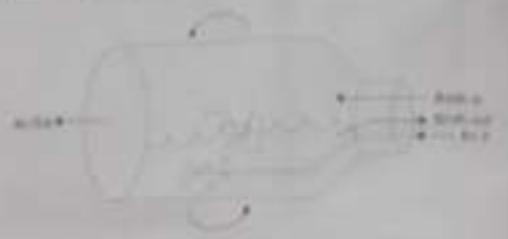


Fig. 3.14. Diagram of a Rotating Drum Bioreactor.

It essentially comprises of a horizontal rotating drum strategically fitted on rollers. It is however pertinent to note here that the rotating motion of the drum markedly facilitates the **sparger** *as well as pressure mixing of gas and liquid phases in the bioreactors, whereby accelerating the promotion of efficient oxygen transfer to the respective cells specifically at high values of densities.* **Impressively, the rotating drum bioreactor** *predominantly gives rise to* **diffusive lower hydrodynamic stress.**

Disadvantage : *The cardinal disadvantage of the rotating drum bioreactor is its critical dependence upon its comparatively high energy consumption in commercial scale operations.*

4.3.11. Bubble Column Bioreactor

The **bubble column bioreactor** *represents an unique development in the field of air-sparged stirred bioreactors that have traditionally proved to be highly successful ; and hence being used quickly across the global periphery. Nevertheless, a major negligence of the* **latest bioreactor designs** *are not only very sophisticated (i.e., requires skilled personnels), but also quite complicated (i.e., needs to be operated by only fully trained persons) ; whereas, the* **bubble column bioreactors** *are a lot simple to construct and operate efficiently. They are gaining an immense popularity in usage and adaptation equally in* **biochemical** *and* **chemical industries.** *Just contrary to much of other bioreactors that essentially use many-fold mechanically agitated devices the* **bubble column bioreactor** *comprises of a cylindrical large vessel, preferably of SS, wherein the compressed sterile air or adequately sparged right into the base hole of the liquid culture medium. Obviously, it does not possess any moving parts because relatively high and efficient degree of mixing could be accomplished with the duly sparged gas jet. Besides, these* **bioreactors** *the requisite quantum of energy needed for thorough agitation, and also the much required O₂ replenishment exclusively for the culture medium is duly provided by the sparged air itself.*

Advantages : *A number of cardinal and universally advantages of a* **bubble column bioreactor** *are as stated below :*

(1) *It is basically a highly simple arrangement for producing biochemical products e.g.,* **antibiotics.**

(2) *It does not require energy for driving any mechanically operative devices whatsoever.*

(3) *Complete dismantling of sealing arrangement of the usual stirred shaft assembly (as also* **"bioreactors"** *) is allowed.*

(4) *Absence of any shaft in the head space of the fermentation vessel invariably provides enough room for various entry ports essentially required in such* **bioreactors** *that are mostly having rather small sizes and dimensions.*

(5) *Complete freedom from any sort of 'mechanical arrangements' does help tremendously to maintain the high degree of sterility over extended periods (5-6 days).*

(6) *Absence of shafts also eliminates risk genes, expensive, highly vulnerable and pressure sensitivity factors to a great extent.*

Limitations : *The various limitations of the* **bubble column bioreactor** *are enumerated as under :*

(1) *It is less suited specifically for such processes that essentially make use of highly viscous liquids.*

(2) *It has been observed that the coalescent environment in a bubble column bioreactor results in more aggressive bioorganism sticking whereby the 'bubbles' emerging at the sparger head invariably coalesced outwardly to give rise to relatively large bubbles usually termed as* **'slugs'.** *These slugs eventually rise quickly all along the* **'axis of the column'** *thereby setting the whole body of the liquid into circulation with distinct movement in the* **upward direction** *in the vicinity of the axis of the cylindrical vessel, and movement in the* **downward direction** *near the walls of the same vessel.*

Sparger Specifications : *The* **'sparger'** *actually determines the initial bubble size, shape and dimension in a given liquid. Interestingly, a sparger having small-sized diameter holes e.g., sintered glass plate, perforated plate, perforates appreciably* **'much smaller bubbles'** *in comparison to a* **'single-orifice sparger'** *; and, therefore, provide a distinct, advantageous, and energetic* **higher interfacial gas-liquid contact surface area** *in the close approximity of the 'sparger'.*

Choice of Cultures : *Importantly, a host of large scale fermentative bioprocesses make utilisation of* **shear-sensitive cultures.** *Besides the* **hairy-root cultures,** *which are also shear-sensitive, have been grown quite successfully and gainfully in the* **bubble column bioreactors.** *Ample evidences based on several experimental procedures duly revealed that the usual growth of* **plant-cell cultures** *in various* **bioreactors,** *namely :* **bubble column bioreactors, stirred-tank bioreactors,** *and* **shake flasks** *was shown identical.*

Finally, the **bubble column bioreactor** *depending on its various excellent characteristic features, such as :* **O_2-transfer at low shear, low hydrodynamic stress,** *and* **low operative cost** *render it an exceptional attractive choice for a plethora of articulated scale-up investigative studies in* **biotechnology.**

4.3.12. Commercial Fermentation Plant

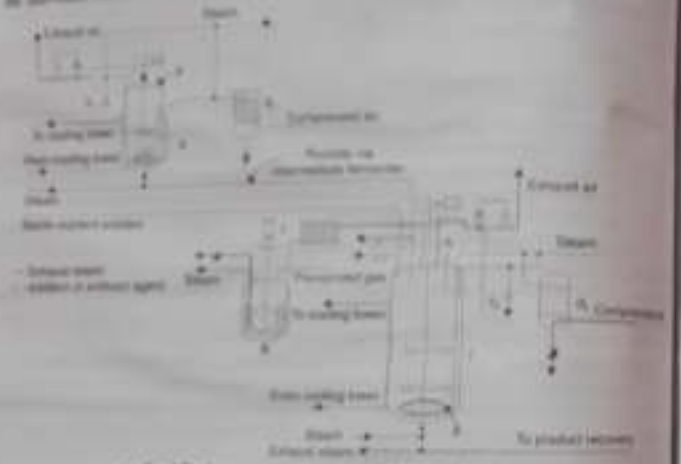
Commercial fermentation plants (or industrial bioreactors) are usually designed in such a manner so as to provide the following three cardinal objectives, namely :

(a) best possible growth,

(b) biosynthesis parameters for industrially important microbial cultures, and

(c) permit ease of manipulations associated with various operations of the bioreactors.

In reality, these **'fermentation vessels'** *(i.e.,* **bioreactors***) should be strong enough to withstand the rousing pressures of huge volumes of aqueous medium. As most industrial fermentations invariably make use of relatively* **'pure cultures',** *the bioreactors should provide adequate provision and means for the control of or prevention of the growth of possible contaminating microorganisms. It is quite obvious at this position to take cognizance of the fact as the instance whereby growth of the microorganism*



c = Seed Tank ; s = Substrate Container ;
b = Inoculation Port ; f = Sterile Filter ;
s_1 and s_2 = Sampling devices ; g = Reaction Tank ;
d_1 and d_2 = Filters ; k = Electrode for Foam control ;
r = Cooling coils ;

Fig. 3.10. Flow Sheet Depicting Layout of a Commercial Fermentation Plant. [Blake and Kastich, *Argew Chem Internat. Edit.*, 5, 653-662, 1966]

Highlights of Commercial Fermentation Plant : These are as follows :

(1) Must have an adequate provision for the intermittent introduction of *antifoaming agent* as and when required by the actual foaming status of the medium.

* **Antifoam Agents** : Crude organic materials viz., **animals and vegetable oils**, such as : lard oil, [illegible] oils of ; **long-chain alcohols**, for instance : octadecanol ; **mixture of oils and alcohols** *e.g.*, lard oil + octadecanol (used for penicillin fermentations) ; **inert surfactant agents**, such as : silicone compounds (very expensive for use in commercial scale).

(3) Maintenance of a constant predetermined temperature in the fermentor for optimum growth of the microorganism.

(3) Adequate provision and means for the necessary withdrawal of culture samples in the course of fermentation process ; besides, the introduction of inoculum at the initiation of the fermentation process.

(4) A suitable supply device for withdrawing samples from the on going fermentation process in the fermentor to ascertain pH values of the prevailing culture medium ; and also for appropriate adjustment of these values by the addition of a calculated amount of alkali/acid to the fermentation medium.

(5) Provision of seed tanks or additional inoculum wherein inoculum is produced and introduced directly to the fermentor without making use of extensive pipe lines, that may usually not only magnify but also give rise to **serious contamination problems.**

5. MUTANTS

Mutants may be defined as — **'variations of genetic structures that eventually breed true'.**

Nevertheless, the actual usage of metabolically blocked mutants of certain microorganisms is relatively quite recent, but do possess an immense importance and recognition. In reality the extensive and intensive study of the **'genetic blocks'** has vitally unravelled largely and specifically the present-day knowledge of microbial genetics. Besides, it has more or less paved the ways and means for directing microorganisms to accumulate comparatively huge quantities of metabolic intermediates which usually by virtue of their 'transient existence' in the metabolic pathways, otherwise fail to get accumulated to any extent in cultures precisely. Interestingly, the actual mechanisms that are intimately associated with a few specific metabolic blocks are being deciphered aggressively and progressively nowadays across the globe.

It has been adequately observed that the mutating mutation of the fermentation organism poses a serious problem in a situation when the resulting **mutants** exert a selective growth advantage in the course of a prolonged incubation ; and simultaneously give rise to an appreciably lesser amount of the desired fermentation product. Therefore, in order to circumvent the phenomenon of 'mutation' it is always preferred to make use of **multistage-continuous fermentation procedures,** whereby the first bioreactor (bioreactor) in the prevailing sequence being remembered periodically. In short, the real overall solution to curtail mutation is to minimise their incidences of occurrence whereby the offending cells may be flushed from the bioreactors before they get an opportunity to multiply once again.

5.1. Isolation of Mutants

In true sense, one may have to look into the means and ways whereby **'mutants'** are actually formed *i.e.*, the phenomenon of **mutagenesis.** In its simplest way one may explain mutagenesis as an essential process in the course of genetic engineering experiments that specifically lead to regulate a region of the gene of interest in order to be able to manipulate it in a highly desired manner. Therefore, to accomplish such manipulation(s), it is absolutely vital as well as necessary that the **'desired gene'** must be fully isolated and subsequently characterised meticulously. One must also have a clear picture with regard to its various important informations, such as : *restriction map* ; *sequence of the entire gene* ; and *sequence of the 'target region'*.

5.1.1. Method of Causing a Mutation

Evidently, the most common methodology usually adopted for effectively causing a mutation is ultimately expose the *culture of the organism* to a particular **mutagen.** For this, nitrous acid (HNO$_2$) is considered to be one of the most widely employed mutagenic (chemicals). The cultures of the bacteria are being exposed gradually to the respective mutagen, the former may be allowed to grow and multiply under several experimental parameters ; and, thus the resulting mutants having **desired phenotypic characteristic features** are isolated. Consequently, the genotype of these organisms is subjected to elaborate characterization so that the *'specific gene'* actually responsible for the altered characteristic features in mutants may be scored without any ambiguity. Nevertheless, such mutations are known to occur random and the resulting **mutants** are invariably recognized by the phenotypic changes in their characteristic features.

5.1.2. Somaclonal Variation

It may be defined as — *the genetic variability present amongst the cultured cells, plants derived from such cells, and progeny of such plants*.

In general, the terminology is broadly employed for describing genetic variability invariably present among all types of cells/plants derived from cells that are cultured *in vitro*. It has been observed that *the plants generated from tissue or cell cultures exhibit predominant heritable changes* associated with both *qualitative* as well as *quantitative* characteristic features.

Examples : A few typical examples wherein somaclonal variation has been described have already been mentioned earlier etc. There are, in fact, two types of variants, namely :

(a) **R_0 Generation :** i.e., those which are obtained in specific hormonal** condition at the *plants usually regenerated from cells cultured in vitro*; and

(b) **R_1 Generation :** i.e., those that are *recovered in the selfed progeny of tissue culture regenerated plants*.

5.1.2.1. Isolation of Somaclonal Variants

It has been well established that **mutants** for several characteristic features may be far more conveniently and easily isolated from respective cell cultures rather than from whole plant populations. Perhaps the above mentioned analogy holds good because a plethora of cells, approximately as 10^6 to 10^8, may be screened for **'mutant traits'** both effectively and conveniently.

However, in actual practice screening of so many plants would prove to be real herculean task and may turn out to be virtually impossible. Importantly, **mutants** could be selected effectively to serve a host of definitive aims and objectives, namely : improvement in nutritional quality and aspects of stress resistance ; adaptation of specific plants to several well-defined stress parameters *e.g.* low temperature, soil conditions (salinity), toxic metals (e.g., Al) ; resistance to various herbicides (chemicals) and to enhance particularly the biosynthesis of plant products usually employed for industrial or medicinal purposes.

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* The expression of the given genes in an individual.

** Produced by specific alleles (i.e., different genes containing specific inheritable characteristics that would corresponding positions (loci) on paired chromosomes).

In short, the major acceptable approaches put forward towards the isolation of **somaclonal variants** may be broadly categorized into *two* heads, namely : (i) screening ; and (ii) cell selection, which shall now be dealt with individually in the sections that follows.

5.1.2.1.1. Screening

It is solely based upon the *observation of a substantial quantum of cells or regenerated plants* for the ultimate detection of certain individuals. Perhaps this particular approach is considered to be the only plausible and feasible technique employed for the isolation of **mutants** not only confined to yield exclusively but also the corresponding ensuing yield traits. Generally, as a common practice the *specific R_1 progeny* (i.e., the progeny of regenerated R_0 plants) are invariably scored for the identification of variant plants, and their corresponding R_2 progeny lines are mostly evaluated for confirmation.

Advantages : Screening has been exploited both profitably and extensively for the categorical isolation of **'cell clones'** which evidently give rise to certain higher amounts of some biochemicals ; besides, computer aided automated cell sorting devices (CAACSDs) have also been introduced overwhelmingly to aid the screening of upto **1000-2000 cells per second** from the assorted cell-pool the desirable variant cells *were segregated via* automatic means.

5.1.2.1.2. Cell Selection

The wonderful **cell selection** *modus operandi* essentially makes use of an appropriate applied **'selection pressure'** that allows the preferential survival or growth of the ensuing variant cells solely.

Examples : A few typical examples of the **cell-selection** are, namely : high salt concentration, presence of herbicides (chemicals), adequate selection of cells resistant to different toxins, and the like.

However, **cell-selection** may be of *two* types, such as :

(a) **Positive Selection.** In this particular instance the selection pressure largely permits only the *mutant cells* to either **survive** or **divide,** and

(b) **Negative Selection.** In this specific case the wild-type of cells usually undergo division ; and, therefore, get killed by a **counter selection agent,** such as : arsenate or 5-BUdR*. Obviously, the *mutant cells* fail to undergo division and consequently they escape the possibility of any interaction with the **counter selection agent.** Naturally, the prevailing cells may be rescued by timely removal of the **counter selection agent.** However, in actual practice the **negative-selection** *modus operandi* is employed exclusively for the **isolation** of **auxotrophic** mutants.**

Nevertheless, the **positive selection** methodology may be further categorized into *four* groups, namely :

(i) Direct selection ; (iii) Stepwise selection ; and

(ii) Rescue method ; (iv) Double selection.

The four groups of positive selection shall now be discussed individually in the sections that follows :

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* 5-Bromodeoxyuridine ;

** Requiring a growth factor that is different from that required by the parent organism.

(c) **Direct Selection** : In this case, the cells that are resistant to the prevailing selection pressure survive and divide to form **colonies**, while the wild type of cells are eventually killed by the selective agent. It is, however, pertinent to state here that it is one of the most adaptable common selection methods , and, therefore, is being employed for the **isolation** of cells that are specifically resistant to herbicides (chemicals), toxins (caused by **pathogens***), the normal salt concentrations, amino acid analogues, antibiotics, and the like.

(d) **Rescue Method** : In this particular instance, the *wild type* cells are virtually **killed** by the corresponding selective agent , whereas, the *variant cells* do remain very much **alive**, but fail to undergo division by virtue of the ensuing unfavourable environment. Subsequently, attempt is made to remove the *selective agent* specifically so as to recover the prevailing **variant** cells. The **rescue method** has been employed frequently to recover the **low temperature** as well as **aluminium resistant variant cells.**

(e) **Stepwise Selection** : In this specific instance, the ensuing **selection pressure** viz., salt concentration, may be enhanced slowly from a relatively *low level* to the *Optimum*** *level* and thus, the *resistant clones* **isolated** at each and *every progressive state* are appropriately subjected to the higher selection pressure. In actual practice, **stepwise selection** approach has *invariably* **favour gene amplification** (an *unstable phenomenon*) or subsequent **mutation** in the *expanded DNA*.

(f) **Double Selection** : In **double selection** approach, it may be absolutely feasible to select cells for usual survival and/or growth on one hand, and affording resistance to the selective pressure on the other.

Example : **Streptomycin** (*mutagenesis*) : It illustrates *double selection* explicitly *i.e.* the selection was based on **cell survival** as well as **colony formation** (*first aspect*) ; and specific **development of green colouration** in the *aforesaid colonies* (*second aspect*) — only green colonies were selected. Interestingly, the double selection approach has been judiciously applied for the selection of *cells* that are found to be resistant to such substances as : amino

→ herbicide , tobacco mosaic virus (TMV) ; and aluminium (Al)

8.1.2.1.3. Variant Traits

It has been observed that the somaclonal variants *isolated via* cell selection approach are invariably **unstable** ; whereas, the perceived frequency of **stable** variants may vary between 0-62%, most probably depending on the species and the selective agent. Besides, there are several **selected clones** that do not exhibit their due resistance in the course of further selection or screening. Evidently, these emerged clones are fairly susceptible, and were wrongly classified as resistant ; and, therefore, rightly termed as **escapes.** Nevertheless, there exists a plethora of *clones* which eventually lose their resistance to the prevailing selective agent after a certain span of growth in the absence of the ensuing selective

* Disease producing microorganisms.

** Destructive to cells.

pressure. Thus, the clones obtained in this manner are commonly known as **unstable variants.** However, the *unstable variants* may be duly accomplished from valid and legitimate alterations achieved from **gene expression*** and from **gene amplification****.

8.1.2.1.4. Molecular Foundation of Somaclonal Variation

The **somaclonal variation** may come into being by virtue of any of the following events that occur at **molecular level exclusively,** such as : gene mutation ; plastogene mutation ; gene amplification ; changes in gene expression ; meiotic crossing over ; alterations in chromosome number and/or structure ; rearrangements in cytoplasmic genes ; and transposable element activation.

It has been observed that a majority of mutants isolated from cell cultures may essentially engage **single-gene mutations,** wherein the *mutant allele* could be either recessive or in dominant form.

Salient Features : A few salient features with respect to the molecular foundation of somaclonal variation are as follows :

(1) Gene amplification has been duly observed in certain variants which are normally recovered *via* stepwise selection of plant cell *in vitro*.

(2) Deamplification may often take place in somaclonal variants, *e.g.*, for rRNA genes.

(3) Transposable elements may be activated during *in vitro* culture.

(4) Cleavage and fusion of chromosomes that take place during culture may aid in augmenting the Ac activity and/or other controlling factors.

8.1.2.1.5. Somaclonal Variations and Induced Mutations

In certain specific instances, *mutagenesis* was observed to be absolutely vital and necessary for the adequate recovery of the specific variant that are being **isolated.** Nevertheless, the phenomenon of *mutagenesis* must be kept at an arm's length in view of the undesirable features intimately associated with such treatments.

It is pertinent to state here that the somaclonal variations are most preferable for the induction of mutations based on a plethora of widely acceptable valid reasons as enumerated below :

(1) **Chimaerism** is a predominant and serious problem in the *induced mutations*, but not so in *somaclonal variations*.

(2) **Induced mutations** are frequently linked with undesirable characteristic features *e.g.*, sterility.

(3) **Both** '*newer breed of mutations*' and '*newer alleles*' have been legitimately **isolated** *via* somaclonal mutations.

(4) Degree of **frequency-useful mutations** is found to be at a reasonably high level in somaclonal variations.

(5) Applicability of **highly specific and effective selection** may be accomplished *in vitro* for a large number of economically viable and important characteristic features which is virtually impossible in the particular instance of **mutation breeding.**

* **Gene Expression** : An overall process by which the information encoded by DNA in a gene is converted into an observable **phenotype** (most probably the production of a protein).

** **Gene Amplification** : An increase in the number of copies of a gene per genome of the organism in comparison to that naturally present.

(b) Astronomically huge number of individuals may be screened quite effectively in vitro.

Special note : Precisely the **conclusion** variation is exclusively applicable to flower species whereby the whole plants may be reproduced from the cultured cells ; whereas **meiotic** breeding may be applicable to all species in general. Furthermore, the **somaclonal** variation is solely dependent upon highly modernized and sophisticated facilities for the tissue culture as well as the procedures.

8.2. Factor influencing Rate of Mutation

After having grasped sufficient understanding of the various aspects of **mutation**, it is now necessary and important to know the various factors that exert their specific influences upon the different type of mutation. A few such aspects are as stated under, namely :

(a) Conditional mutation,

(b) Radiation induced mutation,

(c) Effect of UV radiation,

(d) Chemically induced mutation, and

(e) Beneficial mutation.

These aforesaid aspects shall now be treated individually in the sections that follows :

8.2.1. Conditional Mutation

It has been observed that sometimes the **mutation** is strategically taking place in such a 'genetic locus' that under one particular experimental parameters the organism tends to grow normally, whereas under an altogether different experimental parameters, either the expected growth is far from being normal or the organism fails to grow at all. Thus, such not so usually mutations are usually termed as be conditional mutations. In actual practice, however, the prevailing conditions that invariably permit to 'normal growth' are called the '**permissive conditions**' ; and the other conditions are collectively referred to as either the '**non-permissive conditions**' or the '**restrictive conditions**'.

Now if under the influence of **restrictive conditions** the organism is totally unable to grow in **nature** is known as a **conditional lethal mutation.**

Auxotrophic Mutation : In this case, the *growth media* and the *metabolic conditions* are responsible for the ensuing **expression of mutation.**

Examples : A few specific mutants have the capability to grow very conveniently in the presence of '**glucose**' but a possible replacement of glucose with any other sugar entity would virtually arrest the growth to a complete stand still (i.e., stop).

Mutants may be either *temperature sensitive* (hot or cold) or *suppressor** mutants. In the latter instance the organism is found to be viable in the presence of a *suppressor*, whereas the mutation becomes lethal in the absence of a *suppressor*.

8.2.2. Radiation Induced Mutations

A plethora of '**electromagnetic radiations**', particularly the electromagnetic waves having UV ray or even smaller wave lengths that give rise to the phenomenon of '**ionization**'.

* **Suppressor :** It contains a **gene** which either complements or corrects the defect in the **mutant** (both wild type or dominant).

Examples : The various typical examples are : *X-rays, γ-rays, and cosmic rays.*

Muller (1927) observed the ultimate effect of these rays and concluded that an excessive exposure to X-rays enhanced the incidence of sex-linked **recessive-lethal mutation** in Drosophila particularly. It was further ascertained that there exists a direct relationship between the '**radiation dose lgred**' and the '**incidence of mutation**' articularly.

Examples : The following are some concrete evidences :

Exposure (r)	Occurrence of Mutation (%)
500	15
1000	5

Besides, there are some other vital factors that invariably govern the **incidence of mutation,** for example :

8.2.2.1. Duration of Exposure to Radiation

It has been duly observed that certain mutations may even occur at very low exposure dose but for a relatively longer duration or at high exposure dose but for much shorter span. Therefore, one may infer that there is '**no safe-level of radiation**', and even a very small dose could be usually practically for causing **mutation.** It has been found that the probability of mutation in experimental rats are comparatively much less if a '**chronic radiation**' with a low dose is administered than if the '**same dose of radiation**' is given in one go.

8.2.2.2. Environmental Conditions

In reality, the affect of **environmental conditions** exert a positive effect on **ionization.**

Example : Lower the pressure of O_2 tension given rise to lower incidence of mutation. Likewise, the presence of **higher O_2-tension** at the time of irradiation affords **higher incidence of mutation,** even if the animal is subjected to lower O_2-tension at a later stage. All kinds of mutations are adequately accumulated by ionic radiation. Incidences of *chromosome* abberration of all kinds are duly observed on ionic radiation, such as : duplication, deletion, inversion, and *transversion.*

8.2.2.3. State of Cellular Metabolism

Both the *state of cellular metabolism* and the *phase of cell-cycle* do play a cognizable major role on the remarkable effect of **ionizing radiations.**

Example : In response to the given irradiation to the plant *Trillium*, the observed mutations were **60 times** more prevalent specifically in the **metaphase** in comparison to the **interphase** of the cell-cycle.

8.2.3. Effect of UV Radiation

It has been well established that UV-radiation serves as a '**weak mutagen**'. Besides, the usual normal strengths of UV-radiation in the sun light are not strong enough to initiate and produce mutation. Interestingly, any extent of damage caused to DNA is repaired instantly by the cell. Nevertheless, the exposed UV radiation gets adequately absorbed by both the **purine** and the **pyrimidine bases** respectively ; and, thus, are converted into their corresponding **excitable state** that eventually render them more reactive ultimately.

Importantly, the UV range of either 254 nm or wide known to be highly damaging for **mutation**. *In general, the prevailing relationship between* **the degree of UV radiation** and the **rate of mutation** *is predominantly variable in nature.*

Mechanism of Action : Though the '**mutagenic**' effect of UV radiation has been known for quite sometime, but its exact mechanism whereby it causes **mutations** has been understood only recently. In fact, the irradiation of DNA with UV rays usually gives rise to the actual formation of covalent bonds between **thymine molecules** on the same strand of DNA yielding thereby the **thymine-thymine dimers** as shown in Fig. 3.18.

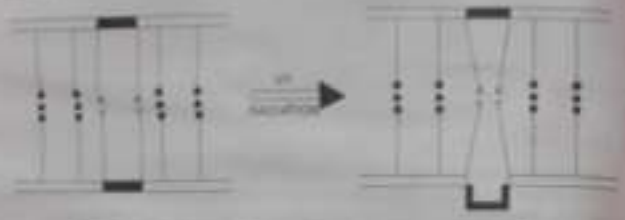


Fig. 3.18. Formation of Thymine-Thymine Dimer by UV Radiation.

It has been observed that *several microorganisms have enzymes which can effect this damage to the DNA* (i.e., **dark repair**). In certain instances the '**repair phenomenon**' *is not done correctly and this may give rise to* **mutations**. Interestingly, as opposed to **dark repair**, the covalent bonds joining the **thymine dimers** *may also be eliminated by the help of light of longer wavelengths, which process is usually termed as* **photo-reactivation**. In our daily, most UV **mutations** are more or less *nuisance type of mutations* and, therefore, are the ultimate result of a change in one or few bases in the structure of DNA.

Besides, *X-rays and γ-rays* are nothing but '**ionizing radiations**' and may cause damage to the prevailing DNA, but no dimer formation takes place at all. The overall net damage frequently caused by these radiations and improper follow up repairs may categorically lead to either *addition or deletion of* bases present in the DNA. This finally gives rise to a change in the *reading frame* (i.e., **frame-shift mutations**).

3.2.4. Chemically Induced Mutations

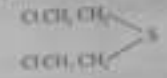
There are a host of pure '**chemical substances**' that are mutagenic in nature. A few such chemicals are used frequently by humans while the others are not so common at all. On a broader perspective the '**chemical substances**' may be classified into two categories depending upon their inherent mode of action, such as

(a) **Chemicals affording mutation to replicating and non-replicating DNA** : A few typical examples of this class are : nitrous acid (HNO_2) and alkylating/hydroxylating agents which would be discussed briefly depending upon their precise '*mechanism of action*' as stated under :

(1) **Nitrous Acid (HNO_2)** : It essentially exerts its mode of action via the oxidative deamination of bases in DNA. Consequently, 'A' gets converted to the corresponding hypoxanthine that may base pair with 'C' during the process of 'replication' and, hence, finally convert an '**A-T pair**' into a '**G-C pair**'. Likewise, 'C' is converted to 'U' that may subsequently base pair with 'A', thereby converting a '**C-G pair**' to an '**A-T pair**'. Then, 'G', on the other hand gets converted to **Xanthine** finally, that may also base pair with 'C'. Therefore, this very change fails to cause a '**mutation**'. In short, because the net effect of HNO_2 is to afford a plausible conversion of an '**A-T pair**' to a '**G-C pair**' and eventually a '**G-C pair**' to an '**A-T pair**', it may be gainfully employed to revert a **mutant** back to the corresponding *wild-type*.

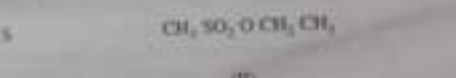
(2) **Alkylating/Hydroxylating Agents** : It has been observed that both the alkylating and the hydroxylating agents help in the transference of a methyl ($-CH_3$) or ethyl ($-C_2H_5$) functional moiety to the corresponding bases.

Examples : Mustard gas (I) and ethyl methane sulphonate (II) usually affect the '*alkylation*' at the N-7 and C-6 positions respectively.



(I)

[Di (2-chloroethyl) Sulphide]



(II)

[Ethyl Methane Sulphonate]

Consequently, these '**modified bases**' usually form a base pair with a *wrong* base.

Examples :

(i) **N-Methyl-N'-nitro-N-nitroso guanidine (NTG)** — is a highly potent alkylating agent and affords a good number of *multiple* as well as related mutations in DNA.

(ii) **Ethylene Sulphonate (EES)** — is another alkylating agent. These two '*chemicals*' help in 7 ethyl 'G' base pair with 'T', thereby converting a '**G-C pair**' to an '**A-T pair**'.

(iii) **Hydroxylamine (NH_2OH)** — is a hydroxylating agent that specifically converts a '**G-C base pair**' to an '**A-T pair**'.

In general, these chemicals, besides effectively a base change may also bring about the following modifications in **mutants** :

- Cross-linking in mutants
- Occasional chromosomal breakage and aberrations
- Activate the repairing mechanism of the cell.

(b) **Chemicals affording mutagenic activity to replicating DNA** : A few befitting examples of this category are acridine dyes and base analogs that would be briefly described along with their respective mechanism of actions as given under :

(1) **Acridines** : It has been observed that the acridine dyes invariably give rise to the '**frame-shift mutations**'. In other words, these dyes *e.g.*, acridine orange, proflavin, ICR 170 and 100 usually intercalate particularly between the stacked base pairs in the DNA and *eventually* are strategically sandwitched between two predominant bases. Consequently, *DNA possesses an enhanced rigidity* and its conformation gets altered. In fact,

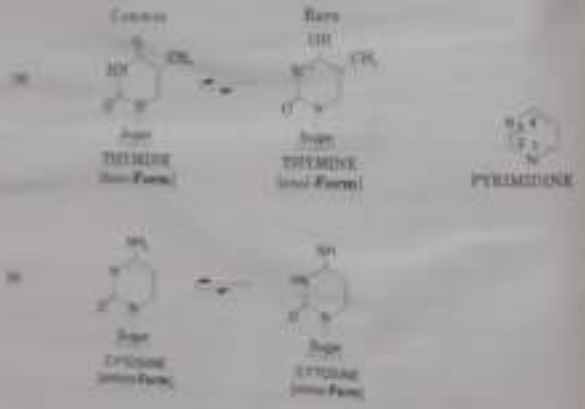
the ensuing conformational change gives rise to the ultimate deletion and addition of one or more bases in the course of replication ; and finally emerges with the **'frame-shift mutation'**.

(2) **Base Analogs** : Extensive and intensive research has made it virtually possible to incorporate several **'modified bases'** in place of a **'normal base'** in the course of DNA replication by virtue of the fact that the prevailing **DNA polymerase** fails to effect a clear differentiation between the normal base and its corresponding structural analog. Nevertheless, given frequently the analog is capable of forming a 'base pair' with an 'alternate base' and thereby gives rise to a replacement change during the next cycle of replication.

Examples :

(a) 5-Bromo-deoxyuridine (BRDU) represents a thymidine analog ; and, hence, may convert a **'G-C pair'** to an **'A-T pair'** only when it is present in an *enol* form and thereby corrects an **'A-T pair'** to a corresponding **'G-C pair'** in its *keto* form as depicted in Fig. 3.17. Interestingly, identical mutation may be brought about by **2-amino purine**. These chemical entities may be strategically and gainfully employed for reverting a possible mutation.

(b) Another set of examples for the **'base analogs'** are : **N⁴-hydroxy CTP*** which is invariably replicated either as a 'G' or an 'A' and results into the replacement of 'G' to 'A' or 'C' to 'T' in 50% of the available molecules.



* CTP = Cytidine triphosphate

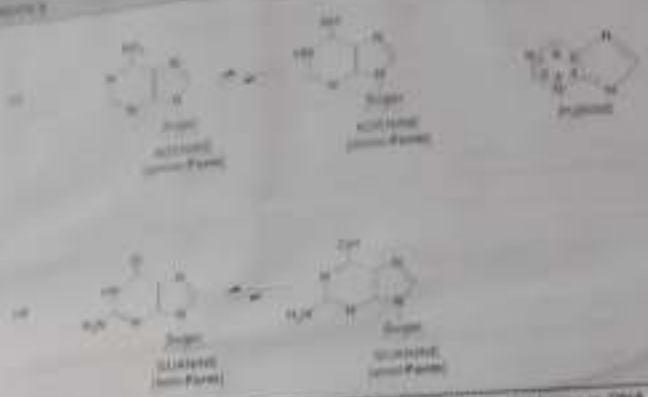


Fig. 3.17: Representation of Tautomeric Isomeric Forms of four common bases in DNA e.g., Thymine (a) ; Cytosine (b) ; Adenine (c) ; and Guanine (d)

It is, however, pertinent to state here that the base-pairing potentials of the above bases, namely ; **prototropic** (a,c) and (b,d) take place due to the shift of H-atoms between — 3 and C-4 positions ; and **partum** (b,c) and (d,a) — occur on account of the shift of H-atoms between N-1 and C-6 positions, which cause the effective change ultimately.

3.2.3. Beneficial Mutation

It has been well established that a major proportion of the **'spontaneous mutations'** are harmful to the organism because they invariably render an organism relatively lesser efficient with respect to its activity profile. In fact, the overall phenomenon of evolution could be made feasible exclusively by virtue of the ensuing rather slow **mutations**. It has been amply demonstrated that a plethora of **mutations** normally permit an organism to be more appropriate for effective survival in an altogether *unfriendly environment*. Therefore, these *newer developed mutations* critically allow the development of certain *newer characteristic features* that ultimately give rise to greater and befitting degree of adoptibility to their immediate surrounding environment.

The actual applicability of such **'beneficial mutations'** have been duly extended and exploited in the domain of *plant sciences*.

Examples :

(1) Induction of useful characteristic features in an organism e.g., high-yielding seeds, stress resistance plants, and pest resistance species.

(2) Development of specific variety of **Penicillium** that would yield definitely higher yields of **penicillin** (an antibiotic), obviously a highly viable and commercially feasible proposition.

6. DESIGN OF FERMENTATION PROCESSES

The very **design** of fermentation processes essentially requires the most predominant component of the media i.e., **water**, wherein the microorganisms tend to grow. The aforesaid phenomenon is quite good in several well-defined **'biotechnological processes'** leading to the commercial production of antibiotics, industrial alcohols, acetic acid, beer, wines and the like. It is, however, pertinent to state here that most the *liquid fermentation processes* have accomplished optimum production, it is often lately necessary to remove **water** as far as possible because it evidently attributes to a major factor in the cost of **bioproduct recovery** and **downstream processing**.

In reality, there are several vital and critical factors that invariably govern as well as play an important role in the media design of various fermentation processes, such as :

(i) Quality of water,
(ii) Quality control of raw materials,
(iii) Nutritional requirements,
(iv) Sterilization practices, and
(v) Media preparation.

The above mentioned factors shall now be treated individually in the sections that follow.

6.1. Quality of Water

The prevalent quality of water is obviously of the greatest importance by virtue of the fact that it not only affects predominantly the ensuing **microbial growth**, but also the *essential production of specific bioproducts*.

In the past, it was usually a practice to store and condition the so called **'traditional brewing water'** *particularly in such locations that enjoy or pass provided natural springs (i.e., natural source so as to obtain very high quality of soft, sweet and potable water without the cumbersome need to use to expensive and expensive* **'pretreatment'**.

However, the present day practice essentially needs the utilization of commercially soft demineralized water plants (i.e., DM-Plants), reverse osmosis plants (i.e., RO-Plants) etc., to meet pure water required for the fermentation processes.

6.2. Quality Control of Raw Materials

Besides, water the other chemical constituents e.g., preservatives, yeast, yeast extract, solvents, acids, acid-etc., should be of relatively better grade and quality so as to obtain flawless reproducible results while yielding specific bioproducts.

6.3. Nutritional Requirements

It has been duly observed that the required fundamental essential nutritional requirements of the microorganisms are, mainly : an energy or carbon source, an available nitrogen source, inorganic salts, trace, and for certain particular cell types specific growth factors. Interestingly, most heterotrophic microbes invariably derive both carbon and nitrogen sources from culture complex administration of plant, animal by-products or products. For instance : glucose, lactose, starch and sucrose (as sources of carbon) molasses providing carbon), and barley, beet molasses, corn steep liquor, groundnut meal, soybean

pharmamedia, rye flour, soyabean meal, and whey powder (as sources of nitrogen), which have been, duly summarized in the following table :

S.No.	Source of Carbohydrate	S.No.	Sources of Nitrogen (% Nitrogen by Weight)
1.	**Glucose** : Pure glucose monohydrate ; hydrolysed starch ;	1.	**Barley** : [1.5 to 2.0]
2.	**Lactose** : Pure lactose ; whey powder ;	2.	**Beet Molasses** : [1.5 to 2.0]
3.	**Starch** : Barley ; oat flour ; rye flour ; groundnut meal ; soyabean meal ;	3.	**Corn Steep Liquor** [4.5]
4.	**Sucrose** : Sugarcane molasses ; beet molasses ; crude brown sugar ; pure white sugar ;	4.	**Groundnut Meal** : [8.0]
		5.	**Oat Flour** : [1.5 to 2.0]
		6.	**Pharmamedia** : [8.0]
		7.	**Rye Flour** : [1.5 to 2.0]
		8.	**Soyabean Meal** : [8.0]
		9.	**Whey Powder** : [4.5]

6.4. Sterilization Practices

The various conventional, time-tested and widely adopted sterilization practices widely meant for the **'biotechnological media'** *should accomplished maximum kill of contaminating microorganisms, thereby maintaining almost best minimum damage caused to the medium components. Of the two* **steam-batch practices** *frequently employed the* **'batch-wise sterilization'** *in the bioreactor is still regarded to be the most widely used method, whereas the various prevalent* **'continuous sterilization'** *methods are virtually gaining not only enhanced acceptability but also adaptability.*

6.5. Media Preparation

The **media preparation** is precisely the backbone of the entire *'fermentation operation'* ; and, therefore, must be carried out with utmost care and precaution. Importantly, the ingredients and made-upon media design may obviously give rise to both improved efficiency of growth as well as concomitant significantly poor product formation.

Based on the enormous evidences available in the literatures the **design of fermentation processes** *may be categorized into the following five techniques, namely :*

(a) Solid substrate fermentation,
(b) Submerged fermentation,
(c) Downstream processing,
(d) Technology of immobilized and plant cell cultures, and
(e) Cell recycle technique.

The above stated techniques shall now be treated individually as under :

6.5.1. Solid Substrate Fermentation

There are a plethora of "**biotechnological processes**" which predominantly make use of the [illegible] growth of desired microorganisms on particular *solid substrates* either in the **absence** or **almost absence** of free availability of water. The **solid substrate fermentation** techniques are exploited abundantly for the specific production of fungal enzymes that essentially includes growth of filamentous fungus on a water-sprayed wheat or rice bran loaded with requisite nutrients to serve as '**substrates**' [illegible] yield alkaline proteases, amylases and pectinases. However, in the course of the fermentation [illegible] the effective management and control of **pH, temperature** and **humidity** is a little [illegible] task, but nevertheless should be maintained as prescribed as far as possible. The following **Table** [illegible] certain typical examples of solid substrate fermentations :

S.No.	Example	Solid Substrate	Microorganisms (to be) Utilized
1.	Mushroom production (Western and Eastern)	Straw ; Organic manure ;	*Agaricus bisporus* ; *Lentinula edodes* ; *Volvariella volvacea* ;
2.	Sauerkraut	Cabbage ;	Lactic acid bacteria ;
3.	Soy sauce	Soyabeans ; Wheat ;	*Aspergillus oryzae* ;
4.	Tempeh	Soyabeans ;	*Rhizopus oligosporus* ;
5.	Ontjom	Peanut press cake ;	*Neurospora sitophila* ;
6.	Cheese(s)	Milk curd ;	*Penicillium roquefortii* ;
7.	Organic acid(s)	Cane sugar ; Molasses ;	*Aspergillus niger* ;
8.	Enzymes	Wheat bran ; Maize bran ;	*Aspergillus niger* ;
9.	Composting	Mixed organic substances ;	Fungi ; Bacteria ; Actinomycetes ;
10.	Sewage treatment	Components of sewage ;	Fungi ; Bacteria ; Protozoa ;

It has been observed that the most regularly and frequently employed solid substrates are, namely : legume seeds, cereal grains, wheat bran, lignocellulose substances *e.g.*, sawdust, straws, or wood chips ; besides, a broad spectrum of animal and plant materials. A good number of such typical chemical entities are mostly '**polymeric molecules**' that are essentially either insoluble or sparingly water soluble, but are, however, invariably inexpensive, easily obtainable, and do represent largely a concentrated source of nutrients strategically required for the microbial growth.

The **Western world** makes use of the solid substrate fermentation processes rather confined to the production of cheese and sauerkraut, silage and mushroom cultivation ; besides, the composting of both animal and plant wastes. In contrast, the **Eastern world** the fermentation processes have virtually centered upon the very production of a wide array of food fermentations, such as : soy sauce, tempeh ; besides, several huge industrial enzyme processes both profitably and effectively.

6.5.2. Submerged Fermentation

The **submerged fermentation** process essentially makes use of **bioreactors** which are very much identical both in design and function to those employed in the antibiotic production. It is usually made of stainless steel having a capacity ranging between 10 to 15 m^3 and adequately provided with such devices as : *mechanical internal agitator* — for mixing the contents of the bioreactor ; *external pumps* — for circulation, loading and evacuation ; *bubble columns* — for aeration of the medium ; and

the *air-lift loop*. Cultivation progressively involves the suspension growth of microorganisms in the liquid environment. Nevertheless, the **sterilization** as well as the **process control** in the **submerged fermentation** are relatively easy and convenient to accomplish.

In general, the pH of the prepared culture medium is first optimized and then pumped right into the previously steam-sterilized bioreactor *via* HTST* sterilization devices. The thermolabile additives are carefully introduced individually into the sterile medium at an ambient temperature. Adequately propagated inoculum must be transferred into the production-stage vessels through sterilized SS pipes/pumps with utmost care and precaution. At this particular stage *sterile compressed air* is introduced into the *fermentation broth* via a strategically positioned sparger** at the bottom of the **bioreactor**. In commercial bioreactors the agitation is done by the aid of **multiple flat-bladed disc turbines** located onto a vertical shaft. Besides, other suitable devices, such as : *counter-current stirrers*, and *axial flow propellers* may also be employed as per the necessary requirements. It is, however, absolutely necessary to maintain the contents of the **bioreactors** (or **fermentors**) to an ambient temperature by two commonly used devices, namely : (*a*) *external heat-exchangers* ; and (*b*) *internal cooling-coils*.

Note : The evolution of CO_2, as a byproduct, in fermentative procedures essentially generate adequate heat energy, which if not taken care of properly would cause serious unwanted microbial processes to triger off thereby lowering the yield of desired products.

Modern **bioreactors** are frequently provided with *in situ sophisticated probes* and *sensitive instrumental gadgets* to monitor as well as stringently control various physical parameters during development of the inoculum in the course of various production stages. It is, however, pertinent to mention here that the different physical parameters like : dissolved O_2, evolved CO_2, redox potential, and pH value must be monitored and simultaneously controlled adequately.

Factors Governing Submerged Culture : In fact, there are *three* major factors that essentially govern the submerged culture, namely : (*i*) temperature ; (*ii*) pH ; and (*iii*) dissolved oxygen tension (DOT). These factors would be treated individually in the sections that follows :

(*a*) **Temperature :** Each fermentative process leading to a particular **bioproduct** essentially requires optimum temperature for the specific production of an enzyme, and hence the subsequent growth of the microorganism, which varies accordingly with the progressive stages of the entire process. Interestingly, the activity of an enzyme for an already utilized substrate gets remarkably influenced by temperature. It is, therefore, almost mandatory to closely monitor the range of temperature during fermentation in such a manner which would encourage cell growth appropriately.

(*b*) **pH :** The stability of an '**enzyme**' is exclusively guided by the pH optima. Therefore, it is quite evident to sustain and maintain pH optima so as to accomplish the maximum growth rate of the specific organism in question. In other words, it is absolutely vital and necessary that not only the '*pH profile in the course of fermentation alone*', but also the '*enzyme production phase*' must be imposed as well as monitored in such a manner so as to achieve the **maximum growth** and **enzyme production** simultaneously.

(*c*) **Dissolved Oxygen Tension (DOT) :** In actual practice, the **dissolved oxygen tension (DOT)** may be maintained stringently by controlling *three* most important physical conditions, such

* HTST : High temperature short time pasteurizers.

** A device for inlet of sterile compressed air *viz.*, single, multiple inlet type or ring type.

... adequate rate of aeration ; agitation [illegible] ; and gas-phase pressure. It has been demonstrated that the 'level of O_2' just lower than its **critical concentration** [illegible] growth of microorganisms adversely. Therefore, it is very essential to maintain '[illegible] aerobic conditions' in the growth culture medium. Interestingly, the desired '**level of** [illegible]' predominantly in the **submerged microbial cultures** may be obtained by adopting [illegible] the two following methods, namely :

(i) Enhancing the mass of O_2 being provided to the **bioreactor** per unit time by [illegible] the overpressure in the head-space of the **bioreactor**, and

(ii) Increasing the [illegible] rate of air supply to the **bioreactor**.

CAUTION : Oxygen-enriched air could attribute to higher oxygen transfer rates.

6.5.3. Downstream Processing

The **downstream processing** is solely related to the extraction and purification of the [illegible] end product from the bioprocess based on the skills of biochemists, chemists, chemical engineers [illegible] process engineers.

The very design and absolute efficient operation of **downstream processing methodologies** [illegible] include two important aspects, namely :

(a) Vital elements in getting the required products into viable commercial usage, and

(b) Reflect the need to lose none of the desired product than is absolutely necessary.

Example : *Humulin®* [illegible] (Eli Lilly, USA) ; *Insulin* : More than 90% of the 200 staff members are actively engaged in the various recovery processes. Obviously, **downstream processing** of biotechnological processes truly represents a major portion of the overall costs directly involved in most processes, but at the same time is also the least glamorous aspect of biotechnology. Summarily, any improvement [illegible] afforded in the **downstream processing** would certainly benefit the overall efficiency and [illegible] costs of processes.

The various stages of 'downstream processing operations' are as stated below :

Stage I : **Separation** — Filtration — centrifugation — flotation — disruption ;

Stage II : **Concentration** — Solubilization — extraction — thermal processing — [illegible] filtration — precipitation ;

Stage III : **Purification** — Crystallization — chromatographic methods ;

Stage IV : **Modification** — Structural analogs ;

Stage V : **Drying** — Under vacuum — spray drying — freeze drying — fluidised — bed drying [illegible] modes.

Salient Features : The salient feature variants of the **downstream processing** are as enumerated [illegible] :

(1) Initial separation of the **bioreactor** broth into a *liquid phase* and a *solid phase*, and [illegible] concentration and purification of the product.

(2) Processing comprises of at least five stages as described above.

(3) Methods either proposed or in use usually range from two extremes *i.e.*, conventional [illegible] modern [illegible] that may predominantly comprise of such well-known techniques as distillation, centrifugation, filtration, ultrafiltration, solvent extraction, adsorption, reverse [illegible] molecular sieves, selective membrane technology, electrophoresis, and affinity chromatography.

Special Note : **In fact, it is in this particular domain wherein a plethora of reasonably potential industrial applications of latest developments in biotechnology have virtually come to grief or rendered problematic by virtue of the following two important draw-backs, namely** :

(a) extraction failed to achieve the expectations of the designers, and

(b) extraction procedure has virtually consumed so much excessive energy input as to render it almost uneconomical.

It has been a practice to maintain the final product of the ensuing downstream purification stages to possess some degree of **stability** for the ultimate commercial distribution. However, **stability** may be best accomplished for a wide range of products by affording some form of *drying*, for instance : **freeze-drying, spray-drying, fluidised-bed drying**. The method of choice is solely dependent on product quality and cost-effective measures.

Dry-form products : include — antibiotics, amino acids, organic acids, polysaccharides, single-cell proteins, enzymes etc.

Liquid-form products : include — products which cannot be dispensed conveniently in a dried form.

Proteinaceous products : to avoid possible changes of '**denaturation**'.

In general, special precautionary measures need to be taken so as to avoid any scope of either deterioration or microbial contamination.

Highlights of Downstream Processing : Operations indulging in downstream processing do possess quite a few outstanding features, namely :

(1) It gives rise to several challenging and demanding aspects of a *wide spectrum of biotechnological processes*.

(2) Hallmarks of *most high value biotechnological products* are solely based upon their **purity and stability**.

(3) Ultimate success of a wide range of biotechnological processes shall entirely depend upon the correct well-defined area of choice and *modus operandi* of such established systems.

(4) The commercial-scale operation measures will solely depend upon the economic viability/feasibility without the least compromise on the final end-product ; and for this the utmost understanding and cooperation should always prevail between the '**bioscientist**' and the '**process engineer**'.

6.5.4. Technology of Mammalian and Plant-cell Culture

Recent literatures have adequately substantiated the glaring fact that the mass cultivation of organisms for a host of biotechnological processes got evolved and subsequently developed invariably not only around the *bacteria, yeasts and filamentous fungi* ; but also around the plant and animal cell cultures.

Plant-cell Culture : *Plant-cell Culture* may be defined as — **'a specific technique encompassing the *in vitro* culture of plant cells, tissues, organs, and even whole plantlets'**.

In actual practice, the application of plant-cell culture techniques have been abundantly extended and exploited for the micropropagation of certain plants. In such instances, plant-cell cultures would meticulously progress *via* several cardinal stages, namely : *organogenesis, plantlet amplification*, and eventual *establishment in soil*.

However, commercial-scale production of multiple suspension-cell cultures of several species has not been accomplished gainfully ; and the ultimate goals of desired products vary much due to the nature of the whole plant have been largely responsible that accounted, such as : **alkaloids, ginseng, shikonin**. Furthermore, the economy of the cell technique to large-scale fermentation propagation may give rise to the legitimate production of commercially acceptable levels of some **high-value plant products**, for instance : **codeine, digitalis, jasmine, spearmint** etc.

Importantly, the plant-cell culture technique is relatively much slower in comparison to the microorganisms, though a large number of the other characteristics, features of fermentation are very much identical. However, the specific volume of an average cultured plant cell could be upto 250-fold more than of a bacterial cell. Although certain plant products are now being marketed ; however, it is not expected to be commercially viable for several years from now.

Note : Animal or human cell cultures could give rise to a host of potentially vital and important organic compounds. Such break throughs have been mostly stalled or hampered due to several encountered problems together with exhorbitant scale-up operations.

Mammalian Cell Culture : In reality, the culture of both mammalian cells and tissues represents a largely explored and widely employed technique in the ever expanding domain of **modern cell biology** and **biotechnology**. In the recent past, the broad range of cells types now grown invariably in culture is both very extensive and progressively increasing ; and essentially includes cells that are typically derived from brain, liver, cartilage, lung, breast, skin, bladder, kidney, ovaries, prostate, uterus and several types of cancers. In actual practice, there has been an enormous growth in the utilization of **animal-cell culture** cultivation for the commercial-scale production of a good number of **high-value products**, namely : life-saving **vaccines** *e.g.*, polio, mumps, measles, rubella, chickenpox, cholera etc., **insulin, hormones, interferons, plasminogen,** and various **antibodies.**

Major Problems Encountered : The major problems that are usually encountered in the mass cultivation of mammalian cells essentially include :

- Extreme sensitivity of cells to impurities in water.
- Cost effective measure.
- Stringent quality control of media.
- Need to discard contamination by more rapidly growing microorganisms completely.

Primary Cultures : Primary cultures may be defined as — **'freshly isolated cultures obtained from the mammalian systems'.**

The **primary cultures** are normally heterogeneous in nature but still closely designate and represent the parent cell types. They also exhibit and involve in the expression of tissue specific characteristic features. It has been observed that after having passed through several sub-cultures upon fresh culture media, the ultimate **cell line** would either prove to be fatal (*i.e.*, die out) or get transformed into **continuous cell line**. It is, however, pertinent to mention at this juncture that the *continuous cell lines* exhibit several variation from the corresponding *primary cultures*, namely :

- Alterations in cytomorphology,
- Enhanced rate of growth,
- Increase in chromosome variation, and
- Increase in tumorigenicity.

Nevertheless, the *in vitro* transformation explicitly designates primarily the valid acquisition of an inflator event of life span.

Cultivation of Anchorage-Dependent Cell Types : It has been amply demonstrated that specifically the animal cells may be grown either in an **unattached suspension culture** or **attached to a solid surface.**

Examples :

(*i*) **Lymphoblastoid Cells** — usually grow in an unattached suspension culture,

(*ii*) **Primary or Normal Diploid Cells** — normally grow only when they are attached duly to a solid surface, and

(*iii*) **Hela Type* Cells** — invariably can grow in either of the two states stated earlier.

It is duly apprehended that most of the future commercial developments with **animal cells** shall be predominantly guided by the prevailing cultivation of anchorage-dependent cell types.

Monolayer Cultivation of Animal Cells : Presently the monolayer cultivation of animal cells is exclusively governed by the existing **'surface-area'** available for attachment. Importantly, the particular design considerations have been solely directed to methods of **increasing surface area.** The most recent sophisticated system has been developed that essentially supports the actual growth of cells **strategically** in coils of **gas-permeable TEFLON**-tubing** (*i.e.*, each tubing with a surface **area** of 10,000 cm^2, and upto 20 such coils may be incorporated into an incubator chamber). A wide spectrum of cells has been cultured under these experimental parameters successfully.

In short, the **'suspension cultures'** have been developed so meticulously and successfully to substantially large **bioreactor** volumes thereby permitting the utilization of all the ensuing engineering advantages of the **stirred-tank bioreactor** that have eventually accrued from an elaborated microbial studies being presently employed to an added advantage. Such studies have been carried out only on batch culture basis.

Recent Innovative Breakthrough : The wonderful recent innovative breakthrough in biotechnological process has been duly accomplished via an unique combination of **attachment culture** and **suspension culture** by the application of *microcarrier beads*. The underlying principle essentially involves the strategic attachment of the *anchorage-dependent cells* to specially designed **DEAE-Sephadex beads** (with a surface area of 7 $cm^2.mg^{-1}$) which are capable of floating in suspension. Thus, in this manner the engineering advantages of the designed stirred **bioreactor** may be employed with anchored cells overwhelmingly.

Examples : Many cell types have been meticulously grown in this way, namely : (*a*) *human interferon* ; and (*b*) *viruses*.

Further developments surely rest upon the **new bioreactor designs** based on the *microcarrier bead concept* that would certainly afford a much wider large-scale development of both human and animal cell types.

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* Cells obtained from a human malignancy.

** Polytetrafluoroethylene.

6.5.5. Cell Recycle Technique

Ethanol has been exploited and used extensively not only as a **fuel supplement** but also as a **chemical feed stock** in the past 2 to 3 decades, thereby enhancing the overall global interest in economically augmenting production in an extremely cost effective manner (*i.e.*, cheaper) and feasible way. Therefore there are ample evidences in the literature that obviously show a broad spectrum of improvements effected in the *traditional batch fermentation*. Hence, one such methodologies researched in the recent past which has attracted tremendous attention is the **cell recycle technique**. In fact, it does not necessitate its involves any appreciable additional expenditure.

The underlying principle of this newly evolved technique is that it essentially involves the recycling of cell mass which is critically produced in the *course of fermentation process*. Thus, it has been established that the aforesaid **cell recycle technique** gives rise to *three* prominent advantages, namely :

(i) Net saving of nearly 5 to 10% of the entire substrate that would have been otherwise utilized for the ensuing cell growth;

(ii) Significant saving in the cost of inoculum and time; and

(iii) **Cell recycle technology** has virtually reduced the total fermentation time drastically upto 40% etc., from 24-36 hours as a batch fermentation reduced to mere 5-6 hours.

7. PRODUCTION OF ANTIBIOTICS (ISOLATION OF FERMENTATION PRODUCTS)

Industrial fermentation industry, across the globe, commendably received its ever outstanding impetus for the most coveted strategic expansion as well as profits with the wonderful advent and exploitation of **antibiotics*** as potential well known "**chemotherapeutic agents**"**. During the World War II the actual demand for penicillin almost reached its peak to save the lives of millions of wounded soldiers ; and later on followed by *streptomycin* and a host of other **antibiotics** in the domain of *global scenario of pharmaceutical industry*. These developments naturally *triggered off extensive* and intensive *research programmes most articularly designed to look for useful microorganisms* that are capable of producing *highly effective, viable, and good antibiotics* ; and executed a tremendous push towards the *whipsaw research* and development for producing antibiotic substances on a commercial scale. Thus, several *elongation* newer refined procedures were devised, developed, and the state-of-the-art technique of *submerged agitated aerated* fermentation using deep-tank fermentors came into being with obvious high rate of success.

Primarily the antibiotics are produced by **bacteria** *and* **fungi** ; besides, several other classes of microorganisms do possess at least limited functions in this aspect.

Examples : (a) *Bacteria : Streptomyces species , Bacillus species* ;

(b) *Fungi (Molds) : Aspergillus species ; Penicillium species* ;

In general, a good many of the known *bacterial antibiotics are polypeptides*, that have proved to be rather unstable, toxic, and difficult to purify. Likewise, the *fungal antibiotics*, with a few notable

* Microbial metabolites or synthetic structural analogues inspired by them which, in small dosage regimens, inhibit the growth and survival of microorganisms without any serious toxicity whatsoever to the patient host.

** In the treatment of disease, the application of chemical reagents that have a specific and toxic effect on the disease-causing microorganisms.

exceptions, generally have been observed to be too toxic for usage in medical practice. However, the **penicillins** (*i.e.*, the penicillin group of antibiotics) produced by various molds stand out to be an obvious exception. The earlier belief and conception that an '**antibiotic**' possesses toxicity, that invariably negates its '**internal administration**' both to the animal and human body, does not obviously restrict its medical usage, because in certain cases the antibiotic may even be judiciously recommended for use as **topical applications**, namely : dermatological preparations, treatment of burns, open cuts and injuries etc.

The following Table includes the names of certain known antibiotics, the related microorganisms, and therapeutic usages :

Antibiotic-Microorganisms-Therapeutic Usage

S.No.	Antibiotics	Microorganisms	Therapeutic Usages (TN's*
1.	Amphotericin B	*Streptomyces nodosus*	Deep-seated mycotic infections (Fungizone®)
2.	Bacitracin	*Bacillus subtilis*	Applied topically as ointment form.
3.	Chloramphenicol	*Streptomyces ven. caelar*	Broad spectrum agent useful in typhoid fever (Chloromycetin®)
4.	Erythromycin	*Streptomyces erythreus*	Many Gram +ve and some Gram –ve organisms (Ilotycin®).
5.	Griseofulvin	*Penicillium griseofulvum P. nigricans , P. urticae ,*	Oral antifungal antibiotic effective against ringworm.
6.	Kanamycin	*Streptomyces kanamyceticus*	Restricted to Gram –ve organisms ; viz., *Klebsiella, Proteus,Serratia,* and *Enterobacter* spp.
7.	Neomycin	*Streptomyces fradiae*	Local infections viz., burns, ulcers, wounds, impetigo, infected dermatoses, furunculosis, and conjunctivitis (Tradomycin®)
8.	Oxytetracycline	*Streptomyces rimosus*	Broad spectrum antibiotic for dysentery, gum infection etc. (Terramycin®)
9.	Penicillin	*Penicillium notatum, Penicillium chrysogenum*	Bactericidal, for most Gram +ve and certain Gram –ve organisms. (Crystalen®).
10.	Streptomycin	*Streptomyces griseus*	Treatment of tuberculosis in conjunction with drugs like : isoniazid and rifampicin (Streptomycin Sulphate®).

* TN = Trade Name

Based on the above statement we facts the antibiotics of fermentation products of the following subsequent shall be described individually in the sections that follows :

(a) *Penicillins*
(b) *Streptomycins*
(c) *Tetracyclines*
(d) *Vitamin* B_{12}

7.1. The Penicillins

Penicillins, the β-lactam antibiotics, have indeed enjoyed the legendary of a long history of application as chemotherapeutic agents since 1929 by the epoch making discovery of Alexander Fleming and even today they legitimately command the reputation for being prescribed more than 50% of all known antibiotics across the globe. The most genuine and remarkable combination of unique properties like *bactericidal property* and *drastically levels of extremely low toxicity* are solely attributed to its ensuing **bacterial cell wall biosynthesis.** In reality, the relatively low cost of these therapeutic agents exclusively based upon the tremendous acclaimed enhancement in fermentation yields which have been gradually accomplished through years of dedicated researches as : strain improvement, fermentation optimization procedures, and above all meticulous refinement of downstream processing. It would be worth while to lay proper emphasis upon the current status of knowledge with regard to the genetics and molecular biology of penicillin biosynthesis.*

7.1.1. Genes in Penicillin Biosynthesis

It has been observed that the biosynthesis of the penicillin group of antibiotics essentially involves a common pathway having some core activities duly protected among all producer microorganisms which have been screened till date both intensively and extensively. Interestingly, most of the producer species embrace a plethora of *filamentous fungi*, such as : members of the genera *Penicillium*, *Cephalosporium*, *Aspergillus*, a number of **actinomycetes** including *Streptomyces*, and *Nocardia* and a few **bacterial species** *e.g. Flavobacterium* and *Lysobacter* spp. It is, however, pertinent to mention here that in every instance, the pathway essentially commences with the condensation of the precursor amino acids, namely : L-α-aminoadipic acid, L-Cysteine, and L-Valine to give rise to a corresponding tripeptide intermediate, δ-(L-(α-aminoadipyl)-L-cysteinyl-D-valine (ACV). Now, ACV gets converted to **isopenicillin N** in the presence of the enzyme isopenicillin N synthase (pcbC), which is further duly modified to yield a variety of end products for instance : **hydrophobic penicillins,** as depicted in Fig. 3.18.

* Aharonowitz Y. *et al. Annu Rev Microbiol.* **46** : 461-495, 1992 ; Jensen SE. *et al.* (eds) : ***Genetics and Biochemistry of Antibiotic Biosynthesis,*** Butterworth-Heinemann, Massachusetts. 230-268, 1988.

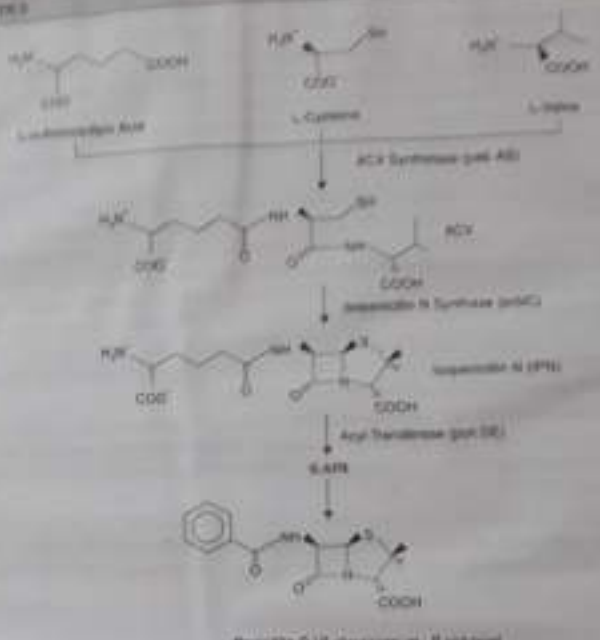

Fig. 3.18. Pathway for Biosynthesis of Penicillins

[ACV = δ-L-(α-aminoadipyl)-L-cysteinyl-D-valine ; 6-APA = 6-amino-penicillanic acid ; IPN = Isopenicillin N ; The genes are shown in parentheses]

A. L-α-Aminoadipic Acid : A common Precursor, but Different Biosynthetic Origins

Nevertheless, a predominant point of difference does exist between the *bacterial* β-lactam and *fungal* producer species with respect to the formation of L-α-aminoadipic acid, which is one of the three precursor amino acids of the **penicillins.** However, this difference actually comes into being via the two separate and distinct pathways specifically for the **lysine metabolism** as could be observed in prokaryotes* and eukaryotes.**

* An organism of the kingdom Monera with a single, circular chromosome, without a nuclear membrane, or membrane bound organelles (i.e., mitochondria and lysosomes). Included in this classification are bacteria and cyanobacteria (formerly the blue-green algae).

** An organism in which the cell nucleus is surrounded by a membrane.

The two alternate routes of biosynthesis shall now be treated briefly :

In Prokaryotes : In this instance, lysine gets biosynthesized via a pathway without the utilization of L-α-aminoadipic acid* ; and, therefore, **the prokaryotic β-lactam producing species** have worked out an altogether different strategy to yield L-α-aminoadipic acid. It has been observed that in the two different species, namely : *Streptomyces* spp., and *Nocardia lactamdurans*, the production of L-α-aminoadipic acid by the **catabolism of lysine** occurs in a two-step pathway, viz.**

First, lysine gets converted to 1-piperidine-6-carboxylic acid, duly catalyzed by the enzyme *lysine-ε-aminotransferase* (LAT). As LAT is exclusively present in **penicillin-producing actinomycetes,** and is apparently absent in corresponding nonproducers, and because the '**gene** responsible for encoding this particular enzyme (LAT) is eventually associated with other penicillin biosynthetic genes in *Streptomyces clavuligerus**** and *Acremonium lactamdurans*****, in such a way, LAT is regarded to be an integral part of the ensuing penicillin biosynthetic pathway as illustrated in Fig. 3.19.

Secondly, 1-piperidine-6-carboxylic acid gets converted to α-amino-adipic acid by the prevailing enzyme *piperidine-6-carboxylate dehydrogenase*.

In Eukaryotes : In this case, the inclusion of fungal β-lactam-yielding species, *lysine gets* biosynthesized via a distinct metabolic pathway wherein L-α-aminoadipic acid invariably **appears as a** exceptional intermediate****** ; and subsequently, it may be removed carefully for the ensuing penicillin biosynthesis.******

Precisely in fungi, the L-α-aminoadipic acid is made available via the prevailing **lysine biosynthetic pathway,** it may also be obtained alternatively via a **lysine catabolic pathway,** very much identical to the one frequently observed in the actinomycetes ; and ultimately routed and channelled into **the penicillin biosynthesis.**

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* Wang LC *et al. Biotech Ad.* **8** : 159-183, 1990.

** Shiffrin E *et al. J. Bacteriol.*, **171** : 299-302, 1989.

*** Madduri K *et al. J. Bacteriol.*, **173** : 965-969, 1991 ; Tobin MB *et al. J. Bacteriol.*, **173** : 6223-6229, 1991.

**** Coque JJR *et al. J. Bacteriol.*, **173** : 6258-6264, 1991.

***** Blumenthes JC. *Crit Rev. Microbiol.*, **12** : 131-151, 1985.

****** Lorenjo JM *et al. J. Bacteriol.* **168** : 869-870, 1986.

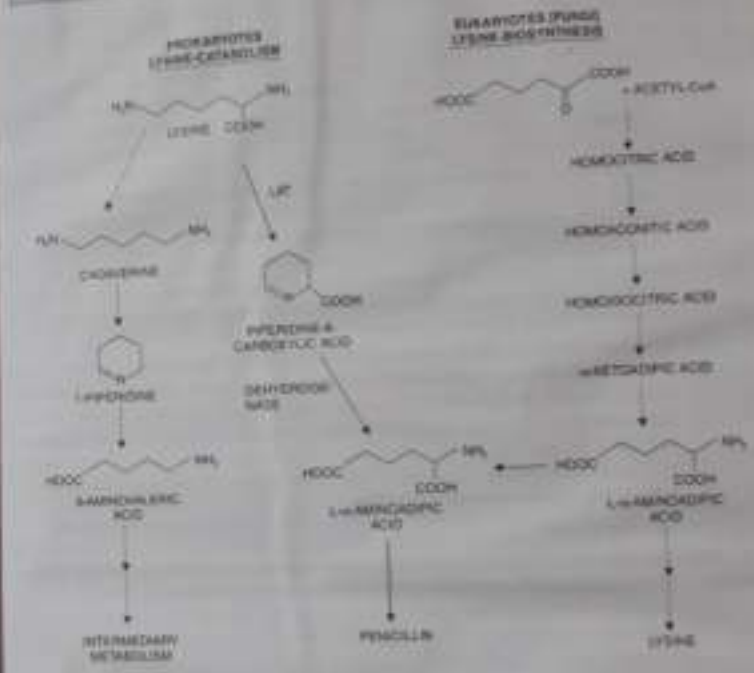

Fig. 3.19. Lysine Metabolism and Relationship to L-α-Aminoadipic Acid Production in Microorganism and Fungi

B. Late Genes in the Biosynthesis of Hydrophobic Penicillins

Extensive research has amply proved and established that isopenicillin N (IPN) may be rightfully regarded as the most crucial **branch-point intermediate** in the penicillin pathway. Furthermore, its subsequent strategic conversion to a wide range of hydrophobic penicillins, such as : *Penicillins G*, essentially designates the ultimate and final step particularly related to the **penicillin-producing segment of the pathway.**

Salient Features : The various salient features involving the fate genes in the biosynthesis of the β-lactam penicillins are as follows :

(1) It has been observed that the L-α-aminoadipic acid moiety first gets dislodged by an enzyme, amidohydrolase, to give rise to the formation of 6-APA followed immediately by reaction with a CoA derivative to result into the formation of the targetted drug penicillin.*

(2) Enzyme actively engaged in catalyzing the reaction [in (1) above], **acyl-CoA-isopenicillin acyltransferase** (ACT), normally occurs as a *heterodimer* usually comprising of two subunits of *29 and 11 kDa*** (*i.e., kilo daltons*)

(3) ACT represents a **'multifunctional enzyme'** which predominantly exhibit the characteristic features of three enzymes, namely **acetyl-CoA-isopenicillin N acyltransferase (IAT), acetyl-CoA-6-APA acyltransferase (AAT),** and **penicillin amidase.**

(4) Interestingly, the aforesaid subunits [in (2) above] are particularly derived from a **40 kDa precursor protein** by the aid of a **post translational processing mechanism ;** and are legitimately encoded by a single gene, *pen* DE, that has been duly *cloned* as well as *sequenced* originating from two **fungal β-lactam producers.*****

(5) It has been shown that the sequences encoding the 11 kDa subunit precisely precede those that encode the 29 kDa subunit, thereby having the *'processing site'* strategically positioned between **Gly-102** and **Cys-103.******

(6) The *pen* DE gene expression along with its various structural analogues critically presents an *E.coli* expression system has virtually suggested that the actual generation of an **active ACT** prominently requires a *cooperative interaction between the two polypeptide segments in the course of their* **synthesis** and **folding.**

7.1.2. The Penicillin Variants

Penicillin is the name assigned to the mixture of naturally occurring chemical entities having the molecular formula $C_9H_{11}O_4N_2SR$, and differing specifically only in the nature of 'R'.

PENICILLIN

The various *penicillin variants* are mainly produced by a variety of strains of *Penicillin notatum* and *Penicillium chrysogenum.* Six **naturally occurring penicillins** have been prepared, characterized, and studied extensively, whose chemical names, other names and the nature of 'R' are stated in the following table :

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* Queener SW and Neuss N. **The Biosynthesis of β-lactam antibiotics.** Moran EB, Morgan M. eds. *The Chemistry and Biology of β-Lactam Antibiotics*, vol. 2. Academic Press, London, pp. 1-81, 1982.

** Tobin MB *et al. J. Bacteriol.* **172** : 3008-3018, 1990.

*** Montenegro E *et al. Mol Gen Genet.* **223** : 322-330, 1990.

**** Aplin R T *et al. FEBS Lett.* **319** : 106-170, 1993 ; Tobin MB *et al. Gene* **132** : 199-206, 1993.

S.No.	Chemical name	Other names	–R
1.	Pent-2-enylpenicillin	Penicillin F or F	$-CH_2CH=CHCH_2CH_3$
2.	Benzylpenicillin	Penicillin II or G	$-CH_2-C_6H_5$
3.	*p*-Hydroxybenzyl penicillin	Penicillin III or X	$-CH_2-C_6H_4-OH$
4.	n-Heptyl penicillin	Penicillin IV or K	$-(CH_2)_6CH_3$
5.	n-Amylpenicillin	Dihydro-F Penicillin	$-(CH_2)_4CH_3$
6.	Phenoxymethyl penicillin	Penicillin V	$-CH_2-O-C_6H_5$

7.1.3. Production of Benzylpenicillin [Penicillin G]

Alexander Fleming's originally isolated strain of *Penicillium notatum* (Strain) afforded actually very low yield of **penicillin.** Vigorous search for improvement of strain revealed the isolation of *P. chrysogenum* which distinctly gave much *higher yields of penicillin.* Importantly, the newer strains of *Penicillium* could even produce upto 160 folds higher yields in comparison to the original isolate that are solely based upon the novel phenomenon of **'mutation'** or the so-called **'genetic engineering'** methodologies.

In actual practice, **penicillin** is commercially produced in submerged vat cultures employing a highly purified and selected strain of *P. chrysogenum,* whereby the ultimate yield of the targetted product (**penicillin**) has been enhanced almost **three folds** *i.e.,* from 10 mcg. mL^{-1} to 30 mcg. mL^{-1}. Interestingly, these modified, researched, purified strains of **Penicillium** do exhibit a number of marked and pronounced characteristic features, such as : high-titre values, improved growth, immense tolerance to the side-chain precursors, acetyltransferase activity, ability to store intracellular requirement(s).

The various steps that are associated intimately with the production of **Benzylpenicillin [Penicillin G]** are stated as under :

7.1.3.1. Inoculum

Penicillium notatum (*i.e.,* Fleming's original/original strain) together with other 'early isolates' afforded exclusively low yields of **penicillin** ; besides, they responded very sluggishly to the **submerged culture techniques** particularly. Contrary to this, an early strain of *P. chrysogenum* (**NRRL, 1951**), duly isolated from the mouldy fruits, was observed to yield much higher yields of **penicillin.** Consequently, the *high yield strain* was duly subjected to careful treatment with a broad-spectrum of time-tested **mutagenic agents,** for instance : UV-radiations, X-rays, and mechlorethamine (MBA)—a nitrogen mustard. Obviously, these mutagenic agents helped a long way in the appropriate selection of several **higher yielding mutants** in particular ; and, in general, the judicious application of these ensuing mutagenic agents in sequence, along with certain repetitive treatments, ultimately gave rise to the newer strain **Q-176,** that essentially had the ability of producing maximum yields of penicillin.

[illegible]

Drawback : [illegible]

Developments in Better Penicillin Producing Strains : [illegible]

Asexual Reproduction : [illegible]

Parasexual Recombination : [illegible]

[illegible]

Formation of Haploid Nuclei****** : [illegible]

[illegible]

7.1.3.2. Production Media

[illegible] **penicillin production media** [illegible] **trade secrets** [illegible] **patented** [illegible] potassium dihydrogen phosphate (KH_2PO_4) [illegible] Jackson (1958)* [illegible] the following composition :

S.No.	Ingredients	Quantity (%)	Remarks
1.	Fermentable carbohydrates :		
	— Corn steep liquor solids	3.5	Organic
	— Lactose	3.5	Carbon
	— Glucose	1	Source
2.	Organic nitrogen source	0.5	
3.	Phenyl acetic acid	0.5	Penicillin precursor
4.	Potassium dihydrogen phosphate (KH_2PO_4)	0.4	
5.	Calcium carbonate	1	Acts as buffer
6.	Edible oil	0.25	
7.	Organic salts	q.s.	Maintains salt balance in medium

Note : (1) **The pH after sterilization is carefully maintained between 5.5 to 6.0.**

(2) **Higher lactose content ranging between 4 to 5% is desired with vigorously increased aeration and agitation environments maintained within the fermentor (i.e., bioreactor).**

(3) **The 'production media' contains both 'lactose' and 'precursor' which are not included in the inoculum media.**

7.1.3.3. Biomass** Production

It has been amply demonstrated that the ensuing production of penicillin exclusively depends upon the prevailing **biomass production** [illegible] **initial O_2-uptake rate** [illegible] **subsequent CO_2-evolution rate** [illegible]

* Jackson T, Development of Aerobic Fermentation Processes. In : **Biochemical Engineering** [illegible], 1958.

** [illegible]

the ultimate penicillin production may be enhanced by suitably engineering and greatly improving **mycelial* biomass,** which could be accomplished appropriately by boosting up both the spread and the *of agitation.*

7.1.3.4. Course of Typical Penicillin Fermentation

The actual course involved in a typical penicillin fermentation on account of several **inter**-*chemical changes is represented explicitly in Fig. 3.20.*

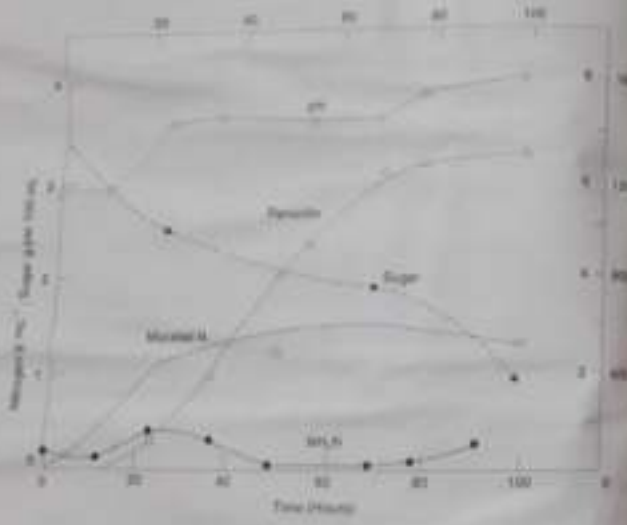


Fig. 3.20. Various Chemical Changes Involved in a Typical Penicillin Fermentation with Added Phenylacetic Acid Precursor

[Adapted from : Brown WE and Peterson WH, *Ind. Engg. Chem.* **42**, 1773, 1950]

Salient Features : The various salient features intimately associated with the chemical changes recorded in Fig. 3.15 are enumerated below :

(1) At the initial stage of fermentation pH remains rather constant, whereas the corresponding liquor cakes entities, glucose, and ammonia are being utilized simultaneously.

* The mass of filaments (**hyphae**) which constitutes the vegetative body of fungi, such as : molds.

(2) Optimum pH range for penicillin production rises maximum between 7 to 7.5 by a sequence of events, namely : carbon compounds (i.e., carbohydrates) utilized and depleted — portion of lactic acid (from corresponding liquor) being consumed — ammonia (NH_3) released by deamination of aminoacids from corresponding liquor.

(3) At this critical point in time pH remains virtually steady and constant as the method makes use of the lactose to form **penicillin.** Further rise in pH arrested due to the fact that prevailing mold gets absolutely starved for nutrients.

(4) Completion of fermentation is indicated by pH rise to 8 or even higher by virtue of considerable depletion of **'lactose'** which usually brings about autolysis of the mycelium.

(Note : In usual practice, the penicillin fermentation is arrested and harvested before this specific and critical stage is achieved.)

(5) **First 20 to 30 hours** (*i.e.*, during corresponding liquor solid and carbohydrate consumption) the **fungal growth** turns out to be distinctly thick and heavy due to *three* possible reasons, namely : (i) disperse strands (of DNA) ; (ii) clumps of mycelium ; and (iii) availability of definitive pellets of mycelium (ranging between 0.5 to 2 mm diameter).

(6) Fig. 3.15 reveals vividly that the yields of penicillin are found to be **'linear** even at 22 hours, but in actual practice they range between 48 to 96 hours.

(7) Ultimate yield of **penicillin** varies between 3 to 5% solely based upon carbohydrate as easily consumed, and almost attains a level in excess of **1300 Units . mL.**$^{-1}$.

(8) Sylvester and Coghill* (1954) have arrived at the following statistically averaged estimation with regard to the yield of **penicillin** :

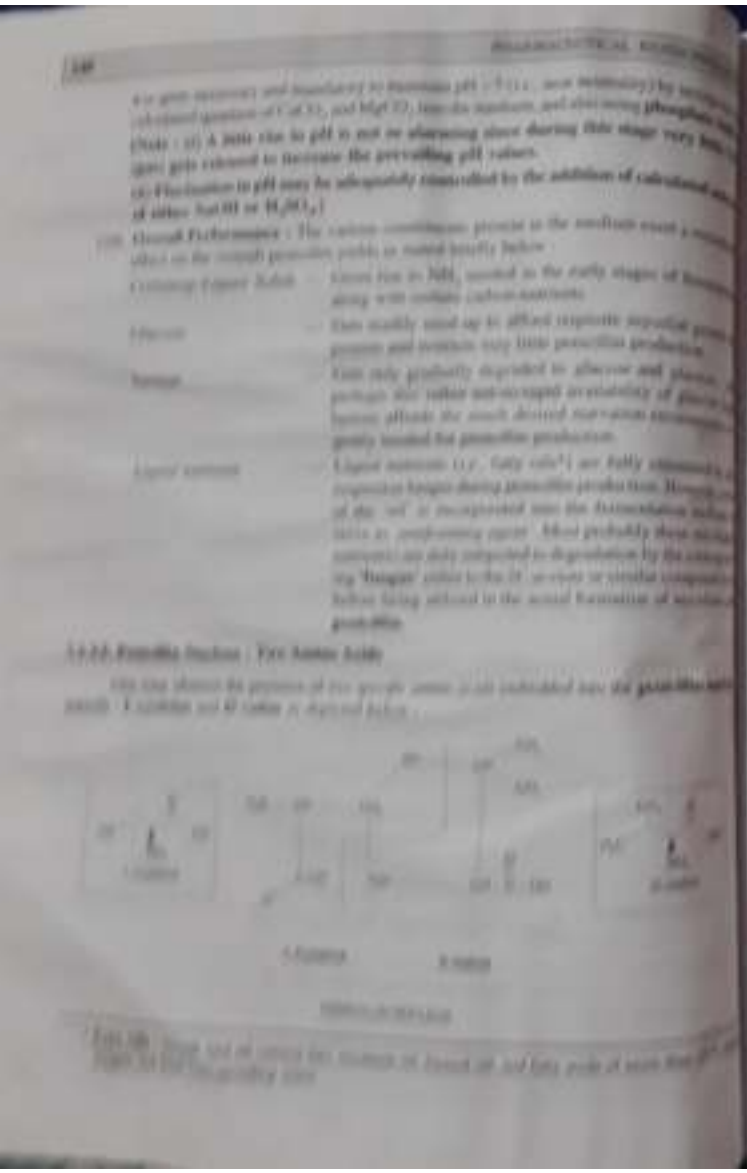
Aim : To produce 1000 Gallons of fermented culture (approx. equivalent to 5-6 lbs of penicillin) by submerged-culture process.

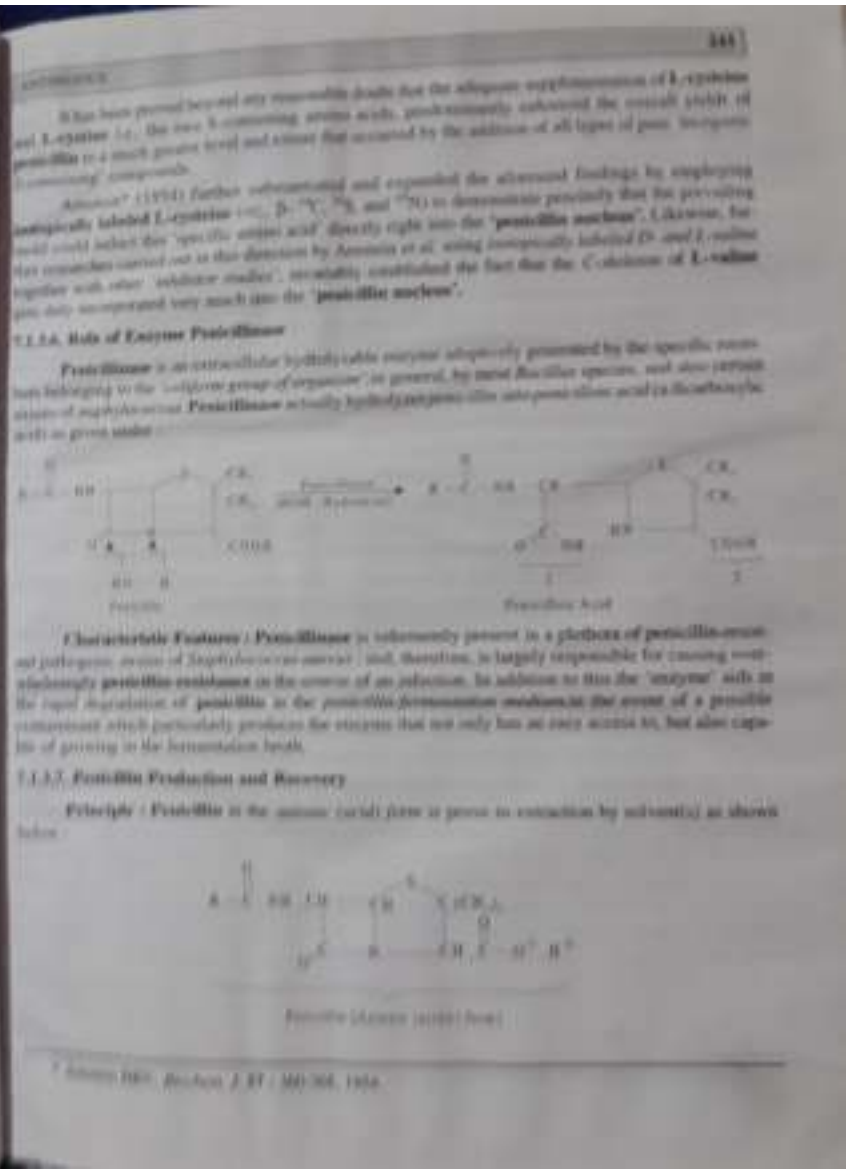
S.No.	Requirements	Quantity	Unit
1.	Various nutrients (*e.g.*, corresp-liquor solids, lactose, glucose etc.)	500	lbs
2.	Live LP-Steam	7500	lbs
3.	DM-Water	10,000	Gallons
4.	Electricity	1,000	kwh
5.	Air (Compressed and Sterile)	250,000	

LP = Low Pressure ; DM = Demineralized Water ; Gallon = 4.5 L or Imperial Gallon = 3.75 L ; kg = 2.45 lbs ;

(9) pH plays an extremely vital and critical role particularly in the course of *penicillin fermentation* since **penicillin** is quite sensitive to relatively low pH values. Besides, **penicillin** is equally sensitive to pH values above 7.5, specifically in the presence of NH_4^+ ion. Therefore,

* Sylvester JC and RD Coghill : **The Penicillin Fermentation.** In : Industrial Fermentation, Vol. II (Underkofler LA and RJ Hickey eds). Chemical Publishing Co., Inc., New York, pp. 219-263, 1954.





The corresponding solution in an organic solvent may be back-extracted conveniently to its corresponding salt into an aqueous solution. This, in fact, constitutes the fundamental basis for the '**recovery**' as well as subsequent means of '**purification**' of **penicillin** from the respective duly harvested culture broths.

Production and Recovery : A general and basic flowsheet diagram for the large-scale recovery and purification of 'antibiotics' is illustrated in Fig. 3.21. The various steps that are usually followed in a sequential manner are described as under :

(1) Once the entire fermentative procedure is accomplished i.e., at harvest, the completed penicillin fermentation culture is subjected to filtration by the help of **heavy-duty rotary vacuum filter** so get rid to the mycelium plus other unwanted solid residues.

(2) The pH of the clear filtered fermented broth is carefully brought down between 2 to 2.5 by the addition of a calculated amount of either *phosphoric acid* (H_3PO_4) or *sulphuric acid* (H_2SO_4) so as to convert the resulting **penicillin** to its *organic form*, as shown above.

(3) The resulting fermented broth (pH 2 – 2.5) is **extracted immediately** by using a **Podbielniak countercurrent solvent extractor*** with an appropriate organic solvent *e.g.*, *amyl acetate, butyl acetate, or methyl isobutyl ketone*.

(4) Penicillin, thus obtained, is back extracted into aqueous medium from the corresponding organic solvent by the careful addition of requisite quantum of *KOH* or *NaOH* to give rise to the formation of the corresponding **potassium or sodium salt of the penicillin**.

(5) The resulting aqueous solution, containing the respective salt of penicillin, is again acidified and reextracted with the organic solvent **methyl isobutyl ketone**.

(6) In fact, these shifts taking place between '**aqueous**' and '**solvent**' medium help in the ultimate process of purification of the **penicillin**.

(7) The resulting solvent extract is finally subjected to a meticulous back-extraction with aqueous *NaOH* preferably, a number of times till extraction of penicillin is completed ; and from the combine of aqueous extractions different established procedures are adopted to affect the penicillin to crystallize out either as **sodium or potassium penicillin**.

(8) The crystalline penicillin thus obtained is washed, dried under vacuum, and the final product must conform to the requirements/specifications laid down by various **Official Compendia**.

*Casida L.E. Jr. **Industrial Microbiology**, New Age International Publishers, New Delhi, p 244, 2004

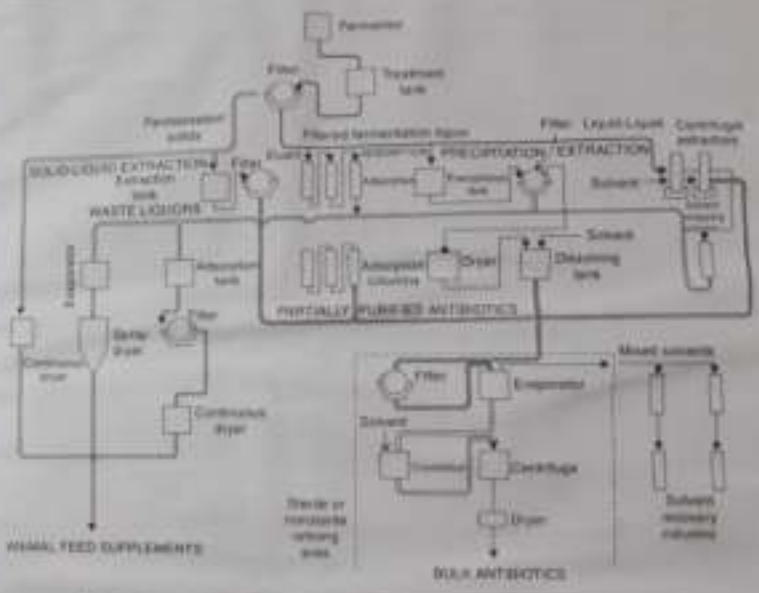


Fig. 3.21. General and Basic Flowsheet Diagram for Large-scale Recovery and Purification of Antibiotics

[Adopted From : Casida L.E. Jr. Industrial Microbiology, 2004].

Fig. 3.22 illustrates a typical '**antibiotic**' fermentation plant. In actual practice, the culture medium could be conveniently batched as well as sterilized in the fermentor itself. Nevertheless, most of the fermentors are attached to a batching vessel and subsequently to the respective sterilizers as given in the above figure. The various feed vessels duly connected to the final-stage fermentor are invariably employed to supplement both *nutrients* and *precursors* during the on going fermentative process. Importantly, the **seed fermentor** and the **final-stage fermentor** should be operable under stringent aseptic environments. The **bioreactors** are made of SS, having a capacity ranging between 30 and 300 m^3, agitation by 2/3 flat-paddled impellars, aeration done with compressed sterile air injection, generated heat dissipated by employing chilled-water cooling coils (maintained at 26 ± 2°C). Sterilization of the system done with live-steam injection ports adequately.

Fig. 7.25. A Typical Antibiotic Fermentation Plant

[Adapted from : Gupta PK. Biotechnology and Genomics. 2004]

7.2. Streptomycin

Streptomycin is produced by either of the two *Streptomyces* species, namely : *S. griseus* and *S. griseolus*. The antibiotic is particularly active against Gram –ve bacteria e.g., *Mycobacterium tuberculosis*. Besides, it is also found to exert its activity against Gram +ve bacteria, and used therapeutically for curing infections inflicted by such organisms that distinctly show resistance to **penicillin.**

The wonderful epoch making discovery of **streptomycin** was meticulously carried out by three prominent researchers : Schatz, Bugie and Waksman (1944) ; and one of their first and foremost acts written (Training No. 18.18) derived from *S. griseus* was, in fact, a mixture proven still being employed

largely as **industrial strains** across the globe even today. However, constant endeavour in selective screening and purification of various strains articulately helped to enhance excellent practically achievable yields of today.

7.2.1. Chemical Structure

The chemical structures of two basic compounds, viz., **streptomycin** and **dihydrostreptomycin** are as given below :

STREPTOMYCIN

Dihydrostreptomycin may be prepared by the chemical reduction of the carbonyl moiety present in the *L* streptose segment of the **streptomycin** molecule as shown above.

The various salts of **streptomycin** and **dihydrostreptomycin** are as stated below :

Streptomycin

(i) Trihydrochloride : $C_{21}H_{39}N_7O_{12} \cdot 3HCl$

(ii) Trihydrochloride-calcium : $(C_{21}H_{39}N_7O_{12})_2 \cdot CaCl_2$
chloride double salt

(iii) Pamothenate : $C_{21}H_{39}N_7O_{12} \cdot C_9H_{17}NO_5$

(iv) Sesquisulfate : $(C_{21}H_{39}N_7O_{12})_2 \cdot 3H_2SO_4$

Dihydrostreptomycin

(i) Trihydrochloride : $C_{21}H_{41}N_7O_{12} \cdot 3HCl$

(ii) Sesquisulfate : $(C_{21}H_{41}N_7O_{12})_2 \cdot 3H_2SO_4$

(iii) Pamothenate : $C_{21}H_{41}N_7O_{12} \cdot C_9H_{17}NO_5$

Besides **streptomycin**, some other forms also exist as the ensuing fermentation products by *S. griseus* as given below :

S.No.	Streptomycin Variants	Organism Used	Remarks
1.	Streptomycin	*S. griseus* ; *S. bambergiensis* ; *S. reticuli*	Highly active
2.	Mannosidostreptomycin* (Streptomycin B)		Less antibiotic activity
3.	Hydroxystreptomycin (Reticulin)	*S. reticuli*	—do—
4.	Mannosidohydroxy streptomycin	*Streptomyces* spp.	—do—

7.2.2. Choicest Medium

The choicest medium for the fermentative process of streptomycin production essentially consist of :

(a) **Carbon Source** : *e.g.*, dextrin, glycerol, glucose, starch, and similar economically cheap substances.

(b) **Nitrogen Source** : *e.g., naturally occurring processed agricultural products* : cotton seed meal, soyabean meal, corn/crep liquor solids, casein-hydrolysate, yeast and its products, meat extract ; and *pure inorganic salts* : ammonium sulphate [$(NH_4)_2$ SO_4], ammonium nitrate [NH_4NO_3].

(c) **Vegetable/Animal Fat** : *e.g.* soyabean oil, linseed oil, fatty acids having more than 4 carbon chain lengths plus their corresponding esters, and lard oil.

Two research groups, almost over a gap of a decade suggested typical industrial media best suited for the fermentation of streptomycin as given below :

S.No.	Ingredients	Woodruff and McDaniel[1]	Hockenhull[2]
1.	Soyabean meal	1%	—
2.	Glucose	1%	2.5%
3.	Sodium chloride	0.5%	0.25%
4.	Extracted soyabean meal	—	4%
5.	Distiller's dried solubles	—	0.5%
6.	pH (Before Sterilization)	—	7.1-7.5

1 : 1954 ; 2 : 1963 ;

* Depending on the choice of strain of organism actually employed or on the production medium used, in normal fermentative process a small quantum of mannosidostreptomycin is produced specifically in the initial stages of fermentation ; however, the same gets mostly enzymatically degraded by organism 1 prior to **Streptomycin** by the time of harvest.

7.2.3. Inoculum

High yielding *Streptomyces griseus* spores accomplished through meticulous mutation procedures are stringently maintained either **soil stocks*** or duly **lyophilized** in an appropriate carrier, for instance *sterile skimmed milk*. Consequently, the spores obtained from these **stock cultures**** are carefully inoculated into a *sporulation medium* strictly under aseptic conditions. It has been observed that *6 duly caters* for sufficient **'sporulated growth'** to gainfully initiate the much desired **liquid build-up of** mycelial inoculum in flasks or inoculum tanks respectively.

7.2.4. Streptomycin Production

Preamble : Streptomycin production outputs in **bioreactors** invariably respond overwhelmingly to relatively high degree of aeration as well as agitation. It has been duly established that the **'optimum production parameters'** for **streptomycin** are :

Fermentation temperature	: Varies between 25-30°C (~ 28°C)
pH	: Ranges between 7-8 (Max. between 7.6-8)
Duration	: Varies between 5-7 days (yield > 1200 mcg . mL^{-1})

Importantly, **streptomycin** is *fairly rough and tough* and hence hardly gets destroyed by the presence of contaminating microorganisms as is the case with *penicillin*. Nevertheless, contaminants definitely minimize yields to a considerable extent.

Note : (1) The actinophage infections may prove to be harmful and serious in nature for both the inoculum and production vessels, because the *streptomycete* rapidly undergoes cleavage thereby reducing yields substantially.

(2) Development and application of *'mior-made'* strains of *S. griseus*, specifically resistant to certain more common phages, are being used nowadays globally.

Production : The classical and widely promulgated commercial fermentation operation for the production of **streptomycin** essentially passes through *three* cardinal phases, namely :

Phase-I : It extends upto only *24 hours* wherein the rapid growth commences producing the large proportion of mycelium required for the fermentation. The highly *energized proteolytic* characteristic property of *S. griseus* predominantly sets free NH_3 right into the medium from the soyabean meal, and thus the carbon-enriched nutrients present in the soyabean meal are adequately consumed for the vigorous progressive growth. Nevertheless, the glucose up-take of the medium is rather on a very low ebb during this particular phase, and perhaps that could be the reason for reasonably lower (slight) streptomycin production. Interestingly, the ensuing pH of the medium rises from 6.7/6.8 to nearly 7.5 or so.

* One must seriously consider the maintenance of **'stock cultures'** based upon the fact that the high yielding mutated strains of *S. griseus* are **genetically unstable.**

** **Stock Cultures** : These are maintained very carefully (*e.g.*, by lyophilization) that essentially require transfer as infrequently as possible, as repeated transfers may ultimately select only those cells of the organism that are rather poor generators of antibiotic.

Phase-2 : It is the most crucial and critical stage wherein during this phase streptomycin is [illegible] generated at a tremendously rapid rate that usually extends from 1 day to almost 6/7 days of [illegible] under perfect sterile environment. Because there is little growth of mycelium ; and hence, the weight of mycelium almost remains constant. In fact, three events take place precisely in this specific phase, namely : (a) NH_3 is fully consumed ; (b) glucose also being used up to the maximum extent ; and (c) pH stands constant between 7.6 to 8.

Phase-3 : With the virtually complete depletion of 'sugar' from the fermentation medium, the **streptomycin production** almost comes to a standstill situation. At this point in time, the [illegible] fermentation is invariably harvested before the commencement of this phase of **senescence** *i.e.*, the *period of old age*.

Harvest-Recovery-Purification : Once the fermentation attains completion, the **[illegible] mycelium** is duly separated from the ensuing fermented broth by filtration ; and thus, the **streptomycin** is finally recovered by one of the two methods described below based on the specific individual [illegible].

Method-I : The **streptomycin** produced is adequately **adsorbed** from the fermented **broth** onto activated carbon particles, and subsequently subjected to elution from the carbon particles **by means of** diluted mineral acid till **streptomycin gets eluted** almost completely. The eluted product is precipitated by suitable solvents, filtered, and dried under vacuum before further purification.

Method-II : Fermentation broth is first acidified and subsequently filtered and neutralized. The resulting clear broth is forced via a *packed column* of **cation-exchange resin** to allow the **adsorption of streptomycin** on it completely. The **column** is washed with water (DM) thereafter the completion of **adsorption**, and finally eluted with diluted HCl, and the liquid containing **streptomycin** is concentrated under *vacuo* almost to dryness. The crude antibiotic is dissolved in methanol and filtered, and acetone is now added so as to allow the complete precipitation of **streptomycin**. In the final treatment the ensuing precipitate is washed thoroughly with acetone and dried in *vacuo* before being solubilized in MeOH for the ultimate preparation of the desired **streptomycin-calcium chloride complex** in its purest form.

Note : *The final product obtained either from Method I or II must rigidly conform to the stand[ards] of purity and assay as prescribed in the* ***Official Compendia***.

7.3. The Tetracyclines

The epoch-making discovery of chlortetracycline (aureomycin) in 1947 by Duggar paved the way for a number of structural analogues used as broad spectrum antibiotic that belong to the tetracycline family. The tetracyclines which are found to be effective therapeutically are listed in the following table.

7.3.1. Salient Features of the Tetracyclines

Name of Compound	Official Status	Brand Name(s)	R_1	R_2	R_3	R_4	R_5
Tetracycline	BP ; (1973) ; USP ;	Tetracyn® (Pfizer) SK-Tetracycline® (SK & F)	H	OH	CH_3	H	H
Oxytetracycline	USP ;	Terramycin® (Pfizer)	OH	OH	CH_3	H	H
Chlortetracycline HCl	BP, USP ; Eur. P ; Int. P ; Ind. P ;	Aureomycin® (Lederle)	H	OH	CH_3	Cl	H
Demeclocycline HCl	BP, USP ; Eur. P ;	Ledermycin® (Lederle, UK)	H	OH	H	Cl	H
Methacycline HCl	BP (1973) ; USP ;	Rondomycin® (Wallace)	OH	=	CH_2	H	H
Doxycycline	USP ;	Vibramycin® (Pfizer)	OH	H	CH_3	H	H
Rolitetracycline	USP ;	Syntetrin® (Bristol)	H	OH	CH_3	H	$-CH_2-N$ [illegible]

7.3.2. Nomenclatures

Based on the above conventional numbering of various carbon atoms and subsequent labelling of the four aromatic rings present in the tetracycline nucleus, oxytetracycline is chemically designated as :

"4-Dimethylamino-1, 4, 4a, 5, 5a, 6, 11, 12a-octahydro-3, 6, 10, 12, 12a-penta-hydroxy-6-methyl-1, 11-dioxo-2-naphthacenecarboxamide"

Some other members of the tetracycline family may conveniently be named as follows :

Methacycline : 6-Methylene-5-oxytetracycline ;

Doxycycline : α-6-Deoxy-5-oxytetracycline ;

Rolitetracycline : N-(Pyrrolidinomethyl)-tetracycline.

7.3.3. General Characteristics of the Tetracyclines

Following are the general characteristic features of all the members of the tetracycline family :

(a) The tetracycline are obtained by fermentation procedures from streptomyces species or by the chemical transformations of the natural products.

(b) The important members of this family are essentially derivatives of an octahydronaphthacene, *i.e.*, a hydrocarbon made up of a system of four-fused rings.

(c) The antibiotic spectra and the chemical properties of these compounds are quite similar but not identical.

Tetracycline* is the drug of choice in the treatment of chlamydia, relapsing fever, granuloma [illegible] and infections produced by rickettsia, *Borrelia, Mycobacterium fortuitum and marinum*, and *Chlamydia* [illegible] *psittaci and trachomatis* (except pneumonia and inclusion conjunctivitis).

It may be employed as an '**alternative drug**' in the following two situations, namely :

(i) With silver nitrate in the prevention of neonatal ocular prophylaxis of chlamydia [illegible] gonococcal conjunctivitis, and

(ii) For treatment of actinomycosis, anthrax, chancroid, melioidosis, plague, rat-bite fever [illegible] [illegible] and yaws.

It has also been reported to be beneficial in the treatment of toxoplasmosis.

Tetracycline Production

Tetracycline is *produced on large scale using the submerged fermentation process* by [illegible] dominant strains employed across the globe, namely : (a) *Streptomyces aureofaciens* [**ATCC** : [illegible] 13911 and **NCI. B** 9514]. In actual practice, *the stock cultures* are adequately maintained for [illegible] long duration in the shape of **spores**. Importantly, the resulting spores are maintained [illegible] under liquid N_2 (– 70 to – 80°C) or **lyophilised**.** The '**bioreactor**' being employed is normally [illegible] of stainless steel along with SS connecting pipes, two-or three-way SS gate valves, SS-**pumps** [illegible] **Level** [illegible] provided with adequate agitation and compressed sterile air circulation. Besides, [illegible] have all the necessary gadgets and recording devices meant for round-the-clock **monitoring** [illegible] main fermentation operation. Various physical and physiological parameters of the **culture media** [illegible] **bioreactor** need to be controlled automatically, such as : pH regulation, **supplementation** [illegible] nutrients during the fermentation run etc. In addition, a number of vital and critical tests are [illegible] carried out for the optimum growth of the antibiotic (**tetracycline**), for instance : **strength of** [illegible] morphology and growth of culture, antibiotic production, and sterility conditions.

Note : *Sterilization of the* '**liquid nutrient media**' *is normally carried out at 120°C for a period of 40 minutes.*

Culture Medium : *It has been observed that the overall tetracycline production is solely* [illegible] *erned by the ensuing C : N ratio of the nutrients* (*i.e.*, *nutrients in the culture medium*). However, in [illegible] practice the various components that essentially provide **carbon sources** are, namely : *starch, sucrose and glycerol* ; and **nitrogen sources** are, namely : *admixture of soyabean meal plus mineral salts, ammonium salts, amino acids, casein (milk-protein), meat-extract (animal-protein)* etc. Besides, [illegible] usable medium also comprises of **cotton-seed meal, peanut meal, cornsteep liquor** etc. It is absolutely important as well as necessary to stringently maintain very **low concentration of Cl^- ion** in the medium so as to accomplish **high production levels**. Deionized cornsteep liquor and similar raw materials [illegible] from Cl^- ions may also be used gainfully. The optimum temperature should be 28°C and pH must [illegible] between 5.5 to 6.5 (ideally 5.8 to 6.0).

Inoculum : The basic inherent characteristic features of the ensuing **inoculum** do play a [illegible] role for the biosynthetic production of **tetracycline**, such as : quality of vegetative inoculum or [illegible] (*i.e.*, its threshold age, genetic homogeneity, metabolic status profile etc. Optimum tetracycline [illegible] duly accomplished from a medium that predominantly comprises of **inoculum** for 24 hours, and [illegible] within a range of 2-10% having an optimum pH value between 5.6 and 6.2.

* Kar A., **Medicinal Chemistry**, New Age International Publishers, New Delhi, 3rd. edn., 664-667, 2007.

** Rapid freezing of a substance at an extremely low temperature and then dehydrating the substance in a high vacuum. [Synonym : Freeze-Drying].

Aeration : It is quite necessary and equally important to make available both vigorous and [illegible] [illegible] **aeration** in the submerged cultures of *Streptomyces aureofaciens* right from the very initial stage of [illegible] [illegible] phenomenon. Any observed irregular aeration or interruption in aeration during the **first few hours** invariably give rise to an appreciable extent of production of **tetracycline**.

Tetracycline Production : The tetracycline production is carried out in submerged aerated agitated bioreactor containing adequate sterile culture medium loaded with genetically homogenous and metabolically reactive inoculum. It essentially comprise of *three distinct and vital stages*, namely :

Stage-1 : Growth Phase : It is mainly characterized by instant fast utilization of incorporated nutrients. There is a distinct enhancement of the **cell mass**. The phosphate (PO_4^{3-}) ion concentration has an enormous influence upon the prevailing culture medium. Interestingly, during the on-going production phase of the tetracycline fermentation procedure the **secondary mycelium** (*i.e.*, *the thin hyphae*) is found to modulate the specific phosphate ions present in the culture medium. However, the '*production type of the mycelium*' is hardly generated particularly in the overwhelmingly presence of the PO_4^{3-} ions.

Stage-2 : Production Phase : In this particularly phase the maximum quantum of the antibiotic is actually generated. Thus, the overall rate of growth of the concerned microorganism **gets decreased** substantially and almost ceases in due course.

State-3 : This is, in fact, the last phase wherein the **production of the** tetracycline almost attains the lowest ebb. The mycelium undergoes due fragmentation and the process of cleavage commences apparently.

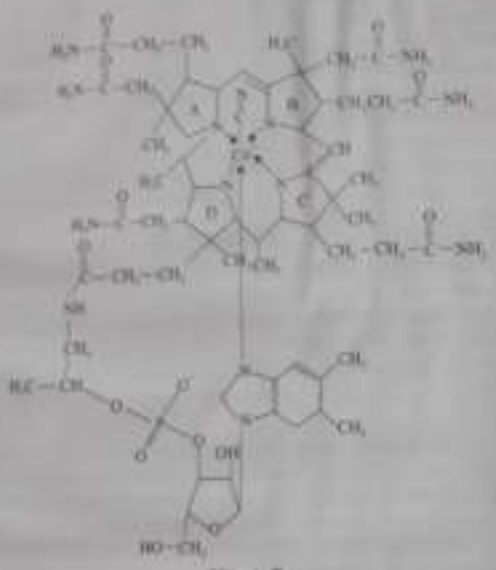
Isolation and Purification : **Tetracycline** is invariably obtained from the **clear filtrate** obtained from the acidic medium (*i.e.*, the fermented broth) by the help of sterilized SS Plate Type Filter Press or Podbielniak Counter Current Extractor (as mentioned under 'penicillin'). The clear filtrate is subjected to a process of adsorption upon an active substance *e.g.*, activated carbon, and elution subsequently. The eluted liquid is treated with a requisite amount of salts of alkaline earth metals to obtain the **precipitate of tetracycline** as its corresponding salts. The resulting salt is poorly water soluble, but fairly soluble in several organic solvents. Once the organic phase is separated, the **tetracycline** is adequately pushed into the aqueous phase (by the addition of diluted HCl). Finally, the **purified form of tetracycline** is salted out or crystallized carefully, and dried **under vacuo**.

7.4. Vitamin B_{12} (Cyanocobalamine : Cobamide)

Vitamin B_{12} is produced commercially by the aid of a **direct fermentation procedure** using *Streptomyces* species, for instance : *Streptomyces olivaceus*.

Rickes *et al.** (1948) first and foremost recovered the active crystalline **vitamine B_{12}**, as given below from a *S. griseus* culture (that also eventually produced the antibiotic **resin**).

* Rickes EL *et al. Science*, **108** : 634-635, 1948.



Vitamin B_{12}

Prolonged intensive and extensive researches have adequately proved and revealed that a too *small quantum* of vitamin B_{12} could be synthesized by a host of microorganism variants belonging specifically to *actinomycetes* and bacteria ; and that relatively *'large quantum'* was prevalent particularly amongst the microorganisms pertaining to the *intestinal habitats*.

In general, cobamides (vitamin B_{12}) essentially comprise of a *'porphyrin nucleus'* to which its **ribose** and **phosphate** residues are attached strategically. Nevertheless, the cobamide variants do differ in their corresponding **purine, benzimidazole** or other base located in the **nucleotide-like segment of the molecule** ; and besides, in the chemical functional moiety duly attached to the Co atom.

The various steps involved in the production of Vitamin B_{12} are enumerated sequentially as stated below :

(1) *S. olivaceus* is allowed to grow with adequate constant aeration at 27°C *preferably* in a nutritionally rich crude-medium having **glucose** as a major source of **carbon.**

(2) A potential source of cobalt (Co) between 2-10 ppm is duly incorporated into the above medium in the form of its salt cobalt chloride ($CoCl_2 . 6H_2O$) to serve as a **precursor.** These

the *actinian organisms* meticulously scavenges low levels of cobalt from the prevailing medium, whereas the respective higher levels are proved to be toxic in nature.

(3) Total duration of the fermentative process lasts between 3 to 4 days or until such time when **mycelium lysis** commences to take place. In this manner, a major segment of the vitamin B_{12} produced remains very much concealed within the microbial cells until **autolysis*** comes into force ; and, therefore, the recovery of the vitamin from the **'fermentation broth'** is tremendously simplified by actually initiating harvesting before autolysis has turned out to be adequately extensive, a situation where **vitamin B_{12}** is still lodged within the mycelium actually.

(4) At harvesting stage, both the whole and the mycelium are duly *filtered* or *centrifuged* to separate them from the ensuing **'fermentation broth'**, and dried subsequently for an appropriate usage in the form of either **vitamin B_{12} enriched** animal or poultry feed supplements.

(5) **Alternative Method of Recovery** : In this specific instance, the **vitamin B_{12}** is predominantly released from the concerned cells by several tried and tested methodologies, namely : *alcohol treatment, heat, acidification* etc.

Example : The adequately completed **'fermentation broth'** is first acidified, treated with sodium sulphite so as to protect the vitamin, and finally the admixture of *culture* and *fermentation* broth is subjected to careful heating by employing steam heated coils with agitation or passing low-pressure steam slowly with proper agitation.

(6) The *'solid residues'* as well as *'spent mycelium'* are duly separated either by 'filtration' or *'centrifugation'*, and the resulting clear fluid thus obtained is meticulously evaporated strictly **under superb vacuum facilities.**

Note :

(1) Main bulk of vitamin B_{12} is used as such in solid dosage forms and liquid preparations.

(2) Relatively small proportion of the vitamin B_{12} is further purified and crystallized for the exclusive treatment of pernicious anemia and other vital medicinal usage via the IV route of administration.**

Latest Method of Production : The latest method adopted for the present-day commercial production of **vitamin B_{12}** is usually carried out by adequately aerated submerged bacterial fermentation making use of strains of *Propionibacterium* or *Pseudomonas* with a beet-molasses based culture medium and the adequate supplementation with requisite amount of the cobalt salts.

Vitamin B_{12} may also be produced on a large-scale by using *Bacillus megaterium*, and the overall recovery from this fermentative procedure almost approaches quite similar to those obtained from the *Streptomyces* organisms.

The details of commercial production from *Propionibacterium shermanii* and *Pseudomonas denitrificans* are described as under :

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* The self-dissolution or self-digestion which occurs in cells by enzymes in the cells themselves.

** A chronic, macrocytic anemia marked by achlorhydria. It mostly occurs in 40 to 60 year old northern Europeans with fair skin, but has been reported in other races as well and ethnic groups. It is rare in blacks and Asians.

7.4.1. Vitamin B_{12} from *Propionibacterium Shermanii*

There are, in fact, *three* different types of medium that are skillfully used in the production of vitamin B_{12} from *P. shermanii*, such as : (a) *maintenance medium* ; (b) *seed-culture medium* ; and (c) *main-culture medium*. All these media shall now be treated individually in the sections that follow.

(a) **Maintenance Medium :** The **maintenance medium** for *P. shermanii* essentially includes per lt. the various ingredients as : sucrose 50 g ; yeast extract — 10 g ; filtered horse-radish juice — 200 g ; and agar 10 g. The pH of the prepared medium is adjusted to 7.2. The inoculated media is duly incubated for a duration of 96 hours at 30°C.

(b) **Seed-Culture Medium :** The **seed-culture medium** is usually of different types which are prepared according to the following two stages, namely :

(1) **First Stage Medium :** It is very much identical in composition to the maintenance medium and is precisely devoid of agar. It is unusually incubated for a duration of 48 hours at 30°C without any agitation whatsoever.

(2) **Second Stage Medium :** The exact composition of the second stage medium, lt. is as follows : containing liquor solids — 20 g ; glucose — 90 g ; and the pH is maintained at 6.5. In general, the medium is duly incubated for 24 hours at 30°C devoid of any aeration, and pH is adjusted to 6.5.

(c) **Main-Culture Method :** The main-culture (*i.e.*, production) media essentially comprises of the following ingredients in lt. : containing liquor solids — 40 g ; glucose — 100 g ; cobalt chloride [$CoCl_2 \cdot 6H_2O$] — 0.02 g ; and the pH adjusted to 7.0. It is usually incubated at 30°C. Nevertheless, the first phase of 80 hours is allowed to carry on without aeration, but with slight introduction of N_2 with agitation. Later on, a slight aeration to the tune of 0.1 v/v/m is activated, and pH is adjusted to 7.0.

It has been observed that *propionibacteria* are invariably grown/cultivated upon carbohydrate-based media specifically and that too in an **anaerated environment.** However, the cobalt supplement is an absolute necessity for vitamin B_{12} production. Besides, it also solely depends upon either the external generation or external supply of **5, 6-dimethyl benzimidazole** (or 5, 6-DBI). Importantly, the mutant strained *P. shermanii* are capable of synthesizing their own 5, 6-DBI, which ultimately enhances the yield of vitamin B_{12} to an extent of 65 mg . L^{-1} in a pilot scale.

It is, however, pertinent to state here that the 'aeration' definitely augments the formation of 5, 6-DBI, whereas it distinctly lowers the vitamin B_{12} biosynthesis at one of its various steps. Therefore, it is quite necessary and equally vital that the very first stage (80 hours) the fermentative process in the bioreactor must be carried out predominantly in an **anaerobic environment,** but a little agitation is still necessary until the main bulk of the carbohydrate present in the media is fully consumed for the growth and the ultimate formation of **cobamide.** Of course this kind of sequential steps will exert hardly any undue effect. The subsequent follow up stage (next 48 hours) is supplemented with *moderate* agitation and slight aeration. The aeration afforded at this stage essentially reduces the biosynthetic pathway of 5, 6-DBI, whereby the resulting cobamide gets converted ultimately to **cobalamin.**

7.4.2. Vitamin B_{12} from *Pseudomonas Denitrificans*

Quite recently a plethora of mutant strain variants have been duly developed that are prominently based upon the original wild-type of *P. denitrificans* used for the large-scale production of **vitamin B_{12}**

biosynthesis, it essentially requires three different types of media, such as :

(a) **Laboratory-Scale Medium :** Precisely the medium required externally for the laboratory-scale fermentative process for producing vitamin B_{12} from *P. denitrificans* comprises in lt. the following constituents : beet molasses — 60 g ; brewer's yeast — 1 g ; N_2 amine — 1 g ; ammonium phosphate [$(NH_4)_2HPO_4$] — 2 g ; magnesium sulphate [$MgSO_4 \cdot 7H_2O$] — 1 g ; manganese sulphate [$MnSO_4 \cdot 2H_2O$] — 2 g ; zinc sulphate [$ZnSO_4 \cdot 7H_2O$] — 0.02 g ; manganese sulphate [$MnSO_4 \cdot 2H_2O$] — 2 g ; zinc sulphate [$ZnSO_4 \cdot 7H_2O$] — 0.02 g ; sodium molybdate [$Na_2MoO_4 \cdot 2H_2O$] — 51.5 g ; agar — 25 g ; and pH adjusted to 7.4. The inoculated laboratory-scale medium is duly incubated at 28°C for 96 hours.

(b) **Seed-Culture Medium :** It has almost the identical composition as stated in (a) above, but it is devoid of agar. It is incubated at 28°C on a '*rotary shaker*' for a duration of 72 hours.

(c) **Production-Culture Medium :** It consists of the following ingredients in lt., namely : beet molasses — 1000 g ; yeast — 2 g ; ammonium hydrogen phosphate [$(NH_4)_2HPO_4$] — 5 g ; magnesium sulphate [$MgSO_4 \cdot 7H_2O$] — 3 g ; manganese sulphate [$MnSO_4 \cdot 4H_2O$] — 0.3 g ; cobalt nitrate [$Co(NO_3)_2 \cdot 6H_2O$] — 0.168 g ; zinc sulphate [$ZnSO_4 \cdot 7H_2O$] — 0.02 g ; sodium molybdate [$Na_2MoO_4 \cdot 2H_2O$] — 51.5 g ; 5, 6-DBI — 0.025 g ; and pH is adjusted to 7.4. The prepared media is duly incubated at 29°C for a duration of 96 hours with constant agitation of 420 RPM and rate of aeration at 1 v/v/m.

Source of Carbohydrate

In actual practice, the sugarbeet molasses loaded with 5 to 10% **betaine** (*i.e.*, trimethyl glycine) serves as an ideal source of carbohydrate. It also stimulates the production of **vitamin B_{12}** by promoting the synthesis of *in synthesize*. It has been duly observed that it specifically aids in the production of **δ-aminovulinic** acid (*i.e.*, the very first and foremost intermediate in the **cobalamine biosynthesis.**

It is pertinent to mention here that the aforementioned **cobalamin biosynthesis** by the organism *P. denitrificans* essentially require the external supply of 5, 6-dimethyl-benzimidazole [*i.e.*, 5, 6-DBI] and cobalt salt.

Fermentation Phenomenon

The process of fermentation involving the growth of the *Pseudomonas* and the ensuing biosynthesis of the **vitamin B_{12}** particularly needs moderate aerated environment along with adequate agitation in the **'bioreactor'.**

8. FUTURE PROSPECTS

Biotechnological processes may be broadly viewed from two distinct and acceptable angles, namely : *first*, to a **small extent** which grossly need to be confined within a specifically well-defined area or system ; and *secondly*, to **large extent** wherein the ultimate eventful grand success of a plethora of the processes shall exclusively depend on the rationalized correct choice and meticulous operation of these systems. Most desirously in an industrial environment, the expected level and scale of operation will, certainly for realistic economic reasons, significantly, be substantially enormous ; and, therefore, invariably in all instances the ultimate success will evidently requires the **closest cooperation** between the *process engineer* and the *bioscientist* thereby proving explicitly the most realistic interdisciplinary nature of the newer **biotechnological processes.**

The latest trend has just gained the momentum for the **overall improvement of various strains** via the spectacular application of **molecular genetics.** It is now possible to manipulate certain rar-

[illegible] **production efficiency** [illegible] **transcriptional** and **regulatory** mechanisms [illegible] of both foreign and native genes [illegible] X-ray crystallographic analysis of the [illegible] permit the synthesis of a much wider range of **bioactive precursors** and **corresponding metabolites**.

[illegible] these **optimistic projections** are [illegible] supported by evidence upon a [illegible] of the application of recombinant DNA technology to the production of **antibiotics**. [illegible] due to the rapid spread of antibiotic-resistant organisms [illegible] new classes of antibiotics [illegible] of discovering and developing these newer breed of antibiotics on the other hand — is [illegible] globally.

In nutshell, [illegible] the problem of prevailing **'antibiotic resistance'** [illegible] research into the development of newer antibiotics [illegible] and the full blast impact of **molecular genetics** shall be brought [illegible] to bear on the problem.

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PROBABLE QUESTIONS

1. (a) What are **'antibiotics'** ? Name the **three** well-known methods for their large scale production quoting specific examples. Elaborate briefly on the **'antibiotic development'**.
   (b) Write short-notes on any **three** of the following :
   (i) **Specific Tests** for identification of pathogens.
   (ii) Laboratory diagnosis for viral infections.
   (iii) Soil as the **'Best Available Source of Antibiotics'**.
   (iv) Detection of Microorganisms by colour change.
   (v) Crowded-plate Technique and its Limitations.
2. (a) Discuss **'Secondary Screening'** and its importance in **Antibiotics.**
   (b) Differentiate between **'Agar-Plate Method'** and **'Liquid Culture Method'** briefly.
   (c) Elaborate the various **'Salient Features'** of Secondary Screening.
3. (a) What are **Fermentors** (or **Bioreactors**) ?
   (b) Give a brief account of the **three** major commercial byproducts derived from the **bioprocessing technology** together with some typical examples.
   (c) Describe the various **'Salient Features'** of **Bioreactors.** Support your answer with appropriate examples.
4. (a) What are the major differences between the **'Anaerobic Fermentation'** and the **'Aerobic Fermentations'** ?