Natubhai V. Patel College of Pure & Applied Sciences
B.Sc. Semester VI
Industrial chemistry/ Industrial chemistry (Vocational)
US06CICH02/US06CICV05: Pharmaceuticals – II
UNIT – 4

SYLLABUS

Microorganisms: Structure, classification, Growth and Usefulness.
Fermentation: General principle of fermentation process and product processing. Manufacturing of Penicillin, Tetracyclins and Vit-B12

FERMENTATION

4.0 INTRODUCTION

The term fermentation (Latin fermentare to boil) formally stood for **decomposition of foodstuff** usually accompanied by **evolution of gas**. The fermentation of sugars to alcohols and carbon dioxide by yeast is one of the oldest examples. The term **fermentation** is now applied to changes brought about by microorganisms. Evolutions of gas are not an essential criterion. In our daily life we see many complex chemical reactions; which are brought about by the agency of living organisms. **Examples** are souring and curdling of milk, putrefaction of meal, production of indigo dye from the compound indicate in the food , curing of tobacco, the development of benzaldehyde or oil of biter almonds from the amygdaline contained in the almond seed, conversion of fruit juice into wines etc. All these **processes** are called **fermentation processes**, in which complex organic material is broken down into smaller substances and decomposition it brought about by the action of living organisms, which secretes the enzyme catalyst, suitable to the process.

Although the fermentation of fruits to alcohol and making of beverage out of fruits and grain have been established for centuries, it is only during the past generation that the wider application of fermentation has been recognized. Now man is directing the life process of microorganisms the yeasts, bacterial and moulds to the production of large number of chemicals, such as alcohol, acetone, acetic acid, lactic acid citric acid and many antibiotics which are of great synthetic as well as industrial important.

4.1 DEFINATIONS

1. **Aerobe:** An organism that can use oxygen as an electron accepter at the terminus of an electron-transport chain can grow at a level of O_2 equivalent to or higher than that present in an air atmosphere (21 %) and has a strictly respiratory type of metabolism.
2. **Agar:** A dried polysaccharide extract of red algae, used as a solidifying agent in microbiological media.
3. **Anaerobe:** An organism that does not use O_2 to obtain energy, that cannot grow under air atmosphere, and for which O_2 is toxic.
4. **Culture:** A population of microorganisms cultivated in a medium.
5. **Inoculum:** The substance, containing microorganism or other material that is introduced in inoculation.
6. **Inoculation:** The artificial introduction of microorganisms or other substances into the body or into the culture medium.
7. **Microorganism:** Any organism of microscopic dimension.
8. **Sterilization:** The process of killing of all forms of life.
9. **Medium (media):** A substance used to provide nutrients for the growth and multiplication of organisms.
10. **Lyophilisation:** The preservation of biological specimens by rapid freezing and rapid dehydration in a high vacuum.
11. **Incubation:** The subjecting of cultures of microorganisms to conditions favourable to their growth (e.g. Temp).

4.2 PREREQUISITES OF A GOOD FERMENTATION PROCESS

- A microorganism that formed a desired end product. This organism must be readily propagated and be capable of maintaining biological uniformity, thereby giving predictable yield.
- Economical raw materials for the substrate, e.g. starch or one of several sugars.
- Acceptable yield.
- Rapid fermentation.
- A product that is readily recovered purified.

4.3 NUTRIENTS FOR MICORORGANISMS

All living organisms, microorganisms are the most versatile and diversified in their nutritional requirements. Human and other animals require certain type of complex carbon containing compounds as nutrients, while microorganisms may not. Some microbes can grow with just a few inorganic substances as their sole nutritional requirements, while other microorganisms are like higher organisms in their need for complex organic compounds. But all living organisms share some common nutritional needs, like the need for carbon, nitrogen, and water.

Water is particularly important to microorganisms, besides most of microorganisms can absorb nutrients only when the chemicals are dissolved in water.

4.3.1 Chemical elements as nutrients

All the microorganisms need a variety of chemical elements as nutrients. These elements are necessary for both the synthesis and the normal functions of cellular components. The **main elements** for cell growth include carbon, nitrogen, oxygen, sulphur and phosphorous.

1. Carbon

Carbon is one of the most important chemical elements required for microbial growth. Carbon forms the backbone of three major classes of organic nutrients: Carbohydrates, lipids and proteins. Such compounds provide energy for cell growth and serve as building blocks of cellular material.

2. Nitrogen

All organisms also require nitrogen in some form. This element is an essential part of amino acids that together form proteins.

3. Hydrogen, oxygen, sulphur and phosphorous

Hydrogen and oxygen form part of many organic compounds. Sulphur is needed for the biosynthesis of amino acids cysteine, cystine and methionine. Phosphorous is essential for the synthesis of nucleic acid and adenosine triphosphate (ATP), a compound that is extremely important for energy storage and transfer.

Many **other essential elements** are required, though in smaller amounts than the elements already listed. These may facilitate the transport of materials across cell membranes e.g. Na^+ is required by the permease that transport the sugar meliboise into cells of Escherichia Coli.

4.4 CONDITIONS FAVOURABLE FOR FERMENTATION OR FACORS AFFECT THE RATE OF FERMENTATION OR PHYSICAL CONDITION FOR CULTIVATION OF MICROORGANISMS

Four main conditions influence the physical environment of a microbe are temperature, pH, gaseous atmosphere and osmotic pressure.

The successful cultivation of a various type of microorganisms requires a combination of the proper nutrients and the proper physical environment.

4.4.1 Temperature

Temperature has great influence on the growth of microorganisms. This is not surprising, since all the processes of growth are dependent on chemical reactions that are affected by temperature. Unlike mammalian cells, which grow within a relatively narrow

temperature range (close to 37° C), microorganisms require rather broad range of temperature. However, this range may be wider for one than for others e.g. the range for **Bacillus subtilis** is from 8 to 53°C, a 45^0C range; for **Neisseria genorrhoeae** is from 30 to 40°C, a 10° C range

At the most favourable temperature for growth, the **number of cell division** per hour, called the **growth rates** generally **doubles for every temperature increase** of to that of most enzyme catalyzed reactions, supporting the principle that growth is the result of series of integrated, enzyme-based chemical reactions. The temperature at which a species of the microorganisms grow most rapidly is the optimum growth temperature.

For many particular microbes the **three important temperatures** are the minimum, optimum and maximum growth temperature. These are known as the **cardinal temperature** of a particular microbial species may vary with the stage in the life cycle of the microorganisms and with the nutritional contents of the medium.

The optimum temperature for microbial species does not lies midway between the minimum and maximum temperatures. Instead, it is nearer the upper limit of temperature range because the rate of **enzymes reaction increases** with **increasing temperature** until a point where the enzymes are damaged by heal and cells stop growing.

Microorganisms may be divided into **three groups** on the basis of the temperature range in which they grow best.

1. Psychrophiles or cold living microbes.
2. Mesophiles or moderate temperature living microbes.
3. Thermophiles heat living microorganisms

4.4.2 Gaseous atmosphere

Microorganisms in their natural habitats require varying amount of gases such as oxygen, CO_2, N_2 and methane. In order to cultivate microbial, plant and animal cells in the laboratory, the proper gas atmosphere must be present. Some gases are used in cellular metabolism; other may have to be excluded from a culture because they are toxic to cells. CO_2 and O_2 are the two principle gases that affect the growth of microbial cells.

On the basis of their **response to gases oxygen**, microorganisms are **divided** into **four physiological groups** as under.

1. **Aerobic microorganisms**

   Microbes that normally require oxygen for growth and can grow in a standard air atmosphere of 21% O_2 are classified as aerobes. E.g. of aerobic microbes are Mycobacterium and legion Ella.

2. **Facultative microorganisms**

   Facultative microorganisms are those that grow in air atmosphere and can also grow an aerobically. They do not require O_2 for growth, although they may use it for energy-yielding chemical reactions.

3. **Anaerobic microorganisms**

   Anaerobic microorganisms are those which may be poisoned by O_2, cannot grow in an air atmosphere and do not use O_2 for energy yielding chemical reactions e.g. Methanobacterium and Methanospirillum are strict anaerobes.

4. **Microacrophilic microorganisms**

   Microacrophilies are organisms which like aerobes, can use O_2 for energy yielding chemical reactions. However, unlike aerobes, they cannot withstand the level of oxygen (21%) present in air atmosphere and usually grow best at O_2 levels between 1 to 15%.

4.4.3 pH

In contrast to optimum temperature, **optimum pH** for microbial growth lies approximately in the **middle of pH range** over which growth will occur. The optimum pH is usually well defined for individual species. Different species are adapted to grow a various pH values. But to grow in acidic or basic environment microorganisms must be able to maintain its intracellular pH at about 7.5 regardless of the external pH. Living cells has the ability, within limits, to keep a constant internal pH by expelling hydrogen ions or by taking hydrogen ions into the cells. Different **species of microbes** have **different pH tolerance**.

For most **bacteria**, the pH normally is about 7 with pH 9 as the maximum for growth. The optimum pH normally lies between 6 to 8 but some species of bacillus e.g. can grow at

pH values as low as 0.5 and are found in acidic drainage water from mines where sulphur and iron are present. **Molds and yeasts** have a broader pH range than the bacteria. In addition their optimum pH for growth is lower than that of bacteria.

However **microorganisms** may have other requirements. An example is the photosynthetic microbes that must have light water, which accounts for 80 to90 % of a cell, is another environmental factor that affects microbial growth.

4.4.4 Concentration

High concentration of a solution renders an enzyme inactive. Thus solution used for fermentation should be sufficient diluted to favours the process

4.4.5 Presence of other substance

Certain inorganic salt solution acts as food for the ferment cell.

4.4.6 Absence of preservatives

Preservative are those substances, which destroy the ferment and retard the fermentation reaction. Hence these substances should be absent.

4.5 CULTURE DEVELOPMENT

Scientists have learned **to cultivate** many types of microorganisms, getting them **to grow and maintain their viability**. As you have already learned microorganisms are activated on media, which provide nutrients. The proper physical environment must be provided for optimal growth. Microorganisms show wide differences with respect to the physical conditions required for growth. Some species grow at temperature near the freezing point of water other grow at temperatures as higher as 110° C. Oxygen is essential to some poisonous to others. Most bacteria grow best at or near neutral pH, but the preferred pH for growth among microbes varies from alkaline to acidic.

Once the chemical and physical requirements are satisfied, it is possible to study the mode of reproduction and growth of species of microorganisms. It is important to remember that the behaviour of the species in pure culture in the laboratory may not be the same as its growth is characterized in nature.

4.6 DEVELOPMENT OF INOCULUM

The culture used to **inoculate for fermentation should satisfy** following criteria.

- It must be in a healthy, active state thus minimizing the length of the lag phase in the subsequent fermentation.
- It must be available in sufficiently large volumes to provide inoculums of optimum size.
- It must be in a suitable morphological form
- It must be free of contamination.
- It must retain its product forming capabilities.

The process adopted to produce an inoculums meeting this criteria is called **inoculums development**. A critical factor in obtaining suitable inoculums is the **choice of the culture medium**. The **quantity** of inoculums normally used is between 3 and 10 % of the medium volume. A relatively large inoculums column is used to minimize the length of the lag phase and to generate the maximum biomass in the production fermentation in as short as possible, thus increasing vessel productivity. Starting from a stock culture, the inoculums must be built up in a number of stages to produce sufficient biomass to inoculate the production stage fermentor. This may involve two or three stages in a sleek flasks and one to three stages in fermentor.

A **typical inoculums development** program will now be described in detail.

- The **master culture** is reconstituted and plated on to solid medium approximately ten calories of typical morphology of high producers are selected and inoculated on to slopes as the **sub-master cultures**, each sub-master culture being used for a **new production run**. At this stage, shake flasks may be inoculated to check the productivity of these cultures, the results of such tests being known before the developing inoculums **eventually reaches the production plant**. A sub master culture

is used to inoculate a shake flask (250 or 500 cm^3 containing 50 or 100 cm^3 medium), which in turn is used as inoculums for a large flask or a **laboratory fermentor** which may then be used to inoculate a **pilot-scale fermentor**.

- Culture purity checks are carried out at each stage to detect contamination as early as possible.
- Although the **results** of these tests may not be available before the culture has reached the production plant at least it is known at which stage in the procedure contamination occurred.

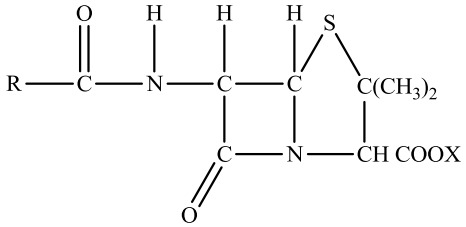
4.7 CHARACTERISTICS OF ENZYMES

- They do not take part in the reaction, but act **catalytically** i.e., they are organized catalyst of nature.
- Enzymes, like inorganic catalyst, **do not disturb** the **final state of equilibrium** in a reversible reaction.
- The **rate** of an enzyme reaction is **proportional to concentration of reactant**, provided the concentration is small. At high concentrations, the rate of the reaction is independent of the concentration.
- A **small quantity** of enzyme can bring about the decomposition of large amount of the substrate. For example, urease extracted from soyabean, will catalyst the hydrolysis of urea when present only to the extent of 1 part in 10 million parts.
- Their **action is highly specific**, i.e. a particular enzyme can bring about a particular reaction. For example, urease catalyses the hydrolysis of urea, but it has no effect on the hydrolysis of methyl urea.
- An enzyme is most **reactive** at a particular temperature, called **optimum temperature**. In many cases this temperature is 30-40^{0}C.
- Enzymes, like other catalyst are **influenced** by the presence of **other substances**. Some enzymes, called co -enzymes act as promoters, while HCN, CS_2, etc., poison them.
- They are **destroyed by** the ultra violet light and by heat. At temperature too low (0°C) and too high (70-80° C) the enzyme are most inactive.
- They **bring** about many **complex reactions** e.g. oxidation, reduction and hydrolysis

Some, of the well-known enzymes are **diastase** (origin: malt, liver), **catalase** (origin: plant juice, blood) **zymase** (origin: yeast), **urease** (origin: soybean), **invertase** (origin: small intestine yeast), **lactic bacilli** (origin: curd).

4.8 PENICILLINS

Penicillin is the name given to the mixture of natural compounds which have molecular formula $C_9H_{11}N_2O_4SR$ and differ only in the nature of R. The general structure of penicillins is



The **thiazolidine ring nucleus** (A) is **fused to β-lactam ring**(B) which is **attached to** a **side chain** (R-CO-). Any chemical modification of β-lactam or thiazolidine rings destroys the anti-bacterial activity of the molecule, e.g., penicillinase breaks the β-lactam ring.

In the above structure of penicillin, X is sodium, potassium, aluminium, procaine, benzathine or free acid.

4.8.1 Properties of Penicillin

- The purified penicillin are white or slightly yellowish white crystalline powders, some of which have unpleasant tastes
- All the natural penicillin are dextro-rotatory.

- The penicillin are only sparingly soluble in water. However, their sodium and potassium salts are soluble in water. Some of these salts are hygroscopic and hence they have to be stored in sealed containers.
- It is soluble in most organic solvents
- It is fairly strong monocarboxyl acids with a pH valve of 2.8.
- It is in the form of free acid has not been obtained crystalline and they undergo rapid decomposition in the presence of moisture.
- It undergoes hydrolysis readily and the nature of the product formed depends on the nature of the hydrolyzing agent. For example, If the hydrolysis of penicillin is carried out in alkaline medium, it yields penicilloic acid which loses carbon dioxide to form penilloic acid.

If the **hydrolysis of penicillin** is carried out in **acidic medium**, the **amide side chain** is involved with the **opening of** the **β -lactam ring**. This causes loss of its activity. Due to this reason, **natural penicillins** are **not very effective** when **given orally**. However, this defect has been partially overcome by introducing an electron-attracting group, particularly in the alpha position in the amide side-chain as in penicillin (V) which is more acid resisting. Further, the stomach juices contain the enzymes penicillinases. These enzymes also cause hydrolysis of penicillin as resulting in the opening of the lactam ring followed by the loss of activity. When these enzymes are present insignificant amounts, a resistance to penicillin is produced.

4.8.2 Activity of Penicillin

Penicillins are found to be active against Gram positive stains. However, these are ineffective against Gram negative organisms. Organisms sometimes develop resistance to penicillin.

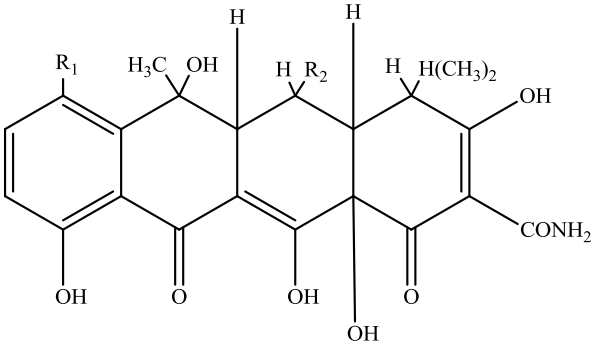
4.8.3 Toxicity (Side-reactions)

Penicillins generally have **low toxicity** in comparison to the sulpha drugs used earlier. However in some cases diarrhea and allergic reactions may result in. Penicillin is hence injected after a test prick is given.

4.9 TETRACYCLINE

In **1947** in study of thousands of **microscopic fungi** among the **actinomycete**s, **Duggar** discovered a soil organism for which the name **streptomyces aureofaciens** was proposed. When grown in culture broth a golden yellow antibiotic was elaborated that was **named Aureomycin**. In **1950 Finlay** and his associates **isolated** a new **actinomycete, Streptomyces rimosus**, from a soil sample that produced in antibiotic named **terramycin**. In **1952**, the **unique chemical structures** of these **two antibiotics** were determined and a third compound was prepared that possessed antibacterial properties. As a class, these antibiotics are called the tetracyclines, and one member of this class is named tetracycline. Many derivatives of the tetracyclines have been made synthetically.

The tetracycline antibiotics contain hydronaphthacene skeleton as a characteristic structural unit. The structures of the tetracycline antibiotics are given as follows.



Chlortetracycline $R_1 = Cl; R_2 = H$
Oxytetracycline $R_1 = H; R_2 = OH$
Tetracycline $R_1 = R_2 = H$

All of the tetracyclines used as antibiotics in man have various substituent groups on carbon atoms 4, 5, 6 or 7.

Aureomycin was isolated from cultures of Streptomyces aureofaciens. Terramycin was isolated from cultures of Streptomyces rimosus. **Tetracycline** is prepared by dechlorinating chlortetracycline by catalytic dehydrogenation with palladium. It can also be

made by fermentation. The stereochemistry of aureomycin had been partly established from the chemical work but it was completely determined by the X-ray analysis of its hydrochloride. Shemyakin et al. have established the absolute configuration of the tetracyclines by means of optical rotatory dispersion studies.

4.9.1 Oxytetracycline (terramycin)

This was the first member of the tetracycline group of antibiotics whose constitution established (Woodward et al. 1953).

Oxytertracycline is a **yellow, odourless, crystalline amphoteric substance**. One gram of this dissolves in about 2000 ml of water. One gram of hydrochloride is soluble in 2 ml of water. Solutions of the base and hydrochloride are not stable at pH below 2 and are rapidly destroyed by alkali hydroxide solutions.

4.9.2 Clinical Property

The tetracyclines are **active against** the **majority of gram positive organisms**, some gram negative bacteria, spirochaetes, rickettsial infections (such as typhus sad Q fever). Mycoplasma and against the lymphogranulomaprittacosis group of virus infections. By and large, they have no action against the viruses.

The tetracyclines are **drugs of choice** for treating **brucellosis, whooping coughs, typhus, Q fever, psifttacosis** and **lymphogranuloma venereum**. The tetracyclines are also useful for treating **respiratory tract bacterial infections** and particularly exacerbations of **chronic bronchitis**. For **urinary tract infections**, tetracyclines are moderately useful but the organisms acquire resistance rapidly.

The tetracycline antibiotics are poorly absorbed from intramuscular injection sites. Chlortetracycline is not absorbed after intramuscular injection and it is not administered by this route.

4.10 FURTHER READING

Synthetic drugs by Gurdeep R. Chatwal