FOOD AND INDUSTRIAL MICROBIOLOGY

Industrial Microbiology

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Introduction

Industrial microbiology is basically associated with the commercial exploitation of microbes for the benefit of mankind. These microbial products may have direct or indirect impact on the economics, environment and social parameters of the society. The use of microbes for the production of industrially important metabolites is not a recent event. Mankind has been producing alcoholic beverages and dairy products since the beginning of the civilization but they were oblivious of the role of microbes in the production of these products. The exact role of microbes in the fermentation process was convincingly shown for the first time by Louis Pasteur in 1850s. With the refinement of technology to obtain pure cultures, the association of specific microbes with specific products gained momentum. Table1 lists some of the historical events in the development of industrial microbiology.

In 1930s accidental discovery of penicillin by Alexander Fleming boosted the importance of Industrial Microbiology in the eyes of mankind. The association of an industrially important metabolites with microbes revolutionized the industry to look for novel microbes that produced various kinds of antibiotics, enzymes, anti-cancerous compounds and other chemicals. With the developments in the fields of biochemistry, purification process brought down the cost of recovery of industrial products made with the help of microbes. Industrial microbiology received a shot in the arm with the advent of genetic engineering in 1970s. The cloning of genes of industrial importance, from plants and animals into microbes and from one type of microbe e.g. fungus into another genetically stable and highly characterized microbe e.g. *E.coli* resulted in very high yields of the products at minimal cost. By the use of site directed mutagenesis of genomic DNA, scientists could bring out the permanent change in the gene and hence the gene product. During the last decade, industrial microbiology has been revolutionized by the use of high throughput screening, DNA microarray system and bioinformatics.

Concepts of Industrial Microbiology

Industrial microbiology deals with the production of microbial biomass or microbial products by a process called fermentation (latin verb *fervere* – to boil). During the growth of microbes, later on identified as yeasts, in the extracts of fruits/cereals there is evolution of CO_2 gas which bubbles out of media thus giving the appearance of a boiling broth, hence the name fermentation. In strict terms, fermentation is an energy generating process in which organic compounds act as electron donor as well as electron acceptors. However from microbiology point of view, fermentation is described as any process that involves the production of biomass/bioproducts by the use of microbes. The fermentation process basically consists of three parameters: a) Microbes, b) Fermentation media, and c) Fermentors.

Microbes

These are tiny organisms which require the use of microscopes for their visualization. Microbes are highly versatile organisms. They have many positive features which are responsible for their uses in the field of industrial microbiology.

  i)   Microbes grow and multiply very rapidly. Billions of cells can be produced in a single day e.g. *E.coli* doubles itself in 15-20 minutes under optimal conditions of growth.
  ii)   Microbes require cheap nutrients for growth which are available throughout year.

iii) Microbes have great biodiversity. They can grow under extreme conditions of temperature, pH, salts, pressure etc.
iv) The genotype of industrially important microbes is fully known. This aspect helps us to understand the biosynthetic and regulation of a desired gene product. In addition, scientists can either shut off undesirable biosynthetic pathways or boost the yield of desired gene product.
v) Microbes can be stored for years together without any effect on their productivity/desired characters.
vi) Microbial process is eco friendly.
vii) Microbial biomass left after the fermentation can be used as excellent fertilizer.

Table 1: Landmarks in the development of industrial microbiology

Landmark	Date
Fermentation to produce alcoholic beverages	6000-2000 BC
Vinegar formation	Pre-3000 BC
Production of beer	4000 BC
Visualization of microbes by Leeuwenhoek	mid 17th century
Discovery of fermentation by yeasts	1818
Involvement of microbes in the production of lactic acid	1881
Discovery of fermentation enzymes from yeast	1897
Microbial process for the production of butanol and acetone	1915-1916
Solid substrate fermentation (of citric acid)	1922
Microbial transformations	1937
Commercial production of penicillin	1941-1944
Discovery of other antibiotics	1950
Production of single–cell protein	1960
Use of immobilized enzymes for high-fructose syrup	1960
Commercial use of genetically engineered microbes	1982
Designer enzymes, combinatorial approach	1995
Cloning of secondary metabolite operons	1990
High throughput screening of industrially significant metabolites	2000

In addition the selected microbe, chosen for the production of a bioproduct, should have the following properties as well:
i) It should not be pathogenic (except when used for the production of vaccine/toxin) and should not cause any allergic reaction.
ii) It should not lose its desired property on sub culturing.
iii) It should be genetically stable.
iv) It should be amenable to mutation as and when desired.

Fermentation medium

The growth medium (liquid or solid) in which microbes grow and multiply is called fermentation medium. The selected microbe should be able to utilize and grow on cheap sources of carbon and nitrogen. Usually these sources are waste products of industrial processes e.g. molasses, whey, corn steep liquor etc. Care is taken to avoid the use of such microbes which require expensive nutrients like vitamins for their growth.

Fermenter

For production of a desired microbial product, it is of utmost importance to optimize physical (pH, temperature, aeration etc.) and chemical (carbon, nitrogen, mineral sources etc.) composition of the fermentation medium. To maintain these stringent conditions, microbes are grown in containers called as fermenters or bioreactors. The capacity of bioreactors may vary from 10 liters to 100,000 liters depending on the byproduct.

In general, fermentation process is divided into two parts i.e. Up Stream Processing (USP) and Down Stream Processing (DSP, Fig.1). USP is basically involved in the a) preparation of a seed culture (i.e. inoculums preparation), b) preparation of fermentation medium and its sterilization and c) the fermentation process for optimal production of a metabolite. DSP is concerned with the recovery of product, its concentration and purification and disposal of biowaste generated out of the fermentation process.
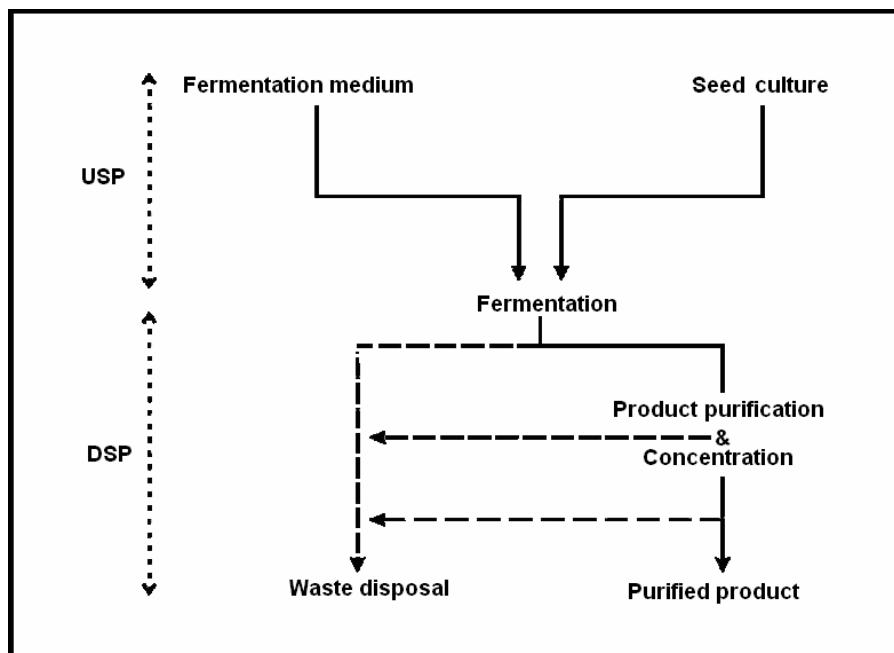
USP: Up stream processing; DSP: Down stream processing

Fig. 1: Diagrammatic representation of a fermentation process

Microbial Products of Industrial Use

A vast range of industrial products which were earlier made by chemical processes are now being made with the help of microbes. Contrary to the belief that microbes are harmful to humans, majority of microbes are either harmless or provide beneficial products to humans.

Microbe based industrial products have made inroads in all walks of life i.e. health sector, food sector, agriculture sector, industrial chemical sector and environmental sector. These products can be categorized into four main groups (Table 2).

Table 2: Microbial products of industrial importance

Category	Product
Primary metabolites	Enzymes, amino acids, nucleotides, organic acids, ethanol, butanediol
Secondary metabolites	Antibiotics, gibberellins, hormones, pigments, alkaloids
Microbial biomass	Baker's yeast, single cell protein (SCP), probiotics, vaccines
Recombinant products	Insulin, streptokinase, interferons, Interleukins, growth hormones, vaccines

Primary metabolites

These are those metabolites which are produced when the cells are growing actively in the presence of sufficient amount of nutrients (Fig. 2). This phase of growth of cells is called **trophophase**. Most of the primary metabolites (amino acids, nucleotides, proteins, nucleic acids, lipids, carbohydrates) are essential or almost essential for the growth and survival of the organisms. Some of the primary metabolites have found industrial applications e.g. ethanol, citric acid, glutamic acid, lysine, vitamins etc.
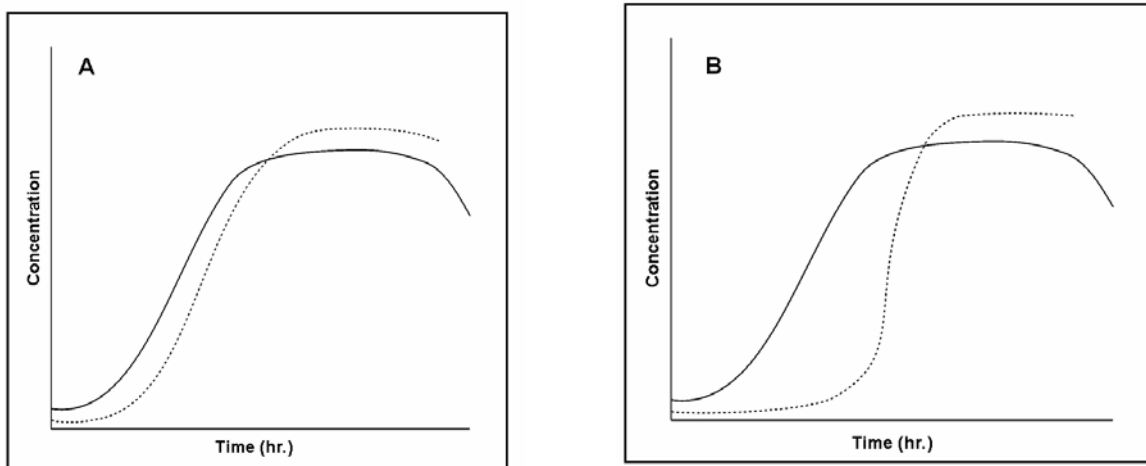
Growth of cells (——), Metabolite production (······)

Fig. 2: Correlation of microbial growth with production of primary metabolites (A); and secondary metabolites (B)

Secondary metabolites

Secondary metabolites are produced by the cells after the active growth of cells has ceased. This phase of growth of cells is called **idiophase**. Cells do not divide but are metabolically active. Secondary metabolites are not essential for the survivability of the cells. Not all classes of microbes exhibit secondary metabolism. It is commonly seen in fungi, yeast,

actinomyces but is absent in a few bacteria like *E.coli, Salmonella, Shigella, Proteus, Klebsiella* etc. The microbes produce secondary metabolites when their growth rate either slows down or there is complete cessation of growth. Both conditions result in the production of secondary metabolites by microbes. Practically the cells exhibit secondary metabolism when there is insufficient availability of nutrients or suboptimal physical conditions like lack of oxygen or deviation of pH of the surrounding environment from its optimal pH of growth. Such conditions are normally encountered by microbes growing under natural environment. In other words, during natural conditions of growth, it's the idiophase that prevails rather than the trophophase.

In the cells there is correlation between primary and secondary metabolism. Secondary metabolism succeeds primary metabolism (Fig.3). Alternatively, secondary metabolites are produced from intermediates and end products of primary metabolites. A particular secondary metabolite is produced by only a few selective microbes e.g. an antibiotic penicillin is produced only by *Penicillium* spp.

Microbial biomass

In a few instances the cells i.e. biomass of microbes, has industrial application as listed in Table 3. The prime example is the production of single cell proteins (SCP) which are in fact whole cells of *Spirullina* (an algae), *Saccharomyces* (a yeast) and *Lactobacillus* (a bacterium). SCP is essentially rich in amino acids which are either absent in vegetarian food or present in low amounts e.g. lysine, threonine, methionine, leucine, isoleucine etc.

Recombinant products

With the advent of gene cloning techniques, many industrially important genes from plants, animals and microbes have been cloned in a few selected microbes like *E.coli* and *Saccharomyces* and *Pichia*. The basic idea behind all these clonings is to produce large amount of proteins and scientists have been highly successful in achieving this goal. Table 4 lists some of the recombinant products.

Important microbial products

In addition to above mentioned microbial products, following Tables (5-8) will expose you to yet more application of microbes or their derivatives in the commercial sector.

Microbial enzymes: Enzymes are biocatalysts which are primarily protein in nature though certain RNA molecules have also been shown to possess catalytic activity. Enzymatic processes are fast replacing chemical processes because such technology is eco-friendly, stereo specific and generate less undesirable waste products. Table 5 lists the microbial enzymes of industrial importance.
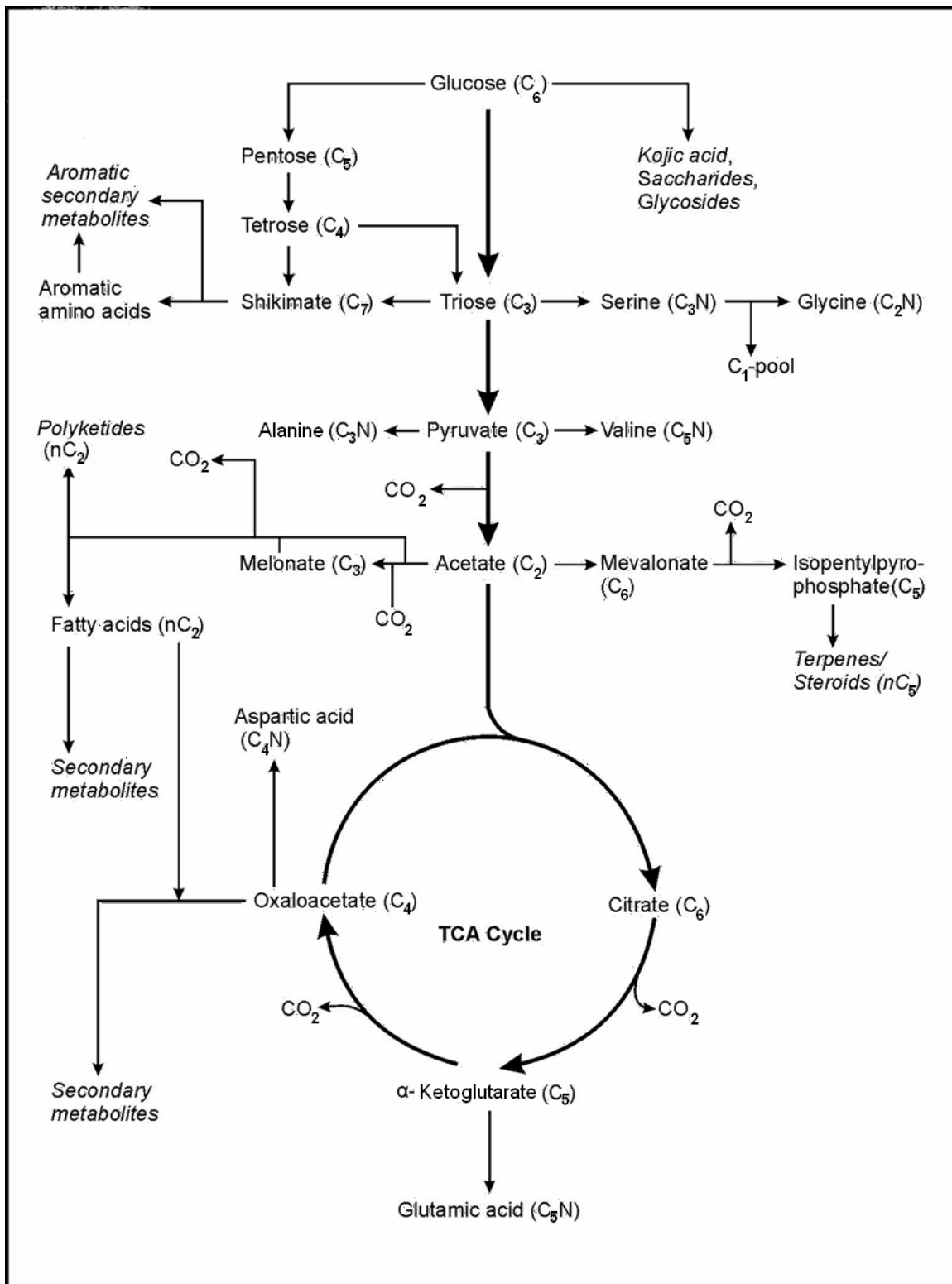
Fig. 3: Biosynthetic pathways for the production of primary & secondary metabolites

Table 3: Microbial biomass production

Product	Source	Use
Baker's yeast	*Saccharomyces cerevisae*	Production of bread, Single cell protein
Pruteen	*Methylophylus methylotrophus*	Single cell protein
Spirullina protein	*Spirulina maxima*	Single cell protein
Mushroom	*Agaricus bisporus*	Single cell protein
Bioleaching agent	*Thiobacillus ferrooxidans*	Solubiliztion of metals from ores
Bioinsecticide	*Bacillus thuriengensis*	Control of cotton eating insects

Table 4: Recombinants of industrial importance

Gene	Original source	Gene expressed in	Usage
Insulin	Human pancreas	*E.coli, Pichia pastoris*	Control of diabetes
Chymosin	Calf stomach	*E.coli*	Cheese production
Human growth hormone	Human pituitary gland	*E.coli*	Increase in height
Clotting factor VIII	Blood cells	*Saccharomyces cerevisae, Pichia pastoris*	To stop bleeding
Tissue plasminogen activator	Blood cells	*Saccharomyces cerevisae, Pichia pastoris*	Dissolution of blood clots
Streptokinase	*Streptococcus equisimilis*	*Pichia pastoris*	Dissolution of blood clots

Table 5: Microbial enzymes of industrial importance

Bulk enzymes		
Enzyme	**Source**	**Uses**
Protease	*Bacillus, Lactococcus, Aspergillus , Rhizomucor*	Biological detergents, dough preparation, beer manufacture, cheese production, leather baiting and tendering, tenderization of meat, recovery of silver from photographic films
Lipases	*Bacillus, Aspergillus, Rhizopus, Rhodotorulla*	Biological detergents, removal of fat during leather processing, cheese ripening and flavour enhancer
α-amylase	*Bacillus, Aspergillus*	Biological detergents, starch hydrolysis during brewing and baking, textile manufacture
β-amylase	*Bacillus, Streptomyces, Rhizopus*	Starch hydrolysis during brewing and baking, production of maltose syrup

Glucoamylase	Aspergillus, Rhizopus	starch hydrolysis during brewing and baking, production of glucose syrup, wine and fruit juices
Lactase (β-D-galactosidase)	*Bacillus, Kluyveromyces, Candida*	Whey syrup preparation, milk and dairy product processing and manufacture of ice creams
Glucose isomerase	*Actinoplanes, Arthrobacter, Streptomyces*	Manufacture of high fructose syrup
Invertase	Kluyveromyces, Saccharomyces	Production of sweets and confectionary products like soft centered chocolates.
Pectinase	Aspergillus, Penicillium	Preparation of fruit juices, extraction of oils and juices from plants, coffee fermentation
Cellulases	Tricoderma, Penicillium, Bacillus	Wood pulp processing, fruit juice preparations, malting of grains
Hemicellulases	Cryptococcus, Trichosporon	Wood pulp processing, baking, brewing, animal feedstuff, nutraceutics
Catalase	Aspergillus, Corynebacterium, Micrococcus	Bleaching of textiles , cheese processing
Phytase	Pichia	Animal feed supplement
Urease	Lactobacillus	Wine production, manufacture of ceramics
Penicillin G acylase	*E.coli,* Bacillus	Biotransformation of penicillin G into 6-amino penicillanic acid

Analytical and diagnostic enzymes		
Alcohol dehydrogenase	*Saccharomyces cerevisae*	Ethanol estimation
Cholesterol esterase	*Pseudomonas fluorescens*	Cholesterol estimation
Glucose oxidase	*Aspergillus niger*	Glucose estimation
Uricase	*Arthrobacter globiformis*	Gout diagnosis
Creatinase	*Pseudomonas putida*	Creatinine estimation
Therapeutic enzymes		
Urease	*Lactobacillus fermentum*	Removal of urea from blood
α-amylase	*Aspergillus niger, Bacillus subtilis*	Aids in food digestion
Asparaginase	*E.coli, Serratia marcescens*	Cancer treatment
β-lactamase	*Bacillus cereus, E.coli*	Treatment of penicillin allergy
Rhodanase	*Tricoderma sp.*	Treatment of cyanide poisoning
Streptokinase	*Streptococcus equisimilis*	Blood clot buster
Molecular biology enzymes		

Restriction enzymes like BamH1	*Bacillus amyloliquefaciens*	Cut DNA at specific site
Taq polymerase	*Thermus aquaticus*	DNA synthesis
DNA ligase	*E.coli*	Joining DNA fragments
RNA Polymerase	*Salmonella typhimurium*	RNA synthesis
Immobilized enzymes		
Penicillin G acylase	*E.coli*	Production of 6-amino penicillanic acid from penicillin
Glucoamylase	*Aspergillus niger*	Glucose production from starch
Glucose isomerase	*Bacillus coagulans*	Production of high fructose syrup
Hydantoinase	*Flavobacterium ammoniagenes*	Amino acid production
Lactase	*Aspergillus oryzae*	Preparation of lactose free milk
Naranginase	*Penicillin decumbens*	Debittering of citrus fruit juices
Invertase	*Saccharomyces cerevisae*	Production of invert sugar (glucose + fructose)

Table 6: Applications of microbes in healthcare products

Product	Source	Use
Penicillin	*Aspergillus chrysogenum*	Antibacterial activity
Cephalosporin	*Cephalosporium acremonium*	Antibacterial activity
Bacitracin	*Bacillus subtilis*	Antibacterial activity
Streptomycin	*Streptomyces griseus*	Antibacterial activity
Erythromycin	*Saccharapolyopora erythraea*	Antibacterial activity
Gentamycin	*Micromonospora purpurea*	Antibacterial activity
Amphotericin B	*Streptomyces nodosus*	Antifungal activity
Nystatin	*Streptomyces noursei*	Antifungal activity
Lentinan	*Lentinus edodes*	Anti tumour activity
Funiculosin	*Penicillium funiculosum*	Antiviral activity
Cyclosporin	*Cylindrocarpum lucidum Tolypocladium inflatum*	Immunosuppresent
Zaragozic acid	*Sporomiella intermedia*	Cholesterol inhibitor.
Prednisolone	*Arthrobacter simplex*	Biotransformation of hydrocortisone into Prednisolone
11α-	*Rhizopus nigricans*	Biotransformation of progesterone to 11 α-

hydroxyprogesteron		hydroxyprogesteron
Anthrax vaccine	*Bacillus anthracis*	Protection against anthrax
BCG vaccine	*Myobacterium tuberculosis*	Protection against tuberculosis
DPT vaccine	*Corynebacterium diptheriae, Clostridium tetani*	Protection against diphtheria, pertusis and tetanus
Typhoid vaccine	*Salmonella typhi*	Protection against typhoid

Table 7: Applications of microbes in food and beverage fermentations

Product	Source	Uses
Enzymes for alcoholic beverages like beer, wine , whisky:		
α-amylase	*Bacillus, Aspergillus*	starch hydrolysis
β-amylase	*Bacillus, Streptomyces, Rhizopus*	starch hydrolysis
Glucoamylase	*Aspergillus, Rhizopus*	starch hydrolysis
Protease	*Aspergillus niger*	Prevention of haze formation
Glucose oxidase	*Aspergillus niger*	Removal of oxygen from beer
Acetic acid (vinegar)	*Acetobacter aceti, Gluconobacter oxydans*	Conversion of ethanol into acetic acid
Yoghurt	*Streptococcus thermophilus, Lactobacillus delbrueckii*	Production of organic acids esp. lactic acid
Cheese	*Streptococcus thermophilus, Lactobacillus, Lactococcus, Propionobacterium, Penicillium*	Production of organic acids esp. lactic acid, flavour enhancers and stabilization of cheese.
Probiotics	*Lactobacillus, Saccharomyces cerevisae, Bifidobacterium*	Stimulate immune system, Stabilizes gut microflora.
Bread	*Saccharomyces cerevisae,*	Rising of bread
Sauerkraut	*Lactobacillus, Leuconostoc plantarum*	Lactic acid production

Table 8: Applications of microbes in food additives and supplements

Product	Source	Use
Glutamic acid, Lysine	*Corynebacterium glutamicum*	Feed supplement,
β- carotene	*Blakeslea trispora*	Bio-colour
Astaxanthine	*Mycobacterium lacticola*	Bio-colour
Vanillin	*Saccharomyces spp.*	Flavouring agent
Monosodium glutamate	*Corynebacterium glutamicum*	flavour enhancer
Ascorbic acid	*Acetobacter suboxydans*	Vitamin C
Riboflavin	*Ashbya gossypii*	Vitamin B_{12}
Citric acid	*Aspergillus niger*	Acidulant
Xanthan	*Xanthomonas compestris*	Thickner and stabilizer for foods
Nisin	*Lactococcus lactis*	Antimicrobial agent

Safe microbes

For any microbe to be used at commercial level, it is imperative that it should not be pathogenic and the final purified product should be free from toxic contaminants. Prior clearance from regulatory authorities have to be sought for their use at industrial level. However, a few microbes have been categorized as GRAS (generally regarded as safe). No permission from authorities is needed for their use at industrial level, as long as they are grown under stipulated conditions. Organisms listed under GRAS category mentioned in Table 9.

Table 9: Microbes classified as GRAS

Bacteria	
	Bacillus subtilis
	Lactobacillus bulgaricus
	Lactobacillus lactis
	Leuconostoc oenos
Yeasts	
	Candida utilis
	Kluyveromyces marxianus
	Kluyveromyces lactis
	Saccharomyces cerevisae
Fungi	
	Aspergillus niger
	Aspergillus oryzae
	Mucor javanicus
	Penicillium roqueforti

Energy Generating Pathways: Fermentations and Respiration

In a living cell, at any point of time, hundreds of chemical reactions are going on simultaneously in a coordinated fashion. The entire spectrum of chemical reactions is collectively termed as metabolism of the cell. For the sake of understanding, metabolism can be divided into two parts: anabolism and catabolism. Anabolism is related with the synthesis of complex molecules and polymers from simpler ones with consumption of energy.

Catabolism is related with the breakdown of polymers and molecules into simple forms with the release of energy. It can be sub-divided into three stages (Fig. 4):

a) Conversion of polymers and complex molecules into their building blocks i.e. proteins into amino acids, polysaccharides into monosaccharides and fats into free fatty acids and glycerol.
b) Conversion of building blocks into pyruvate and acetyl CoA
c) Conversion of acetyl CoA into CO_2 and water via Krebs cycle (also called citric acid cycle or TCA cycle) along with generation of lot of energy rich compounds i.e. ATP molecules.
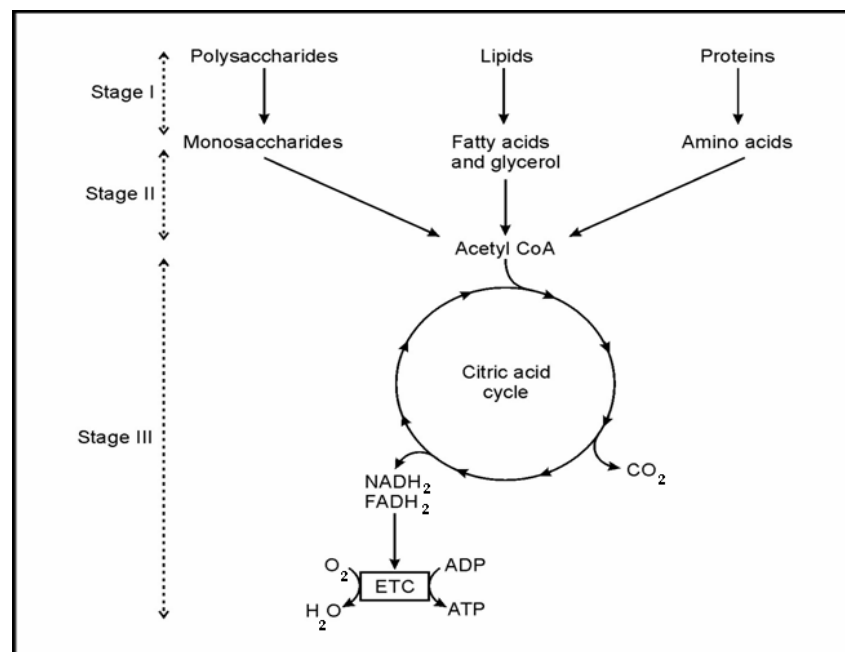


Fig. 4: Different stages of catabolism in a cell

The energy generated by catabolic reactions is utilized by the cells for biosynthetic processes, repair, transport of nutrients across the cell membrane, DNA replication, protein synthesis etc. Most of the microbes used in industry are chemoheterotrophs which catabolize organic compounds (sugars, fats and proteins) to release energy. The microbes are very versatile organisms having the ability to catabolize hundreds of different kinds of organic compounds for generation of energy. The most common form of organic compound used by microbes as carbon and energy source are complex carbohydrates (starch, cellulose, oligosaccharides, tri- and di- saccharides) made up of glucose units. These polymers are first enzymatically broken down to their monomer form, a six carbon sugar called glucose. Glucose is further broken down by various pathways with release of energy which ultimately results in the formation of

energy rich compounds like ATP and reduced coenzymes (NADH, NADPH, FADH$_2$). Various pathways for glucose breakdown are organism specific and are mentioned below.

Embden-Meyerhof-Parnas (EMP) pathway

This is the most common pathway present in all industrially significant microbes. It is functional in the absence or presence of oxygen and involves ten enzyme-catalyzed reactions (Fig. 5) located in the cytoplasm of the cells. Two reactions are energy driven and three reactions release energy, whereas rest of five reactions do not require any energy exchange. Seven steps are reversible and three are irreversible reactions involving hexokinase, phosphofructokinase and pyruvate kinase. This pathway results in generation of pyruvate (a 3-carbon sugar) and energy rich compounds ATPs and NADPH.
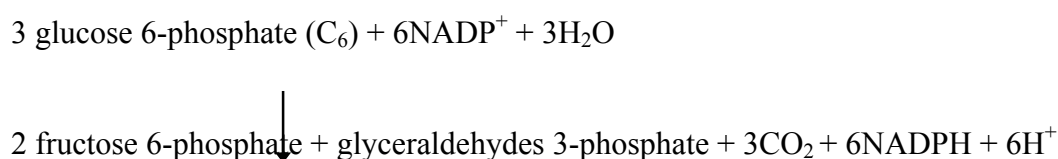
The net reaction of EMP pathway is as follows:

Glucose (C$_6$) + 2ADP + 2P$_i$ + 2NAD$^+$

$\downarrow$

2 Pyruvate (C$_3$) + 2ATP + 2NADH + 2H$^+$
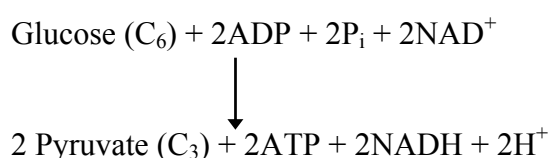
Hexose monophosphate (HMP) pathway

HMP pathway, also called pentose phosphate (PP) pathway or phosphogluconate pathway is also present in many bacteria, fungi and yeasts and functions in conjunction with EMP pathway. Like EMP pathway, HMP pathway occurs in the cytoplasm and functions under aerobic as well as anaerobic environment. It has both catabolic and anabolic roles (Fig. 6).

This pathway performs many important roles for the cells. It generates two important products - NADPH and pentoses needed for the biosynthetic reactions. Pentoses are needed for the synthesis of nucleic acids (RNA and DNA) and nucleotides such as ATP, NAD$^+$, FAD and CoA. NADPH is needed for the synthesis of fatty acids, steroids and a few amino acids. It is also an important component of a reaction involved in the conversion of cell generated H$_2$O$_2$ (a toxic compound) to H$_2$O. HMP pathway also generates four carbon metabolite, eryhtrose-4-phosphate, involved in the biosynthesis of aromatic amino acids.

Overall reaction is as follows:

3 glucose 6-phosphate (C$_6$) + 6NADP$^+$ + 3H$_2$O

$\downarrow$

2 fructose 6-phosphate + glyceraldehydes 3-phosphate + 3CO$_2$ + 6NADPH + 6H$^+$

Glyceraldehydes 3-phosphate may be converted to pyruvate via EMP pathway enzyme or two glyceraldehyde 3-phosphate molecules may join to form one molecule of glucose 6-phosphate.

Entner-Doudoroff (ED) pathway

ED pathway (Fig. 7) is not very common amongst microbes. It is virtually absent in fungi and yeast and is found only in a few species of bacteria. (*Azotobacter, Pseudomonas, Rhizobium, Xanthomonas* and *Zymomonas*) in which EMP pathway is absent.
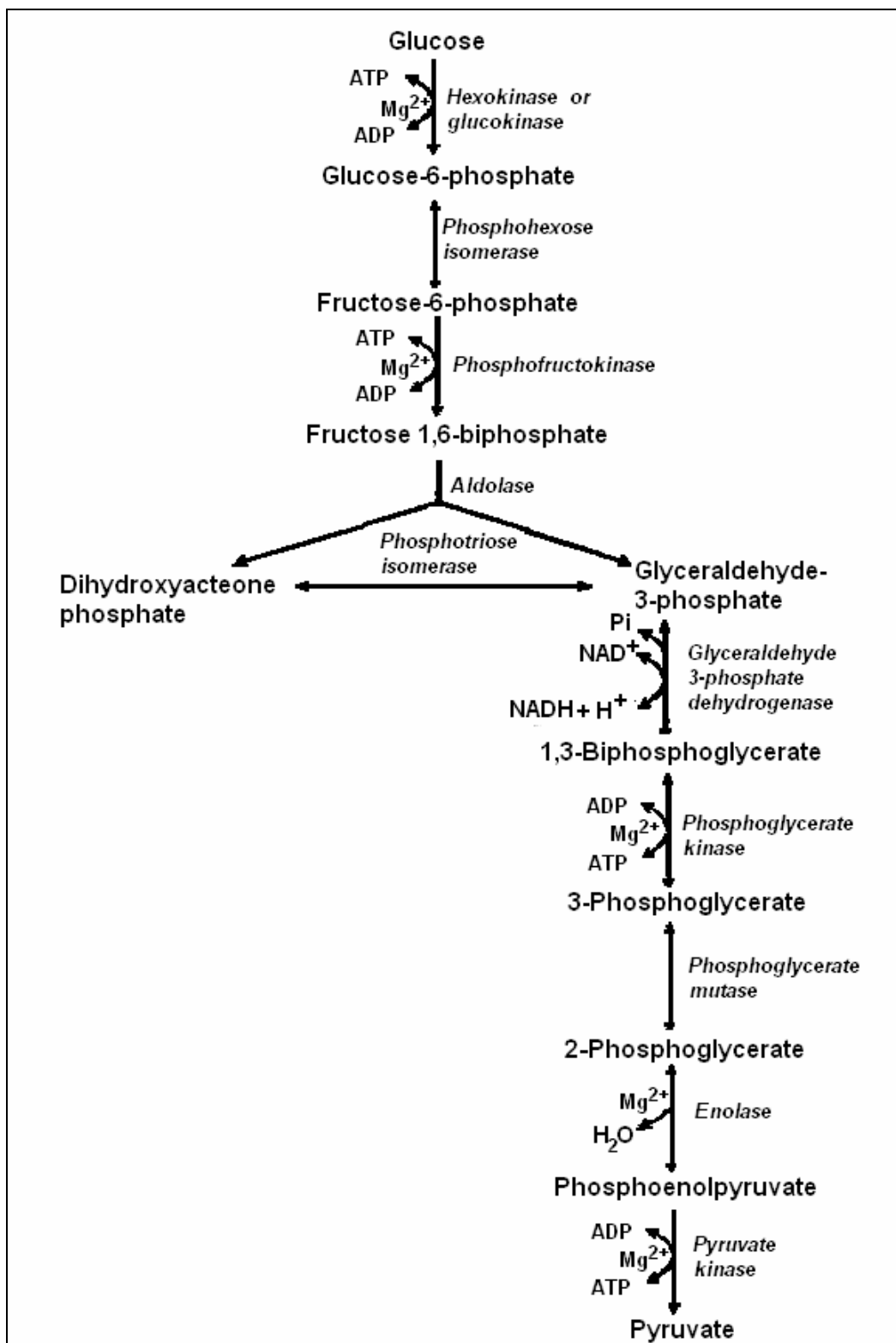
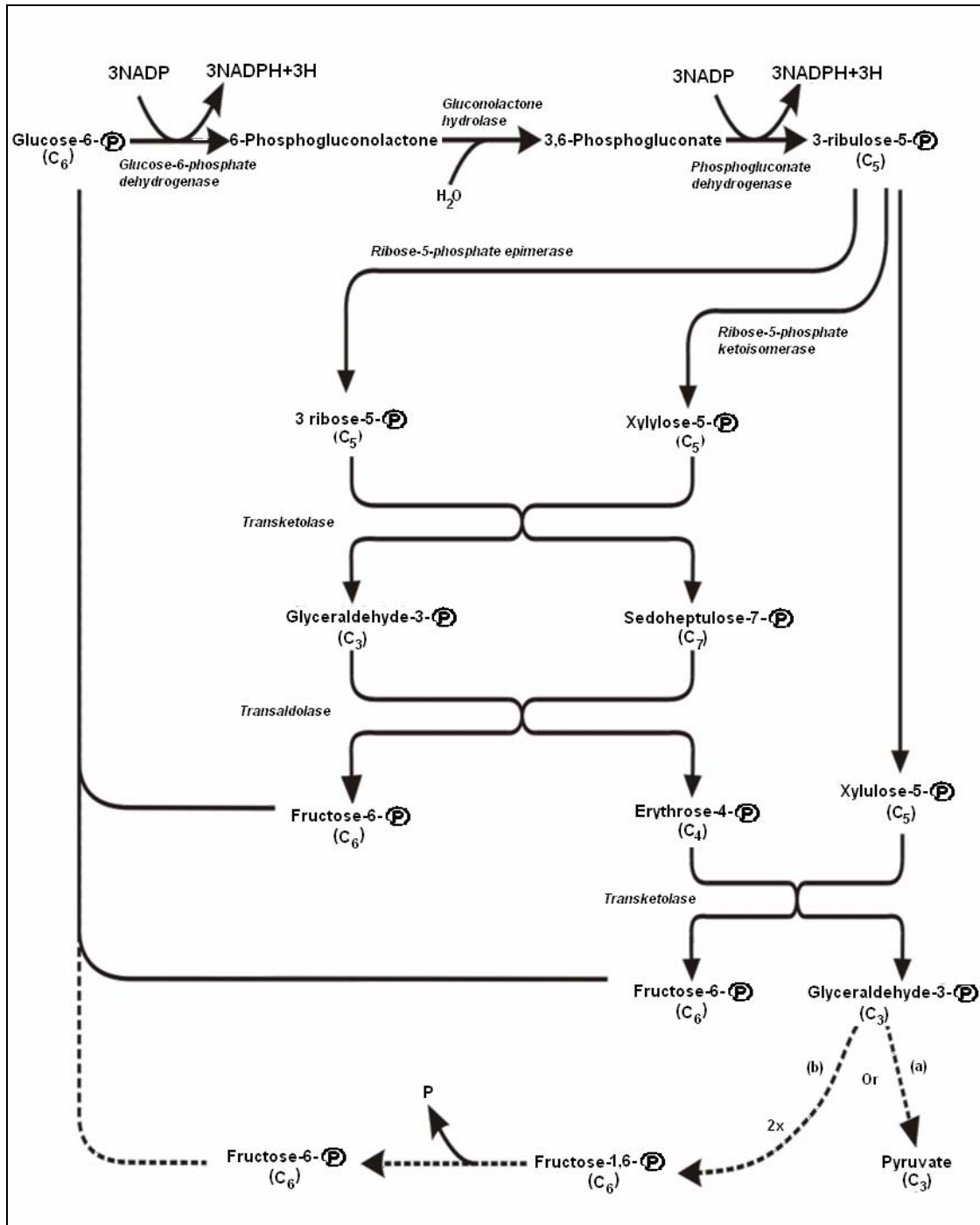**Fig. 5: Embden-Meyerhof-Parnas (EMP) pathway existing in microbes**

Fig. 6: HMP catabolic pathway

This pathway is located in cytoplasm and have a few reactions in common with EMP and HMP pathways. The first two reactions of ED pathway are same as existing in HMP pathway i.e. conversion of glucose into 6-phoshphogluconate. The latter compound is then dehydrated and then cleaved by an aldolase to 3-carbon compounds, pyruvate and glyceraldehyde 3-phosphate. The latter compound is then converted to pyruvate via EMP pathway. Overall reaction converts one glucose molecule into two molecules of pyruvate and one molecule each of ATP, NADH and NADPH. It is quite apparent that energy yielded by ED pathway is less than that of EMP pathway.
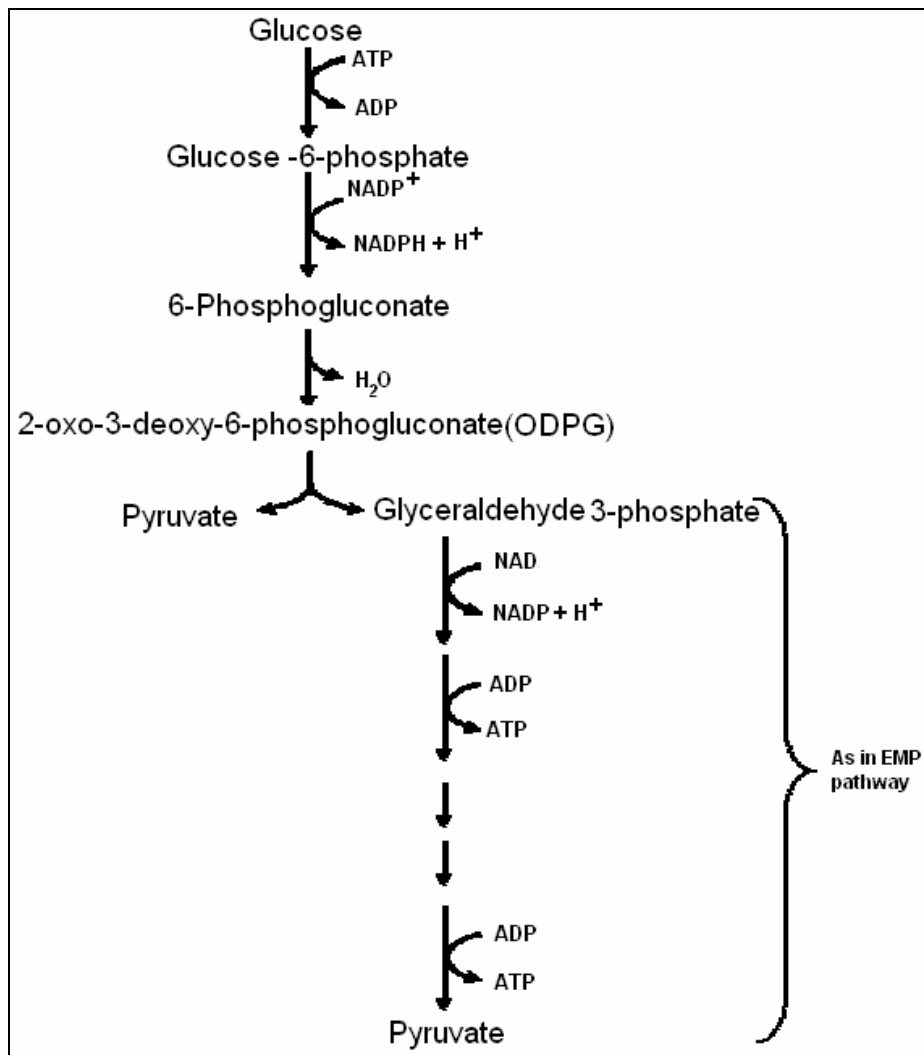
Fig. 7: Entner-Doudoroff (ED) catabolic pathway

Phosphoketolase (PK) pathway or Warburg-Dickens pathway

PK pathway (Fig. 8) is limited to a few bacteria (*Lactobacillus* and *Leuconostoc*) which are important in dairy fermentations. Here, glucose is converted to five carbon xylulose-5-phosphate as it occurs in HMP pathway.

This compound is then enzymatically cleaved by phosphoketolase into three carbon molecule, glyceraldehydes 3-phosphate and two carbon molecule, acetyl phosphate. Glyceraldehyde 3-phosphate is then converted into pyruvate via EMP pathway. Finally, pyruvate is reduced to lactic acid. Acetyl phosphate is also reduced to ethanol involving two intermediates, acetyl CoA and acetaldehyde.
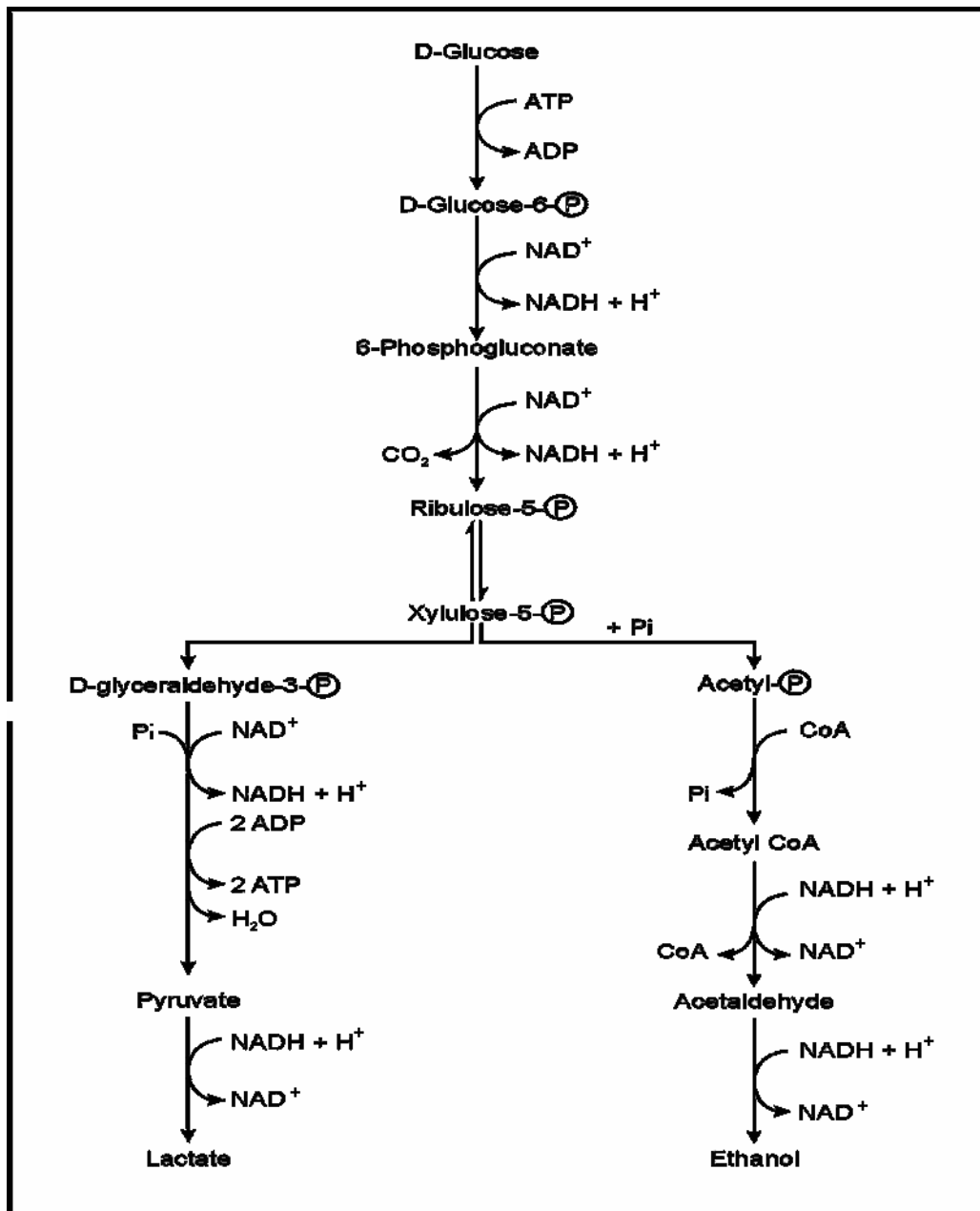
Fig. 8: Phosphoketolase (PK) pathway

Pyruvate - the hub molecule

In terms of fermentation processes, pyruvate is a key molecule. Under anaerobic conditions the enzymatic machinery of the microbes can ferment pyruvate into compounds of industrial importance (Fig. 9) e.g. ethanol, butanol, propanediol, butylene glycol, butanol etc. These fermentations are important for the cell survivability. The steps involved in the conversion of pyruvate into various end products also bring about oxidation of reduced compounds like NADH and NADPH into NAD and NADP. If such oxidations do not occur the cell will be devoid of NAD and NADP, the important coenzymes which are essential for many catabolic pathways including EMP pathway. Because of the incomplete oxidation of organic compounds during fermentation process the yield of energy rich compounds i.e. ATPs is

quite less. Based on the end product formed there are various types of fermentations (Fig. 10).

i)   Alcoholic fermentation: is carried out by yeast (e.g. *Sachharomyces* spp.)   and bacteria (e.g. *Zymomonas* spp.). It's a two step process in which pyruvate is first decarboxylated to acetaldehyde and then reduced to ethanol.
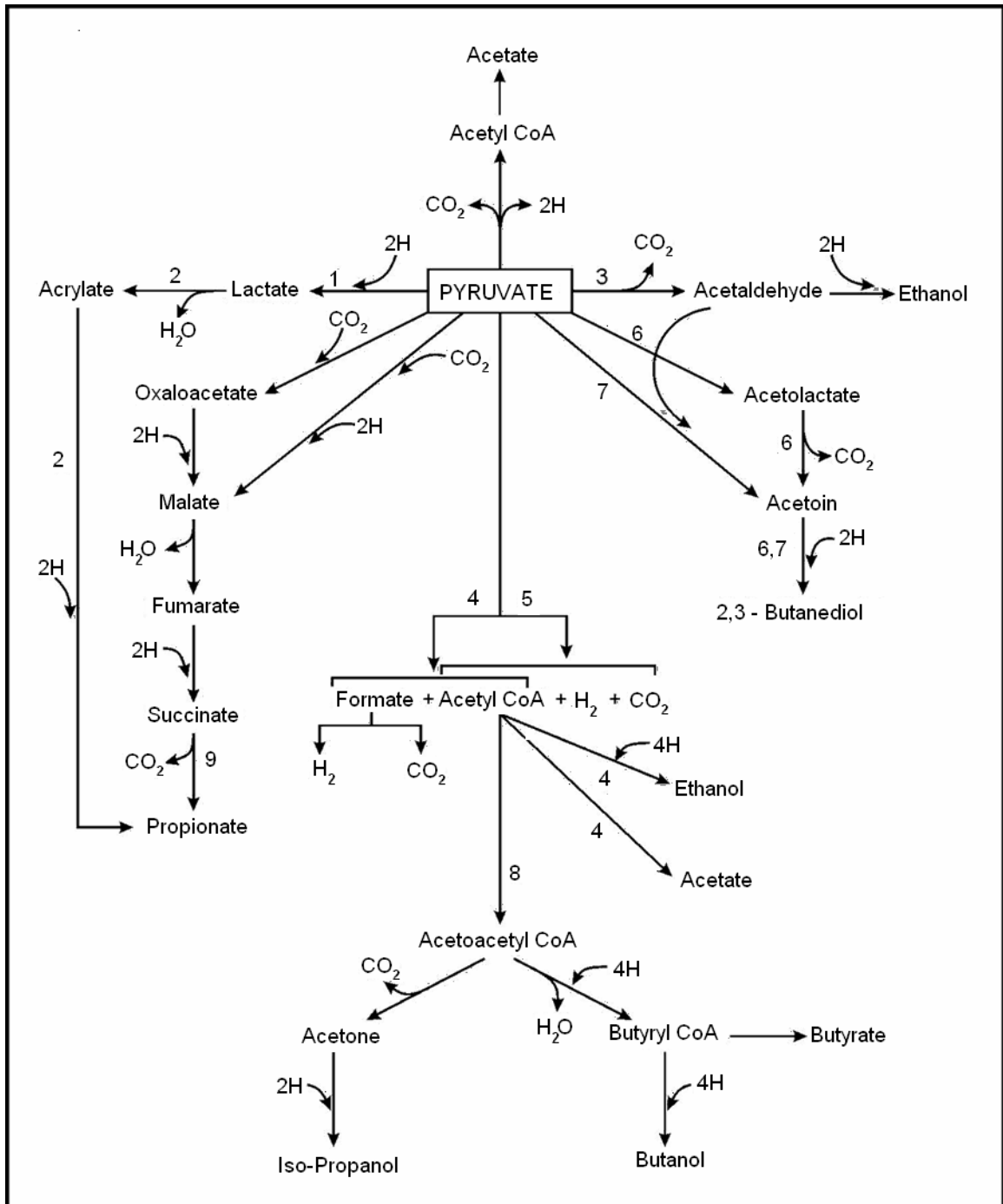
1- Lactic acid bacteria (*Streptococcus, Lactobacillus*); 2- *Clostridium propionicum*;
3- *Saccharomyces, Zymomonas*; 4- *Clostridia*; 5- *Klebsiella*; 6- Yeast;
7- *Clostridium butylicum*; 8- Propionic acid bacteria

Fig: 9: Pyruvate-the hub molecule

A) Alcoholic fermentation

Pyruvate ⟶ CO_2 + Acetaldehyde ⟶ [NADH + H⁺ → NAD⁺] ⟶ Ethanol

B) Homolactic fermentation

Pyruvate ⟶ [NADH + H⁺ → NAD⁺] ⟶ Lactate

C) Heterolactic fermentation

Glucose ⸱⸱⸱PK⸱⸱⸱▸ Pyruvate [+ CO_2] ⟶ [NADH + H⁺ → NAD⁺] ⟶ Lactate

\+ Acetyl phosphate ⟶ Acetyl CoA ⟶ [NADH + H⁺ → NAD⁺] ⟶ Acetaldehyde ⟶ [NADH + H⁺ → NAD⁺] ⟶ Ethanol

D) Mixed acid fermentation

4 Pyruvates —2X⟶ [2NADH + H⁺ → 2NAD⁺] ⟶ 2 Lactate

2X ⟶ Acetyl CoA ⟶ [2NADH + H⁺ → 2NAD⁺] ⟶ Acetaldehyde ⟶ [2NADH + H⁺ → 2NAD⁺] ⟶ Ethanol
or ⟶ Acetate

⟶ 2 Formate ⟶ $2CO_2 + 2H_2$

E) 2, 3-Butandiol fermentation

2 Pyruvate —[CO_2]⟶ Acetolactate —[CO_2]⟶ Acetoin ⟶ [NADH + H⁺ → NAD⁺] ⟶ 2, 3-Butandiol

F) Propinoic acid fermentation

Pyruvate —[CO_2]⟶ Oxaloacetate ⟶ [NADH + H⁺ → NAD⁺] ⟶ Malate —[H_2O]⟶ Fumarate ⟶ Succinate —[CO_2]⟶ Propionate

G) Butyric acid fermentation

2 Pyruvate ⟶ Acetoacetyl CoA ⟶ [NADH + H⁺ → NAD⁺] ⟶ Butyryl CoA ⟶ (or) ⟶ Butanol

Butyryl CoA ⟶ [NADH + H⁺ → NAD⁺] ⟶ Butyrate

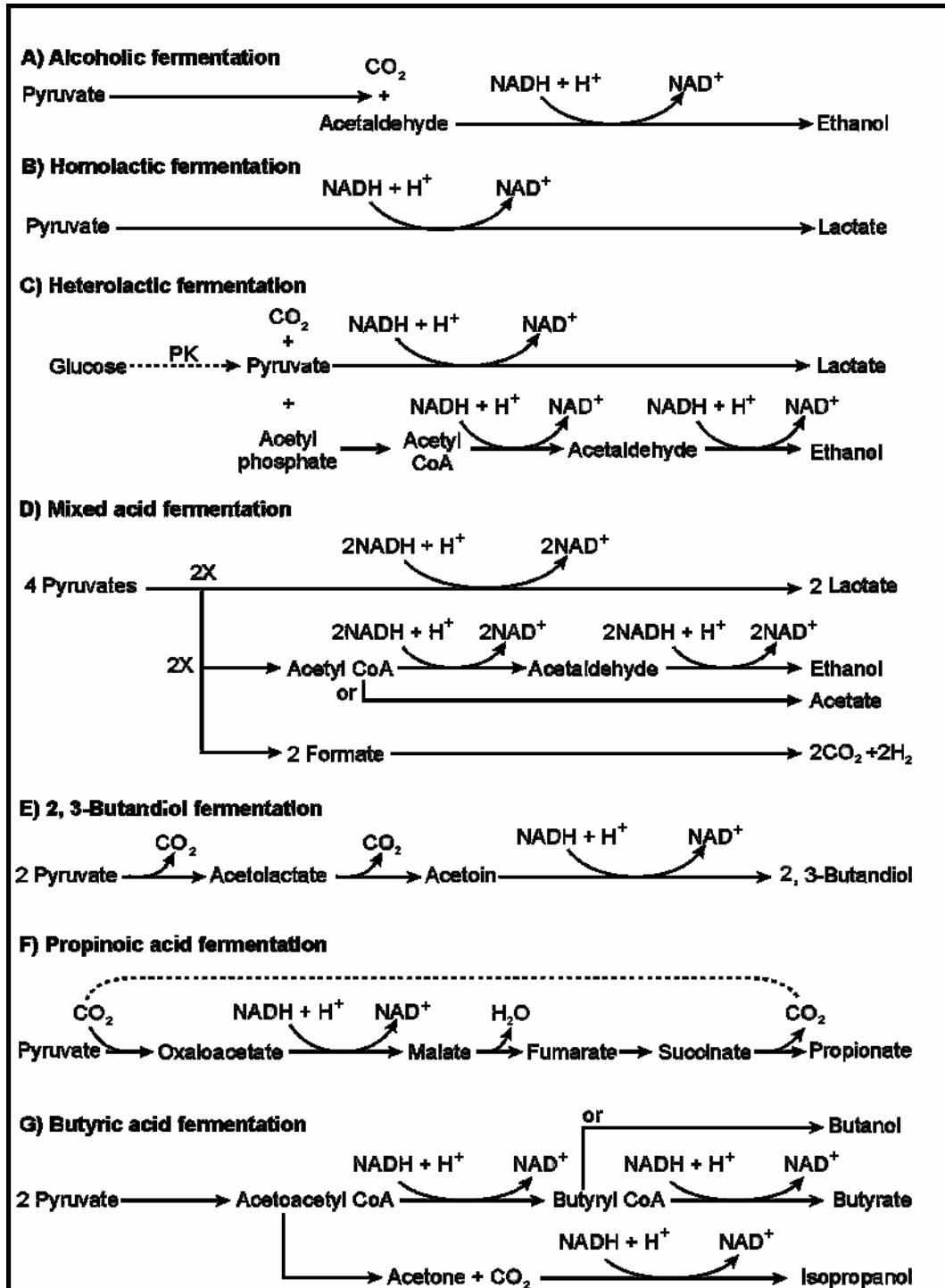⟶ Acetone + CO_2 ⟶ [NADH + H⁺ → NAD⁺] ⟶ Isopropanol

Fig. 10: Anaerobic microbial fermentation products derived from pyruvate

ii)   **Lactic acid fermentation:** is mainly carried out by bacteria e.g. *Streptococcus, Lactobacillus, Lactococcus* and *Leuconostoc.* If the end product formed is only lactic acid then it is called homolactic fermentation. Examples of homolactic fermenters are *Lactobacillus acidophilus* and *L.casei.* If along with lactic acid other end products are formed then it is called heterolactic fermentation. Examples of heterolactic fermenters are *Lactobacillus brevis* and Leuc*onostoc mesentroides* which produce lactic acid as well as ethanol.

iii) **Mixed acid fermentation:** is carried out by bacteria *Escherichia coli*, and *Enterobacter* spp. Pyruvate is fermented to many products like ethanol, acetic acid, lactic acids along with gases like CO_2 and H_2.

iv) **2,3-Butanediol fermentation:** is performed by *Enterobacter, Erwinia, Klebsiella* and *Serratia* organisms. It produces many end products as in mixed acid fermentation but also produce significant amount of 2,3-butanediol.

v) **Propionic acid fermentation**: Many microbes of intestinal flora including *Propionobacterium* spp. convert pyruvate into propionic acid. This type of fermentation also produces many end products including 2,3-Butanediol.

vi) **Butyric acid fermentaion**: It is also a mixed type of fermentation carried out by anaerobic bacteria *Clostridium*. End products include butyric acid, butanol, acetone, propanol etc.

TCA cycle

Under aerobic conditions pyruvate is further catabolized into various intermediates via enzymes of Krebs cycle, also called TCA cycle (Fig. 11). The latter cycle is present in many bacteria, fungi, yeasts, algae and higher forms of eukaryotic cells. Initially pyruvate is decarboxylated to a two carbon compound as shown under:

Pyruvate (C_3) + NAD^+ + CoA

$$\downarrow$$

Acetyl CoA (C_2) + CO_2 + NADH + H^+
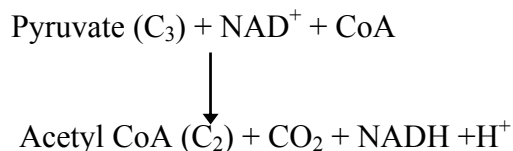
Acetyl CoA then ethers TCA cycle by combining with oxaloacetic acid to form citric acid. The latter molecule is a six carbon compound which gets converted into oxaloacetic acid through a series of reduction and decarboxylztion reactions. The net reaction of TCA cycle is as follows:

Acetyl CoA (C_2) + $3NAD^+$ + FAD + ADP

$$\downarrow$$

$2CO_2$ + 3NADH + $3H^+$ + $FADH_2$ + ATP
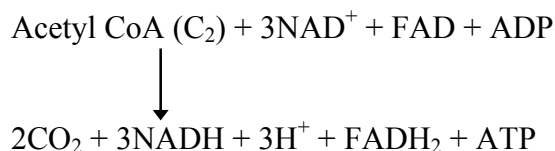
TCA cycle is also a source of generating ATPs though in a limited amount. TCA cycle should not be seen only as a catabolic pathway but also as a major source of C_4 and C_5 intermediates which are required for the synthesis of amino acids and nucleotides. In contrast to fermentation reactions which occur in the cytoplasmic matrix TCA cycle operates in the mitochondrial complex in close proximity to respiratory cycle. The reduced coenzymes like NADH generated during TCA cycle and other fermentation reactions produce ATPs once they enter respiratory cycle as explained under.

Respiration

This is a process in which reduced compounds, NADH and FADH2, generated during various metabolic processes enter electron transport system (ETS) and get oxidized (Fig. 12). This oxidation step is coupled with release of energy that is used for the synthesis of ATP from ADP molecules. The terminal electron acceptor is either oxygen (in case of aerobic respiration) or inorganic compounds like nitrates, sulphates and CO_2 (in case of anaerobic respiration). Theoretically a single molecule of NADH can result in the formation of 7 ATP

molecules. However, in practice the efficiency is only 40% i.e. formation of only 3 ATP molecules per NADPH molecule. The ETS is located in the cytoplasmic membrane of prokaryotes whereas in eukaryotes it is localized in the inner mitochondrial membrane. In case of aerobic respiration the terminal electron acceptor is oxygen which gets converted into water (Fig.). But in case of anaerobic respirations the terminal electron acceptors could be nitrates, sulphates and CO_2 which get reduced to nitrites, sulphites and methane respectively.
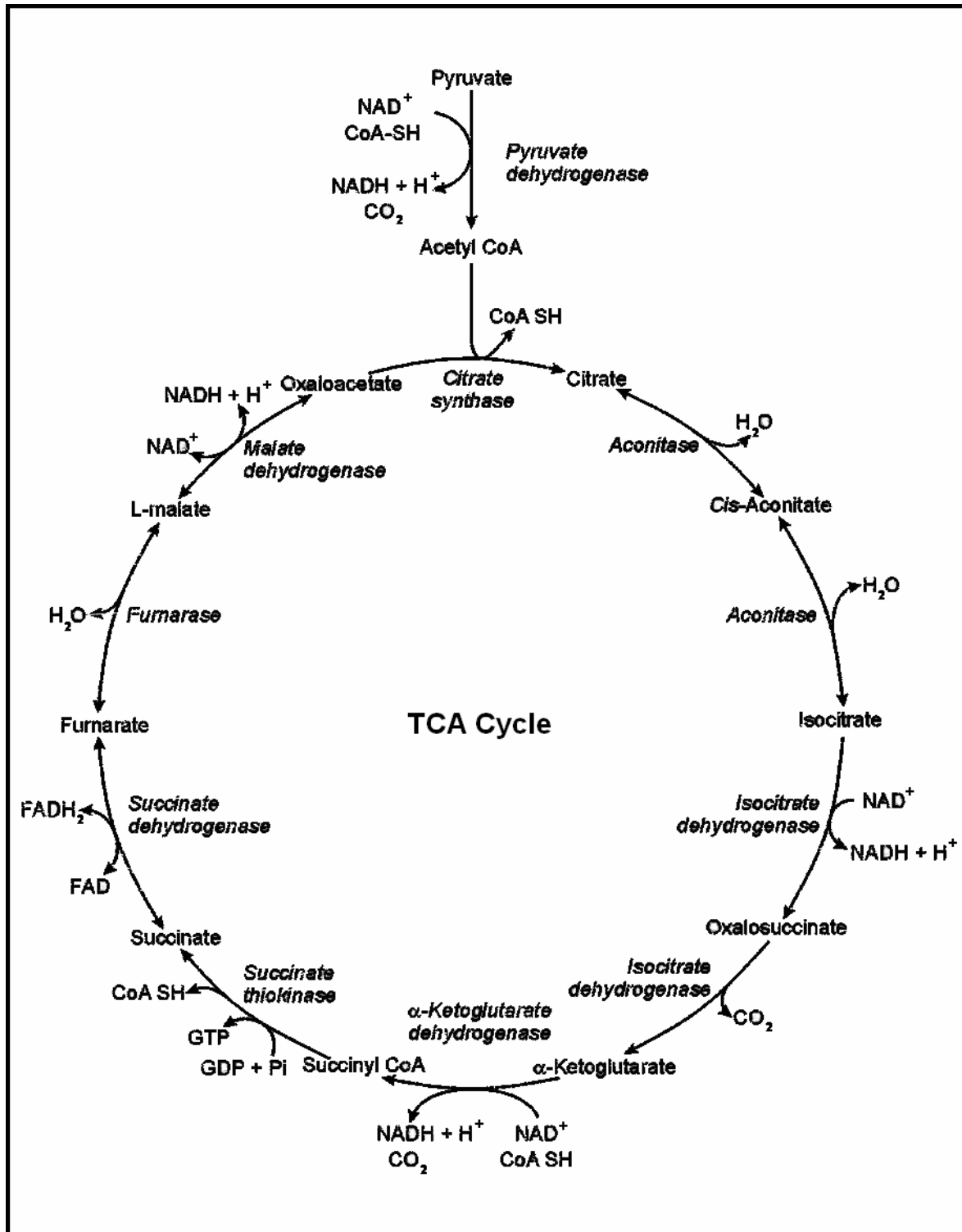

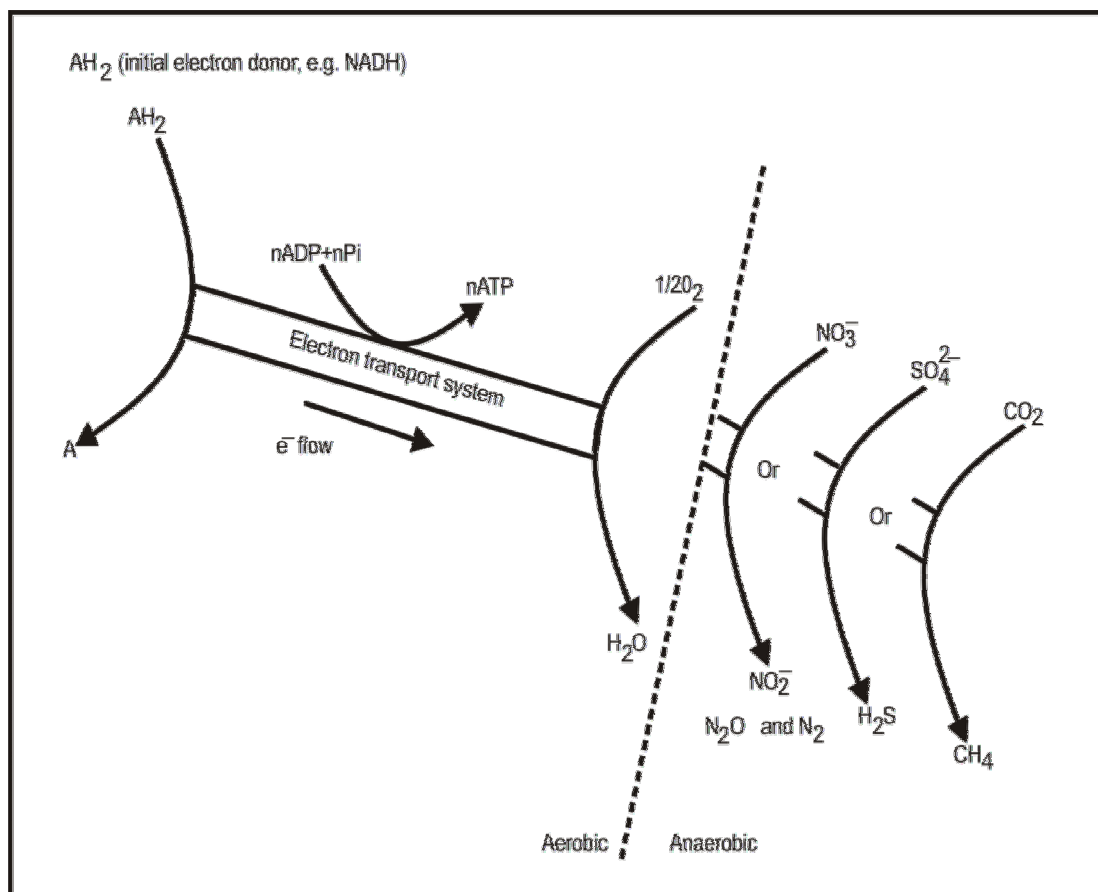**Fig. 11: The Tricarboxylic acid (TCA) Cycle**

Fig. 12: Electron transport chain in microbes

Catabolism of lipids

Lipids are first broken down into free fatty acids and glycerol. The fatty acids are further degraded into two carbon units (i.e. acetyl CoA) via β-oxidation pathway which ultimately enters TCA cycle. There is concomitant generation of NADH and $FADH_2$ coenzymes which enter TCA cycle to generate ATPs. Glycerol is converted to glyceraldehyde 3-phosphate via series of reactions involving phosphorylation, oxidation and isomerization steps.

Catabolism of proteins

Proteins are hydrolyzed to amino acids by proteases and peptidases. Amino acids are converted into keto forms by transamination step in which amino groups of amino acids are removed. The keto acids thus formed join TCA cycle for further oxidation and subsequent formation of ATP as mentioned earlier.

Regulation of Metabolic Pathways

Microbes normally do not overproduce metabolites. There are many checks in the cell which regulate the levels of cellular metabolites. However, overproduction of desired metabolites is of utmost importance for the industry. In order to achieve this, scientists are engaged in understanding the biosynthetic pathways as well as the controls exerted by the cellular

machinery on such pathways. Two major control systems working in a cell are **Feedback inhibition and Feedback repression**. Feedback inhibition is the condition in which the end product of a biosynthetic pathway inhibits the functionality of a key enzyme occurring in this pathway. Inactivation of the enzyme results in cessation of the biosynthesis of a desired metabolite. Feedback repression is a condition in which the end product of a biosynthetic pathway prevents the synthesis of an enzyme/s (Fig. 13). This is achieved by the attachment of the end product or its derivative to the promoter region of the gene. The outcome of this binding is that RNA polymerase is unable to bind to promoter region thus resulting in cessation of transcription process. In general, feedback inhibition and feedback repression act in concert in controlling the biosynthetic pathways. Feedback inhibition is a rapid control mechanism which switches off the biosynthesis of an end product, where as repression is a mechanism to switch off synthesis of temporarily redundant enzymes.
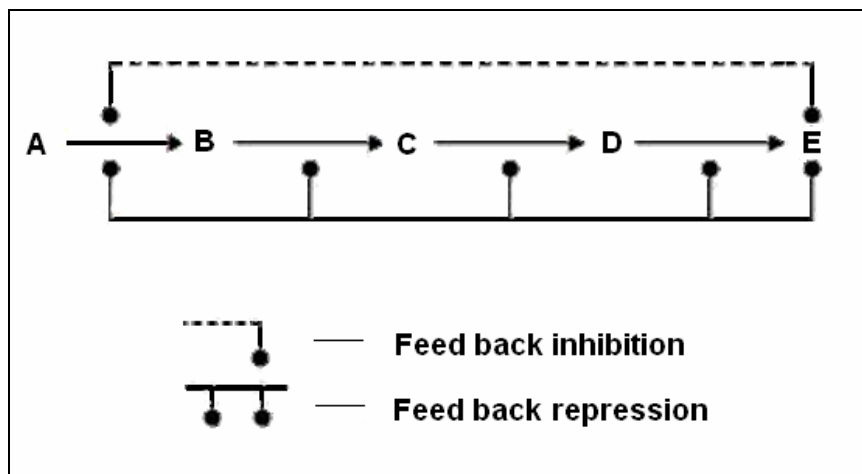

Fig. 13: Regulation of biosynthetic pathways

The biosynthetic pathways of majority of primary metabolites and their regulatory mechanisms are clearly known to us. Production of various amino acids e.g. glutamic acid has been increased tremendously by exploiting the regulatory mechanism of biosynthesis of this amino acid. However, we have limited information regarding biosynthetic pathway of secondary metabolites and their regulations because the number of genes involved in their synthesis is very high. It can go up to over 100 genes. More the number of genes involved, more complex the system is and thus more difficult to understand. A few generalizations have been made in case of production of secondary metabolites:

i) Rapidly utilized carbon sources like glucose and nitrogen sources like ammonia and ammonium chloride results in decreased production of secondary metabolites.

ii) By decreasing the optimal growth rate of an organism, time period for the production of secondary metabolites can decreased drastically.

iii) Phosphates in very small amounts increases secondary metabolite production.

iv) Some of the end products of secondary metabolites result in feed back inhibition.

Screening of Microbes

Isolating microbes from environment is the first and foremost step in screening for an industrial product which may be either a primary metabolite or secondary metabolite.

Although numerous media have been devised for the growth of microbes, there is no single media that supports all kinds of microbes. For example, if a soil sample is plated on nutrient agar (pH 7.0) and incubated at 37°C for 24-48 hours, only a fraction of aerobic mesophiles will be able to grow. We"ll be missing anaerobes, thermophiles, psychrophiles, alkalophiles and acidophiles. Even slow growing mesophiles (e.g. actinomyces) as well as fastidious mesophiles will not get a chance to grow on such media. Also, if a microbe with antibacterial properties is picked up during screening, it may not allow the growth of some/ all of other mesophiles.

Another factor that is quite important for the selection of microbes depends upon existing capabilities of the scientists as well as the existing facilities of the industrial unit. For example the company undertaking the process may excel in handling the bacteria but not fungi. Therefore, these companies would prefer to isolate bacteria (with desired characteristics) and not fungi or algae.

While choosing a microbe for industrial use, specific considerations have also to be kept in kind. The organism to be used should neither be pathogenic (except in case of production of pathogenic factor, vaccine etc.) nor it should be induce allergic reaction. Also its disposal in the environment (at the end of fermentation) should be eco-friendly.

After narrowing down on a specific product to be extracted from the microbes, the first major query which comes to the mind of a microbiologist is, from where to isolate that specific microbe from the environment. The site for isolation of microbes is decided by the type of microbe one is interested in (Table 10).

**Table 10: Preferable sites for isolation of particular microorganisms**

Sr. No.	Product	Site
1.	Thermophiles	Hot spring, thermophilic vents, compost
2.	Alkalophiles	High salinity soils and wells
3.	Acidophiles	Pine growing sites, fruits
4.	Osmophiles	Seas and oceans
5.	Yeast	Wine yards
6.	Phosphate solubilizers	Agricultural soil, rhizosphere
7.	Protease producers	Dairy industry, meat processing units.
8.	Enzymes like cellulase, pectinase, xylanase etc.	Agricultural soil, paper and pulp industry, waste disposal area of fruit juice plants, grain market.
9.	Biogas (methane) producers	Gober-gas plants, effluent collection sites.

Strategies for isolation of desirable microbes

In general, there are two strategies for isolation of microbes from the environment: a) Shot gun approach and b) Enrichment approach.

Shot gun approach: The collected sample is plated on a suitable nutrient medium, e.g. if we are interested in the isolation of a protease producing microbe, a sample from dairy industry (milk, whey, effluent, soil of industrial unit) is plated on skim milk agar and incubated.

Protease producing colonies will show a zone of clearance around them (Fig. 14). This kind of approach is suitable if the desired organism exists in large number in the sample to be tested.
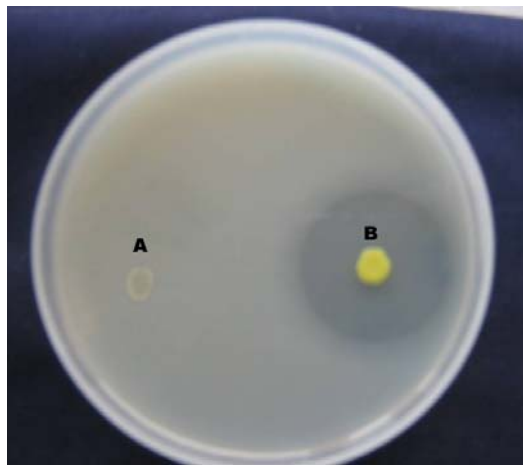
A: Non-proteolytic colony; B: Proteolytic colony

Fig. 14: Protease producing bacterial colonies growing on casein-agar plate

Enrichment approach: If the organism to be isolated exists in less number, it is preferred to kill/suppress the growth of unwanted microbes. This can be done by enrichment process. For example, if one is interested in isolating the organism that can utilize pectin as a sole source of carbon then a synthetic medium is devised which contain pectin as a sole source of carbon. The soil or water sample is added to the synthetic medium and incubated. After 1-2 days of incubation, an aliquot is sub-cultured to a fresh medium and incubated. Four or five sub-culturings ultimately yield organism/s that have the ability to degrade pectin to its monomeric form. The latter form can be metabolized by the organism to produce energy. The organisms which lack the ability to degrade pectin ultimately die as no other carbon source is present in medium.

Choice of a nutrient medium for the growth of microbes

Broadly microbes can be categorized as bacteria, fungi, algae. There is quite a difference in the type of medium required for the culturing of each category of these microbes. Even amongst each category there are thousands of different kinds of microbes which require different kinds of nutrient media for their optimal growth.

Isolation of bacteria: Based on the difference in the composition of the cell walls, bacteria are divided into two parts: Gram positive (G+) and Gram negative (G-). *Bacillus, lactobacillus, staphylococcus, streptococcus* are examples of G+ bacteria. *E.coli, salmonella, shigella, vibrio & pseudomonas* are example of G- bacteria. Most of these bacteria can grow on nutrient agar (NA) medium (Table 11).

Table 11: Composition of nutrient agar (pH 7.0)

Peptone	10.0g
Beef extract	5.0g
Sodium chloride	5.0g
Agar	15.0g
Dist. water	1.0 litre

To suppress the growth of fungi, antifungal agents (cyclohexamide and nystatin in concentration range of 50-100 ug mg/ml) are added to the nutrient medium. For the isolation of G- bacteria, Mackonkey agar (Table 12) is preferred as bile salts present in this medium suppress the growth of G+ bacteria.

Table 12: Composition of Mackonkey agar (pH 7.0)

Peptic digest	2.0g
NaCl	5.0g
Lactose	1.0g
Bile salts	5.0g
Neutral red	0.07g
Agar	15.0g
Dist. water	1.0 litre

For the isolation of spore forming bacteria, the sample is heated around 70°C for 10-15 minutes to kill vegetative cells. For the isolation of organism which can utilize a specific carbon source or nitrogen source a synthetic medium like M-9 is preferred (Table 13).

Table 13: Composition of M-9 medium (pH 7.2)

$Na_2HPO_4.7H_2O$	12.8g
KH_2PO_4	3.0g
NaCl	0.5g
NH_4Cl	1.0g
1M $MgSO_4$	2.0ml
1M $CaCl_2$	0.1ml
20% (w/v) carbon source	20ml
Dist. water	up to 1.0 litre

In this medium carbon and nitrogen sources can be varied. Commonly used carbon source is glucose and nitrogen source is ammonium chloride.

Isolation of actinomyces: This class is a special kind of filamentous bacteria which are slow grower and have the ability to grow in nutritionally poor medium. Although these organisms can be isolated from anywhere but preferable site is an agricultural soil. Their numbers is

quite high during dry seasons and drastically goes down during rainy season. It is desirable to air dry the soil sample for a few days prior to its plating on a media for selection of actinomycetes. For the isolation of this group of bacteria nutrient medium such as Arginine-glycerol-salt medium (Table 14) is well suited. Supplementation of these medium with chloromphenicol (50 ug/ml), nystatin (50 ug/ml), polymixin (50 ug/ml) and penicillin (100ug/ml) help in suppressing growth of fungi and other kinds of bacteria.

Table 14: Composition of Arginine-glycerol-salt medium

Arginine monohydrochloride	1.00g
Glycerol	12.50g
K_2HPO_4	1.00g
NaCl	1.00g
$MgSO_4.7H_2O$	0.50g
$Fe_2(SO_4)_3 .6H_2O$	0.01g
$CuSO_4.5H_2O$	0.001g
$ZnSO_4.7H_2O$	0.001g,
$MnSO_4.H_2O$	0.001g
Agar	15.0g
Dist. Water	1.0 litre

Isolation of fungi: Fungi are ubiquitous in nature and have a diverse nutritional requirement just like bacteria. Hence there is no "all purpose" fungal isolation media. The supplementation of nutrient medium with antibacterial compounds like penicillin, streptomycin, chloramphenicol, kanamycin and tetracycline helps in controlling the growth of bacterial population. Commonly used medium for isolation of fungi are (a) Potato Dextrose Agar (PDA) (b) Rose Bengal Medium (Table 15). The composition of these media are given below:

Table 15: Composition of media for isolation of fungi
(a)   Potato Dextrose Agar

Scrubbed potatoes (peeled and diced)	200.00 g
Dist. Water	1.0 litre
Boil and simmer for nearly one hour,	
Pass it through a sieve so as to retain potato slices.	
Agar	15.0 g
Dist. Water	up to 1.0 litre
Glucose	15.0 g

(b)   Rose Bengal Medium

KH$_2$PO$_4$	1.0g
MgSO$_4$.7H$_2$O	0.5g
Soytone of phytone	5.0g
Glucose	10.0g
Rose Bengal	0.035g
Agar	15.0g
Dist. Water	1.0 litre

In order to isolate fastidious microbes from soil or parts of plants, it is desirable to supplement the medium with soil/plant extract. By and large these aqueous extracts are added to the microbes at a final concentration of 5-10% (V/V). These extracts are filter sterilized (preferably) or heat sterilized prior to addition to the medium.

Metagenomic library

It is believed that microbiologists have been able to cultivate only 1% of all the different types of microbes present on this earth. The inability to cultivate majority of microbes is our lack of understanding of the nutritional requirements of such microbes. From biotechnology point of view, we are interested in a particular metabolite of an organism and not the microbe. Tremendous advancements in the field of biological sciences and technology has led to a unique way of isolating metabolite from non-culturable organism. This technique is known as "Metagenomic technique" which makes the use of PCR (Polymerase Chain Reaction) technique. Briefly, total DNA present in the soil sample is extracted, purified and used as a template for amplification of desired gene whose product has industrial application. The amplified gene is cloned in a vector and transformed into a cell e.g. *E.coli*. Hence, using this technique, the inability of scientists to grow the microbes is taken care off as we have fished out the requisite gene of interest and cloned it in a culturable bacterium like *E.coli*. With this technique many novel lipases have been isolated from the environment.

Screening assays for microbial activities

Depending on the type of metabolite one is interested in, following methods are adopted for the screening of desirable microbes.

Plate assay: This is a very simple method for screening of a microbial metabolite. It is preferably used for screening of extra cellular compounds like enzymes, anti-microbial compounds etc. Lets assume we are interested in the isolation of protease secreting bacteria. For this, a spoonful of soil is added to 5-10ml of sterilized water and vortexed thoroughly so that microbes clinging to soil particles are detached. To aid this process of detachment, sometimes, a non-ionic detergent like Tween 80 (0.01-0.1%, final concentration) is added to sterile water. The soil suspension is allowed to stand for a few minutes for the solid particles to settle down. Various ten fold dilutions of the aqueous sample are made and 100ul of each dilution is plated on a solid nutrient agar medium containing a protein source (casein or skimmed milk, 5%,w/v) and incubated at 30-37°C for 24-48 hrs. The bacterial colonies showing the zone of clearance around them are indicative of protease production.

If one is interested in isolating microbe/s which can utilize specific carbon source e.g. cellulose, or a particular nitrogen source e.g. urea, a synthetic medium e.g. M9 medium is prepared in which the desirable carbon or nitrogen source is added. This medium does not contain any other carbon or nitrogen source. The sample is plated on this medium and incubated. The growth of colonies on such medium indicates that these colonies have the ability to utilize carbon and nitrogen sources present in the medium. To rule out false positive organisms the colonies growing on the synthetic medium are further streaked on fresh M9 plates, incubated and subsequently observed for healthy bacterial colonies.

In order to select for antibiotic producers, the organism is streaked as straight line in the middle of petri plate containing a solid nutrient medium. The organisms to be tested for their sensitivity/resistance to the test organism are streaked perpendicular to the already streaked test organism. The plates are incubated for 24-48 hours. The inhibition of growth of microbes around the streaked culture is suggestive of the sensitivity of that organism towards the antibiotic produced by the test organism.

Colorimetric assay: In this technique a culture is grown in a liquid medium, centrifuged. A small aliquot of the cell free supernatant is added to a substrate solution and allowed to react for a while. The reaction is stopped (either by heat or addition of acid/alkali) and a colour developing agent is added which reacts with the product to yield a colored compound which is measured using a colorimeter or a spectrophotometer.

HPLC technique: To look for the presence of a specific metabolite in a given sample, an aliquot of the sample is injected into an instrument called HPLC {High pressure (performance) liquid chromatography}. The sample flows through a column under high pressure. The solutes present in the sample are resolved into various components. These components are detected by a highly sensitive spectrophotometer and the data recorded in a computer. The column comprises of a stationary phase containing finely divided solid (silica gel, resins, ligands etc.) and a mobile phase (solvent). The movement of the solutes is dependant on their solubility in mobile phase and retention in stationary phase.

Immunoassay technique (Western blotting technique): This test is good for assaying those protein which do have any functional activity, e.g. structural proteins. A small drop of a cell or cell free supernatant is fixed on to a nitrocellulose membrane. About 50-100 samples can be applied to a single membrane. Membrane is then exposed to primary antibodies (raised against the specific protein). Since antigen antibody reaction is highly specific, antibodies will bind only to the specific protein. The membrane is then washed off to remove unbound primary antibodies and then exposed to conjugated secondary antibodies. These antibodies have been raised in an animal in which the primary antibodies have been injected as antigen. The secondary antibodies have also been conjugated with an enzymes like alkaline phosphatase or horse raddish peroxidase. These secondary antibodies will bind only to primary antibodies. After washing the membrane a solution containing substrate is added on to membrane. The bound enzyme converts the colorless substrate into a colored product.

Culture Preservation

The conventional microbiology is totally dependent on the availability of pure and stable cultures. Even in the hands of an adept microbiologist, a culture is prone to contamination, denaturation and death. Since the chosen industrial strain is selected after extensive screening and genetic manipulation, it is prudent to maintain adequate stocks of requisite cultures under

conditions which do not require regular sub-culturing. Its an established fact that serial sub-culturing over 7-8 steps of *Penicillium chrysogenum* and *Streptomyces niveus* results in complete or near complete loss of penicillin and novobiocin production respectively. In order to prevent the loss of production of a desired metabolite and to preserve culture, a variety of culture preservation techniques have been developed. All these techniques have their own advantages and disadvantages. No single method is available for successful preservation of all kinds of microbes. The most prevalent techniques of culture preservation are discussed below.

Storage at reduced temperature

Agar slants/stabs: The culture is streaked over the surface of slope of solidified nutrient medium. It can also be stabbed with the use of sterile straight needle. Usually such cultures are kept in a refrigerator at 4ºC. In order to avoid air contamination, the tops of the tubes are either covered with para film or molten wax. Normally, cultures are viable for 2-4 months depending on the type of organism. The storage period can be enhanced by covering the cultures with a layer of mineral oil. Though this method of preservation is simple and economical but it requires periodic sub-culturing. There is high probability that after a few sub-culturing the cells pick up mutation in the gene of interest and hence become undesirable.

Storage at sub-zero temperature: Microbial cultures can also be stored at -20ºC, -80ºC and under liquid nitrogen (-193ºC). In these cases a heavy growth of microbial cells is suspended in sterile glycerol solution (10-20%, w/v) or DMSO (5%, v/v). Generally the cultures are stored in cryovials which do not crack or break under extreme low temperature. Since glycerol is toxic to the cells, the cultures suspended in glycerol should not be kept long at room temperature. The cultures can be stored for 6-8 months at $-20^{o}C$ and for years at -80ºC and $-193^{o}C$. The disadvantage of this technique is that regular freezing and thawing results in killing of the cells. Also, regular supply of liquid nitrogen may be a problem at some places.

Storage of glass beads: Repeated freezing and thawing of the culture result in their cell death. To overcome this problem cultures are suspended in cryprotectant solution and glass beads are added. A few beads are removed as and when needed and added to sterile nutrient broth. Therefore thawing process is not needed.

Storage in dehydrated forms: *Dried cultures:* This technique is good for spore forming microbes. The sterile soil is mixed with culture of microbes, incubated for a few days and then allowed to dry at room temperature for 1-2 weeks. Dried soil containing spores can be kept at room temperature or in refrigerator. The soil can be replaced by silica gel or porcelain beads. The storage period is very long. Strains of *Actinomyces* have been recovered even after 20 years of storage using this technique. *Lyophilization (freeze-drying):* This is most satisfactory means of preservation of culture. In all culture collection centers (Table 16) cultures are stored in powdered form in glass vials. In this technique, cells are suspended in a buffer or skimmed milk and are frozen. Water is evaporated from this sample by the process of sublimation. Once the water has been removed the culture are sealed under vacuum in vials or ampoules.

Table 16: Important microbial culture collection centers

Culture collection centers	Types of microbes stored
Microbial type culture collection center (MTCC), Institute of Microbial Technology (CSIR branch), Chandigarh. India	Non-pathogenic bacteria and fungi
American Type Culture Centre, Manassa, Virginia. USA	All
Collection Nationale de Cultures de Microorganisms (CNCM), Paris, France	All
Deutsche Sammlung von Mikrooganismen und Zelkulture (DSMZ) Braunscheig, Germany	All
National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Collindale. U.K.	Medical microbes
National Collection of Food Bacteria (NCFB), Aberdeen, U.K.	Food bacteria

Strain Development Strategy

An ideal industrial strain should have the following qualities:
  i) The organism should be able to use wide range of low cost and easily available substrate through out the year.
  ii) The organism should produce large biomass and high amount of industrial product.
  iii) The organism should be non-pathogenic and non-allergic.
  iv) Organism should not degrade with passage of time i.e. it should be genetically stable.
  v) It should be easy to perform genetic manipulation on it.

Unfortunately the organism isolated from the environment for industrial use does not fulfill all these requirements. Any property whose addition or deletion from the strain results in minimizing its cost production comes under the purview of strain improvement. It can be:
  i) Increase in bio-product formation.
  ii) Release of intracellular bio-product by altering permeability of cells.
  iii) Overcoming feedback inhibition/repression phenomenon.
  iv) Simplifying procedure for extraction/purification of bio-product from fermentation medium.

Strain improvement by genetic manipulations

In addition to growth media optimizations, genetic manipulation of the organism have also been used for improving the productivity of the strain. The genetic manipulations can be brought about by a mutations and genetic engineering process.
Mutation: The permanent inheritance change in the genetic make up of the organism is called mutation. The agent which brings about the mutation is called mutagen and the organism in which mutation is carried is called a mutant as it possesses mutated gene/genes. In the absence of mutagen the mutation rate is very low i.e.10^{-8} to 10^{-10} per generation/per gene. However the mutation rate in the cells can be enhanced 100 -100,000 folds with the treatment of cells with a mutagen. Basically there are two types of mutagens: a) physical

mutagens, e.g. radiations (uv-rays, x-rays, gamma-rays) and b) chemical mutagens, e.g. nitrosomethyl guamidine (NTG), ethylmethane sulphonate (EMS), nitrous acids (NHO_2 etc.).

These mutagens can bring about many changes in DNA including base pair substitutions i.e. transitions/transversions and frame shift mutations i.e. addition/deletion of nucleotides. Mutations have been used successfully to increase the production of industrially important product especially antibiotics and amino acids. However, one must keep in mind that mutation carried out with physical or chemical agents listed above is a random process. It can effect any site on DNA thereby bringing undesirable changes in the organism. Also, we might be able to increase the production of a desired metabolite but we are not sure whether mutation has occurred in coding region of the gene or in its regulatory region or somewhere else e.g. secretary pathway. In spite of these drawbacks, many industrial strains have been subjected to mutations with positive results e.g. wild type penicillin producing fungal strain (*Penicillium chrysogenum*) produced 2U/ml of penicillin. By subjecting the fungal strain to various cycles of mutations (uv rays, gamma rays, NTG, EMS etc.) the mutant strain produces 100U/ml of penicillin.

Protoplast fusion: Protoplasts are single cells from which cell wall has been removed. Protoplast fusion provides a unique tool to combine valuable traits beyond the feasibility of normal sexual crossing. It is widely applied in Plant biotechnology, but is gaining importance in microbes, especially fungi. In this technique, cells from genetically related or unrelated species are fused to produce hybrid cells of desirable traits.

The cells are first treated with enzymes to degrade their cell walls. For bacteria, lysozyme and for fungi a mixture of cellulases and chitinases are used. Once the cell wall has been removed the resulting protoplasts are spherical in shape. Protoplast formation is carried out in an osmotic solution so that protoplasts do not lyse in the medium. The protoplasts thus formed are separated from the cells and debris by differential centrifugation. The fusion of protoplasts is carried out by one of the three procedures: a) treatment with calcium ions at high pH, b) Polyethylene glycol (PEG) treatment and c) electrofusion which utilizes low voltage electric current pulses to fuse the protoplasts. PEG is a fusogen. The technique gives high frequency of fusion with reproducible results and involves low cytotoxicity. After the fusion process, cells are transferred to a generation medium in which formation of cell wall takes place. The last step in protoplast fusion process is plating the hybrid cells on a selective nutrient medium which supports the growth of hybrid cells. The final step is the selection of those hybrid cells which are of industrial importance.

Gene cloning: Gene cloning, as the name suggests, is the cloning of a gene of interest. As the genes of all the organisms (microbes, plants and animals) are made up of same genetic material i.e. nucleotides (adenine, guanine, cytosine and thymine), one can easily shuffle the genes of one organism into another. By means of gene cloning the production of many industrially important bioproducts has been increased many folds. Using this technique, not only the copy number of the concerned gene can be increased but the expression of the gene can be enhanced by replacing the original weak promoter of the gene with a strong promoter. The process of gene cloning is explained below.
Lets assume we have isolated a bacterial strain which produces a unique protease of industrial importance. Unfortunately, this organism is slow grower and also requires a highly nutritious and expensive medium for growth. In addition the amount of unique protease produced by this organism is very low. In order to produce this protease in large amounts, we have to clone the protease gene under the influence of a strong promoter in a fast growing organism

like *E.coli* whose nutrient requirements are simple. The protocol used to achieve this goal is as follows:

Genomic DNA of the protease producing organism is isolated and cut into small fragments by the use of a specific restriction enzyme e.g. BamH1. A plasmid present in another organism is isolated and cut with the same enzyme. The DNA fragments and the cut plasmid are mixed together and an enzyme ligase is added which joins the plasmid with DNA fragments. The ligated mixture is then transferred into *E.coli* and plated on a solid nutrient medium in which only plasmid carrying *E.coli* cells will grow. These plates are incubated overnight at 37°C. By this way thousands of transformants are obtained. One of the transformants carry the protease gene. This transformant (or colony) is identified by streaking a small portion of each colony of transformant on casein-agar plate and incubated overnight at 37°C in an incubator. Addition of casein to the solid medium makes it opaque. After incubation, appearance of clear zone around a bacterial colony is indicative of the production of protease producing transformant. Or in simple words, the protease producing gene of slow growing bacteria has been cloned in *E.coli*. The DNA fragment cloned in the plasmid carries the original weak promoter of the gene in addition to non essential DNA portion. In order to find out the location of structural protease gene in the cloned DNA fragment, this fragment is sequenced. The exact location of the structural gene (coding for protease) is determined using bio-informatics tool. The structural gene is sub-cloned in a high copy number expression vector under the influence of a strong promoter and transformed into *E.coli*. Thus we achieve high expression of novel protease gene in *E.coli*.

Microbial Growth and its Kinetics

The growth of microbes in a liquid medium might result in either homogenous suspension (exhibited by unicellular bacteria) or heterogeneous suspension in the form of flocks, pellets or mats (exhibited by filamentous antinomyces and fungi). The growth kinetics of unicellular bacteria can be calculated by mathematical equation and it is of utmost importance when large fermentors are operated upon. A typical unicellular bacteria exhibit four phases of growth when grown in a liquid medium (Fig. 15). These phases are lag phase, log phase, stationary phase and death phase.

In lag phase, although cells are metabolically active but they do not multiply. The metabolic activity of cells is associated with the adaptability of the organisms to new environment (pH, temperature, heat, oxygen tension, sources of carbon and nitrogen etc.). The organisms synthesize new enzymes required for new metabolic activities and shut off those enzymes or proteins which are not needed. In log phase, each cell divides into two daughter cells. The cell division occurs with increasing frequency until the maximal growth rate (μ_{max}) is reached. The cell number and cell biomass increases at a constant rate. This exponential growth can be described by two methods; one is related to biomass (x) and another to cell number (N).

The rate of growth of a cell is dependent on biomass concentration and is calculated as follows:

$$\mu x \quad = \quad dx/dt$$

μx = Specific growth rate / hour.

t = Time in hours

x = Biomass concentration (g/l)
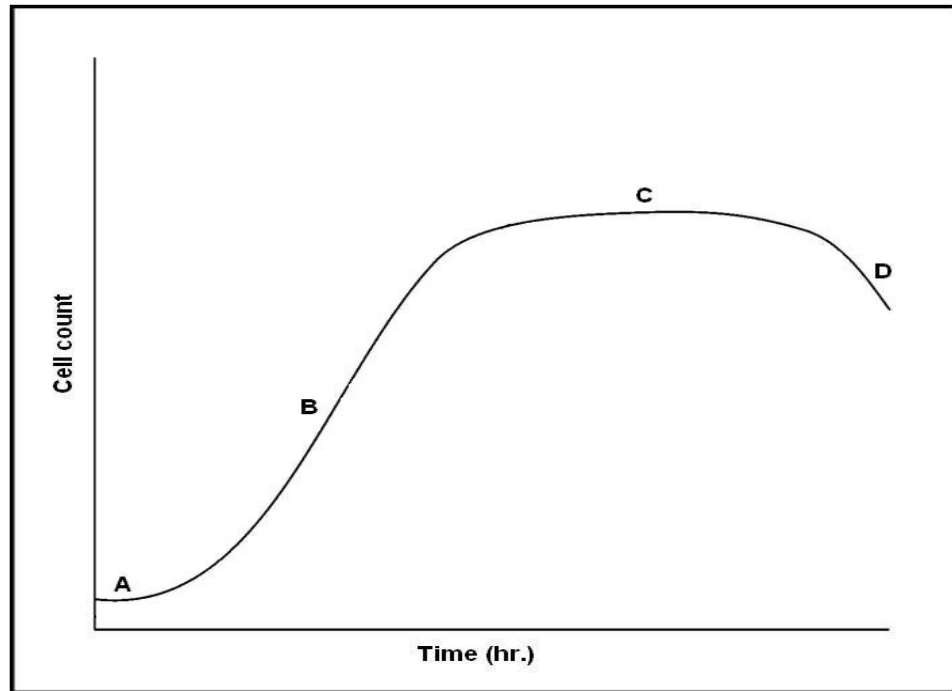
A: lag phase; B: log phase; C: stationary phase; D: death phase

Fig.15: Different phases of growth in bacteria

The specific growth rate (u) can be calculated using the following equation:

$$\mu \quad = \quad 1/x \ X \ dx/dt$$

If a single cell divides into two then growth of the organisms in active phase (log phase) can be calculated by,

$$n \quad = \quad \frac{l_nN_t - l_nN_0}{l_n2}$$

n   =   No. of divisions
N_0 =   Cell number in the beginning i.e. at time t_o
N_t =   Cell number at the end of the time period (t)

Its growth rate (number of division/hour) can be calculated by

$$n/t \quad = \quad \frac{l_nN_t - l_nN_0}{t \ x \ l_n \ 2}$$

Its doubling time (t_d, time taken to double the cell number) is given by:

$$t_d \quad = \quad t/n \quad = \quad \frac{t \ x \ l_n \ 2}{l_nN_t - l_n \ N_0}$$

As long as there is excess of nutrients in the medium, a balanced exponential growth of cells takes place. In other words, there is direct relationship between the specific growth rate (μ) and doubling time (t_d). But as the cells grow there is depletion of nutrients or components of nutrient. This factor along with release of toxins/acids etc. by the cells result in decreased growth rate. The effect of limiting the substrate concentration (of a nutrient) on the growth rate is calculated by using the following equation:

$$\mu = \frac{\mu_{max} \; X \; S}{K_s + S}$$

μ_{max} = max. specific growth (per hour) i.e. all nutrients (substrates) present in excess.

S = Concentration of limiting substrate (g/l)

K_s = Saturation constant (g/l) of limiting nutrient enabling growth at half the max. specific growth rate (i.e. $\mu = \frac{1}{2} \, \mu$ max.).

As per this equation, the cells will grow exponentially as long as the limiting substrate concentration is greater than Ks. When the concentration of the substrate goes down to a level and can no longer sustain μ_{max} the cells enter into the stationary phase. In this phase, number of cells dividing is equal to number of cells dying. So, there is no net increase in the number of cells. As the rate of limiting nutrient/s concentration goes down further, the cell enters death phase. In this phase the cell starts dying exponentially and may undergo lyses (depending on the type of cells).

Fermentor & Fermentation Process

A fermenter, also called a bioreactor, is a vessel in which a particular microbe is grown under controlled conditions to produce a desired byproduct or biomass. The aim of the fermenters is to provide a stabilized condition for growth of cells and better production of a desired by-product. In an ideal fermentation, important parameters of fermentation (pH, temperature, gas transfer, heat transfer, agitation and foaming) are tightly regulated.

In the laboratory, fermentation is carried out in conical flasks as the volume of fermentation medium is small i.e. up to 500-1000 ml. These flasks are made up of glass or plastic material. The flasks are kept in an incubator–shaker in which the temperature and aeration can be regulated.

There are many types of fermenters, but a conventional fermenter (Fig. 16) has the following features: A fermenter is made up of non-corrosive cylindrical steel vessel which can easily withstand a temperature of 150-190°C and a pressure exceeding 380 kPa. The latter conditions are necessary for satisfactory sterilization of the fermenter. The vessel has many inlets for addition of gases, media components, acids/alkalies as well as outlets for escape of gases and sample collection for various tests to be performed during incubation. The vessel also has provisions for ports for various sensors like pH, temperature and dissolved oxygen (DO). The fermentation vessel has a provision for cooling the fermentation broth, if the incubation temperature rises above the requisite temperature. The latter phenomenon is observed in exothermic fermentations like ethanol fermentation. The fermenter is cooled down by passing cold water in the jacket surrounding the vessel or in the coils present inside the vessel. The fermenters are fitted with an agitator for uniform mixing of the media

components and gases, maintaining constant temperature as well as uniform suspension of cells in the fermentation medium.
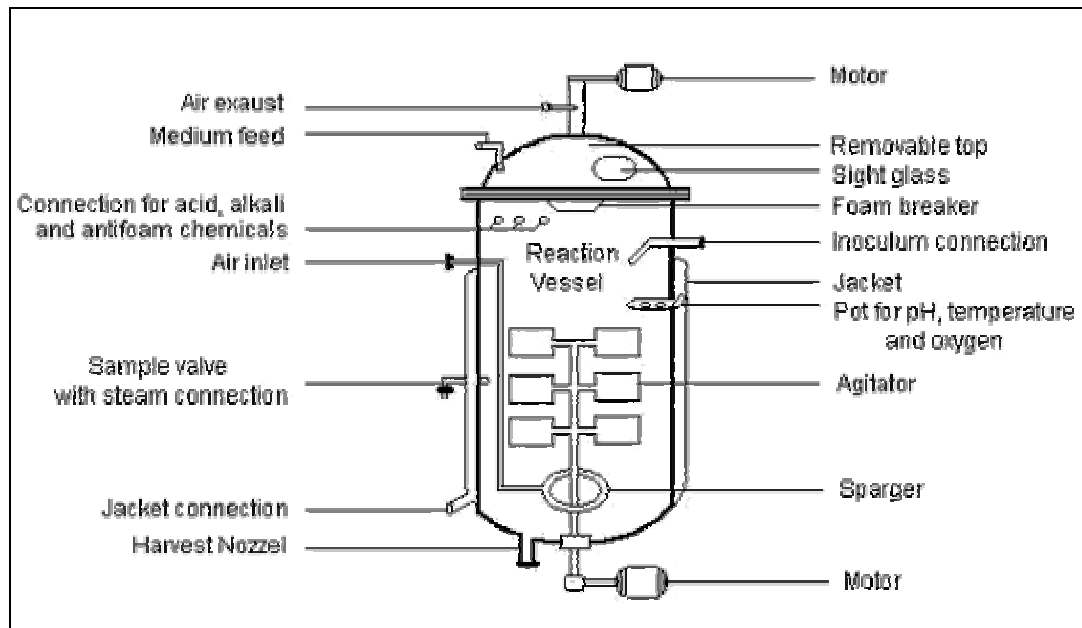

Fig. 16: A typical bioreactor

Types of bioreactors

Based on the design of bioreactors, they can be classified as: a) Stirred tank bioreactor, b) Bubble column bioreactor, c) Airlift bioreactor, d) Fluidized bed bioreactor and e) Packed bed bioreactor

a) Stirred tank bioreactor
It is also called stirred tank reactor (STR). It is fitted with impellors (agitators) which are connected to a motor driven shaft located centrally in the reactor.

b) Bubble column bioreactor
This type of bioreactor lacks impellers. The air is pumped into the fermenter through a plate located at the base of the bioreactor. This plate has many openings through which air or gas is passed in the form of bubbles into the liquid medium contained in the bioreactor. The introduction of air/gas in this manner results in uniform mixing of the fermentation broth.

c) Airlift bioreactor
In this the inside of the bioreactor is divided into two zones, riser zone and downcomer zone, with the help of a baffle or a draft tube. The air enters the vessel through the bottom part of the rizer zone resulting in upward movement of the medium. The upper portion of medium then enters the downcomer zone and flows down to the base of the bioreactor.

d) Fluidized bed bioreactor
In this type of bioreactor the immobilized cells or enzymes, present in the form of beads, are suspended in the lower cylindrical portion of bioreactor. The air is pumped in the vessel from the bottom of the bioreactor. The upper portion of the bioreactor comprises of cell free broth

containing the bioproducts of fermentation. This upper portion is collected in a separate tank as the new medium is pumped in the lower vessel.

e) Packed bed bioreactor
In this type of reactor, the vessel is loosely packed with matrix to which a biocatalyst (cells or enzymes) has been immobilized. The substrate is poured onto the matrix from the top of reactor and the bioproducts are collected from the bottom of the reactor. In a few cases, the substrate is pumped into the reactor from the bottom and bioproduct is collected from the top.

Fermentation processes

There are two groups of fermentation processes: liquid fermentation and solid fermentation. In former case, cells are suspended in aqueous medium where as in latter case, the volume of free liquid is minimal and the cells are adsorbed to a solid and nutrient rich material.

Liquid fermentation

There are many forms of liquid fermentation:

i) Batch Fermentation
The culture is inoculated into the sterile medium contained in a closed vessel. No additional nutrients are added once the fermentation process starts. Physical parameters (pH, temperature and aeration) of the fermentation medium are regulated in this type of fermentation.

ii) Fed Batch fermentation
It is quite similar to Batch fermentation except that the nutrients or one or two components of nutrients are added periodically in the fermentation medium. This type of fermentation lengthens the log and stationary phase of the cells thereby causing increased amount of bioproduct.

iii) Semi continuous fermentation
In this type of fermentation, a fixed volume of fermented medium is taken out from the fermenter and the same volume of nutrients is added so as to keep the volume of fermentation medium same and replenishing the nutrients for growth of microbes. This process also results in maintaining the microbes in same phase of growth.

iv) Continuous fermentation
In this case, there is continuous removal of culture medium as well as continuous addition of sterile nutrient medium. Conditions are predetermined as to what should be the flow rate of incoming nutrient solution so that the volume of fermenting medium remains the same and also fermenting microbes remain in same phase of growth termed as steady state of growth. The latter condition can be achieved by keeping constant concentration of carbon source or nitrogen source or dissolved oxygen content. Such type of fermenter is called a chemostat. The steady state of growth can also be maintained by constantly monitoring the turbidity of the culture medium by use of turbidometer. Such type of fermenter is called a turbidostat.

Solid State Fermentation (SSF)

In some cases microbial growth is carried out on the solid substrates. The amount of free water is almost non existent. The microbes adhere to the solid substrates which are source of nutrients for them. The source of solid material as well as culture is cereal grains of wheat, rice, maize etc. The grains are moistened with water and ground to form a paste. Additional supplements like salts etc. may be added to the solids prior to sterilization. The solid material is then transferred to shallow metallic containers and is steam sterilized. This is followed by the spraying of culture inoculum on to the surface of sterilized medium and incubation is carried out under controlled conditions of temperature, air and humidity. However in some cases like idli, cada, dosa, pickles and jalebi no sterilization is carried out before fermentation. Only when solid fermentation using tray fermentors are used in enzyme production or any such product and pure cultures are used for fermentation then steam sterilization of medium before fermentation is done. Nearly all domestic fermented foods are prepared by use of SSF e.g. idli, vada, pickles and jalebi. Table 17 lists other examples SSF.

Table 17: Products made by the use of solid state fermentations

Product	Substrate	Microbes involved
Soy sauce	Soy beans	*Aspergillus oryzae*
Citric acid	Maize, bagasse	*Aspergillus niger*
Mushroom	Wheat/rice straw	*Agaricus bisporus*
Cheese	curd	*Penicillium roqueforti*

Sterilization of culture media and fermenter

Sterility is of utmost importance to avoid growth of unwanted contaminants and in achieving maximal yield of a bioproduct by the use of fermentation process using known cultures as inoculum. The most common means of achieving sterilization is moist heat. All forms of vegetative microbes are killed by applying heat (60°C) for 10-15 min. The spores are heat resistant and majority of them are destroyed only if exposed to 100°C for 10 min. However, in a few cases, like spores of *Bacillus stearothermophilus,* temperature over 120°C is needed for their destruction. Complete sterility of culture medium is achieved by subjecting the medium to 121°C for 15 min. or equivalent time temperature combination for small volumes of sample. However, the time period is increased as the amount of volume increases. This much temperature is achieved by passing hot steam under high pressure (15lbs per square inch of medium). All batch fermentation media are sterilized by this technique. In case of continuous fermentations, the sterilization of medium is carried out at 140°C for 15-120 sec. depending on the type of medium. Another type of sterilization which is used in the industry is filtration technique. It is applied to such substances which are heat labile e.g. vitamins, serum components, antibiotics. Such solutions are passed through bacterial- filters having porosity of 0.22u.

The fermenters and the accessory pipe lines attached to the fermenter are sterilized by the use of hot steam. The other methods involving radiations and chemical disinfectants are used in a few cases, but they do not guarantee 100% sterility. The gases which are pumped into fermenters are generally sterilized by passing the gas through filters known as depth filters or membrane cartridge filters. The industrial fermenters are made up of high quality steel material which is non-toxic, resistant to corrosion caused by acids/alkali and heat resistant so

that they can be sterilized by pressurized hot steam. Similarly, the associated pipelines which are needed to add inoculums, acids/alkali/air nutrients composed are made up of heat stable material i.e. steel or plastic so that they can be sterilized by passing high temperature steam at a pressure of 15 pounds per sq. inch through them. The fermenters are sterilized either by heating an external jacket covering the fermenters or through internal coils. Now, in recently developed state of art fermenters, cleaning and sterility is carried out using automated jet which are embedded in fermentation vessel.

The associated pipes attached to the fermenters should not be held horizontal as the steam vapours upon condensation settles down in the pipes and might help in promoting the growth of undesired microbes. Butt-welded joints are preferred to overlapping joints so as to reduce microbial contamination. A good fermenter is fitted with pressure safety valves. The function of these valves is to prevent development of too much steam pressure inside the vessels and thus reduces potential safety risk. Safety valves are usually in the form of a metal foil disk held in a holder which is set into the wall of the fermenter. These discs burst at a specific pressure. During the fermentation aerosols are generated which might be toxic/allergic to humans. A good fermenter is fitted with filters which entrap aerosols and permit only cell-free gasses to be discharged from the fermenter. All fermenters are operated under positive pressure to prevent entry of microbes or contaminants.

Control of chemical and physical conditions

Agitation: The agitation in fermentation medium is carried out to mix different phases i.e. solid, liquid and gases. The liquid phase contains dissolved nutrients and metabolites. The gaseous phase is mainly composed of air, CO_2 and O_2. Solid phase comprises of cells and non-soluble substrate. The mixing of these phases is important so as to ensure uniform condition of nutrient availability, constant temperature and equal distribution of gases through out the fermentation medium. The agitation is of utmost importance in case of aerobic fermentation because (1) microbes take up oxygen only from the liquid phase (2) agitation enhances rate of transfer of gases into liquid (3) agitation prolongs the retention of air bubble in suspension (4) reduces the bubble size to increase the surface area for oxygen transfer (5) provides bubbles to coalesce and (6) decreases thickness of foams (made up of cells and/or denatured proteins) at the gas liquid interface. Agitation in the fermentation can be provided either by mechanical movement of stirrer shaft or passing high pressure air through fermenters.

Heat transfer: During the design of fermenters, special attention is paid for the uniform and efficient transfer of heat to each and very part of the fermenter. Heat transfer is important in proper sterilization of the fermneter and maintaining constant incubation temperature in the fermentation medium. During fermentation, heat is generated through metabolic activities of microbes e.g. ethanol fermentation and mechanical movement of the agitators. If this heat is not controlled, the fermentation will shut down because of rise of temperature. The most common means of maintaining constant temperature during fermentation is by passing cool water into the outer jacket surrounding the fermenting vessel or by use of internal coils.

Mass transfer: The growth of microbes is directly dependant on the amount of soluble nutrients present in the medium as microbes are unable to take up solid or insoluble nutrients. Therefore, such nutrients are selected for addition to fermentation medium which are water soluble. Generally the transfer of nutrients from aqueous phase into microbial cells is straightforward as the nutrients are normally provided in excess. However, transfer of gases,

mainly oxygen, is a relatively complex phenomenon. Oxygen is not highly soluble in water and hence becomes a rate limiting factor especially in aerobic fermentations. It is usually provided in the form of air. Sterilized air is added to fermenters in the form of small air bubbles in order to provide larger surface area compared to its volume. This parameter aids in greater oxygen transfer.

During aerobic fermentations, the oxygen mass balance is dependant on a) rate of delivery of oxygen to the fermentation medium i.e. oxygen transfer rate (OTR) and (b) rate of uptake of dissolved oxygen by microbes. It is called critical oxygen demand (COD). During aerobic fermentations, OTR is less than COD. OTR can be measured using the following equation:

$$OTR = \frac{\text{Driving force}}{\text{Resistance}}$$

$$= \frac{\text{Oxygen gradient } (C^*-C_L)}{K_L a}$$

C^* = Saturated dissolved oxygen concentration ($mmol/dm^3$)
C_L = Oxygen concentration at a time t ($mmol/dm^3$)
K_L = Mass transfer coefficient (cm/h) i.e. the sum of reciprocals for the residencies of oxygen transfer from gases to liquid phase.

a = Gas-liquid interface area per liquid volume (cm^2/cm^3)

The transfer of oxygen from air-bubble gaseous phase to liquid phase is also critical & is calculated by:

$$\frac{d C_L}{dt} = K_L a (C^*-C_L)$$

Integration of the equation gives
$$C^*-C_L = e^{-K_L a t}$$

In terms of natural logarithm
$$\ln C^*-C_L = -K_L a^* t$$

$K_L a$ value tells us about the aeration capacity of the fermenter and should be kept above a minimum critical level, so as to supply the oxygen requirement of the cells.

Control and monitoring

In order to achieve maximum yield of biomass or by-product, the fermentation process has to be carried out under stringent condition and various physical and chemical parameters are constantly or regularly monitored. Physical parameters include pH, temperature, aeration and mixing of nutrients. Chemical parameters include constituents of fermentation medium e.g. carbon and nitrogen sources, inducers, inhibitors and precursors, dissolved gases (O_2) and redox potential of medium. Majority of these parameters (pH, temperature, dissolved oxygen)

are checked by sensors. These sensors are positioned in the fermentation medium in the form of electrodes. Such sensors should be heat sterilizable. The pH of the medium is maintained by the addition of acid or alkali. Temperature is controlled by heating or cooling the jacket coil of fermenter. The foaming is controlled by mechanical foam (fabricated in the fermenters) or addition of anti-foam agents.

The physical and chemical parameters can be measured on-line or off line. In case of on-line studies, sensors are connected with computers and the data obtained is downloaded to computer. The computer is attached to feed devices which automatically add requisite amount to ingredients to the fermenters.

In some cases when online estimation is not possible e.g. estimation of proteins/DNA/RNA/total lipids etc. aliquots from fermentation medium are collected aseptically. These aliquots are reacted with chemicals and the products formed are measured using spectrophotometer, HPLC etc.

Fermentation Media

The composition of a nutrient medium is of utmost importance for production of cell biomass or its product. The constituents of the medium must provide the elemental requirements of the concerned organism (Table 18). All microbes require water, source of energy, carbon, hydrogen, oxygen, phosphorus, sulfur and salts like potassium, sodium, magnesium, calcium as well trace elements like cobalt, nickel, molybdenum etc.

Table 18: Elemental composition of microbes

Element	Bacteria (dry weight %)	Fungi (dry weight %)	Yeast (dry weight %)
Carbon	50-54	45-51	40-62
Hydrogen	7-8	7-8	7-8
Nitrogen	11-15	7-11	7-10
Phosphorus	2-3	0.8-3.0	0.4-4.5
Sulphur	0.2-1.0	0.01-0.24	0.1-0.5
Potassium	1.0-4.5	1.0-4.0	0.02-0.5
Sodium	0.5-1.0	0.01-0.1	0.02-0.6
Calcium	0.01-1.1	0.1-0.3	0.1-1.4
Magnesium	0.1-0.6	0.1-0.5	0.1-0.7
Iron	0.01-0.2	0.01-0.6	0.1-0.3

The nutrients used to make up a nutrient medium must fulfill following criteria:

a)   It should produce maximal amount of biomass and concerned bio-product.
b)   It should produce little amount of undesirable product/s.
c)   It should not only be available throughout the year but should also be of consistent quality.

d) It should not interact with other constituents of the medium
e) It should not affect the sterilization process.
f) It should not affect various parameters of fermentation process like aeration, heat transfer, foaming etc.
g) It should not upset the downstream processing as well as waste treatment process.

The above mentioned parameters are important for commercial production of biomass/product but they can be relaxed for laboratory scale fermentations.

Normally there are two kinds of nutrient media: complex and chemically defined. In chemically defined medium, also called as synthetic medium, the type as well as the amount of each constituent is known e.g. M9 medium. Whereas in complex medium, neither all the constituents are known nor is the exact concentration of the constitutes is known e.g. nutrient broth, Luria Bertani broth, trypticase soy broth and brain heart infusion broth. There is another class of nutrient medium known as semi-synthetic medium. Here, composition of some constituents of the nutrient medium are known e.g. Casein-M9 medium. M9 is a synthetic medium, but addition of a carbon source, casein hydrolysate, to it makes this medium semi synthetic because chemical composition casein hydrolysate is not known to us precisely.

Carbon sources

Majority of the microbes used in the industry are chemoheterotrophs i.e. they utilize the carbon compounds not only as energy source but also as organic source. Commonly used carbon sources by microbes are carbohydrates, oils and fats and hydrocarbons.

Carbohydrates: Commonly used carbohydrates are starch, sucrose and lactose. Starch is obtained from cereals like maize, potatoes and cassava. Since starch is a complex carbohydrate, it is generally hydrolyzed to mono and/or disaccharides either by chemical treatment with acid/alkali or by biological treatment with enzymes like amylases especially heat stable amylases. Sucrose is generally added to the medium in the form of molasses which are the residues left after crystallization of sugar solutions obtained from sugar cane or sugar beet. Molasses is dark colored syrup which has over 50% carbon content mainly sucrose, 2-3% nitrogenous substances and some minerals and vitamins. Lactose is added to the medium in the form of whey, which is the milk left after the precipitation of casein from the milk. Malt extract is another source of carbon. It is an aqueous extract of malted barley concentrated to form syrup. Malt extract is rich in monosaccharides, disaccharides, trisaccharides and dextrins. It also has a small percentage of nitrogenous compounds, vitamins and minerals.

Oils and fats: Vegetative oils have been used as a carbon source in the production of antibiotics where they act as primary or supplementary source of carbon. Usually oils have twice the energy of glucose on a per weight basis. Oils have additional advantage as they are carriers of antifoam agents. Plant oils from cotton seed, linseed, maize, olive, palm, rape seed and soy plants have been used in fermentation media. These oils are rich in oleic and linoleic acids. Hard animal fats which are composed of glycerides of palmitic and stearic acids are rarely used in fermentation process.

Nitrogen sources

Most of the industrially used microbes can utilize inorganic or organic sources of nitrogen. Inorganic nitrogen sources are ammonia gas, ammonium chloride, ammonium sulphate and ammonium phosphate. Organic nitrogen sources are amino acids, peptides, proteins and urea. Though inorganic nitrogen sources are added in pure forms, organic nitrogen sources are added in impure form like corn steep liquor, soy meal, peptone, casein hydrolysate, peanut meal, yeast extract etc. *Corn steep liquor (CSL):* It is a byproduct of starch production from corn. It is rich in nitrogenous substances (about 3-5%) including amino acids like alanine, valine, threonine, arginine, glutamic acid along with minerals and vitamins. *Yeast extract:* It is an extract prepared from the yeast biomass generated during high volume fermentations by *Saccharomyces cerevisae* and *Kluyveromyces marxianus* and *Candida utilis*. The biomass is digested (chemically or enzymatically) and the debris is separated out. The aqueous material left out is dried out completely or partially to form a thick paste. *Soy bean meal:* After extracting oil from soy beans, the latter waste material is extracted with water and concentrated. It is rich in carbohydrates and nitrogenous substances which make up 28-35% and 50-55% respectively of the extract prepared. Peptones: These are hydrolytic products of protein rich materials like meat, casein, gelatin, keratin, cotton seeds.

Minerals

Minerals like calcium, magnesium, manganese, potassium, sulphur, phosphorus and chlorides are added along with carbon and nitrogen sources into the nutrient medium. Whereas other minerals, known as trace elements, may be added or may not be added as they are present as impurities in the major components added to the nutrient medium These trace elements are: cobalt, copper, iron, molybdenum, zinc etc.

Precursors and metabolite regulators

Some compounds are added to the fermentation medium to regulate the production of a specific metabolite i.e. main product. Such compounds include precursors, inhibitors and inducers.

Precursors: Some chemicals are added to the nutrient medium, may be at the start of fermentation or much later, which get directly incorporated into the final product. These chemicals are called precursors. The purpose of adding the precursors is to lessen the burden on the metabolic machinery of the cells to synthesize precursors. Table 19 lists the various precursors added to the fermentation medium.

Inducers: A compound which is non-essential for the growth of the cells but its presence is essential for the production of a specific bipoproduct is called an inducer (Table 20).

Generally all hydrolytic enzymes are inducible enzymes. This characteristic of organism is beneficial to the cells because they will produce hydrolytic enzymes only when the substrate is present in the vicinity of the organisms. In this manner the cells preserve their energy by not synthesizing unwanted enzymes. In case of production of bioproducts using genetically modified organisms (GMOs), the hyper production of a recombinant product is usually under the control of an inducible promoter e.g. lactose promoter which is active only in the presence of lactose or its analgue, IPTG in the fermentation medium.

Table 19: Precursors added to fermentation medium

Precursor	Product	Microbe
Phenylacetic acid	Penicillin G	*Penicillium chrysogenum*
Phenoxyacetic acid	Penicillin V	*Penicillium chrysogenum*
Chloride	Chlorotetracycline	*Streptomyces aureopfaciens*
Cyanides	Vitamin B_{12}	*Propionobacterium sp.*
D-Threonine	L-isoleucine	*Serratia marsescens*
L-Threonine	Cyclosporin C	*Tolypocladium inflatum*
Anthranilic acid	L-tryptophan	*Hansenula anomala*

Table 20: Microbial fermentations and enzyme inducers

Enzyme	Inducer	Microbe
α-amylase	Starch	*Aspergillus spp.*
Pullulanase	Maltose	*Acetobacter aerogenes*
α-Mannosidase	Mannans	*Streptomyces greseus*
Penicillin G acylase	Phenyl acetic acid	*Escherichia coli*
Cellulase	Cellulose	*Trichoderma viridae*
Pectinase	Pectin	*Aspergillus spp.*
Lactase	Lactose	*Kluyveromyces marxianus*

Inhibitors: Inhibitors are those compounds which either stop a pathway at a specific point or redirect the metabolism towards a specific target i.e. bioproduct. Inhibitors are added to the growth medium either to stop production of unwanted metabolites which may be the end product of a pathway (in case one is interested in an intermediate of a biosynthetic pathway) or side reactions of major pathway. Table 21 lists the type of inhibitors used in the industrial sector.

Table 21: Inhibitors of fermentation process

Product	Inhibitor	Effect	Microbe
Glycerol	Sodium bisulphite	Repression of acetaldehyde	*Saccharomyces cerevisae*
Citric acid	Alkali metals, phosphates	Repression of oxalic acid synthesis	*Aspergillus niger*
Glutamic acid	Penicillin G	Cell wall permeability	*Micrococcus glutamicus*
Rifamycin B	Diethyl barbiturate	Repression of other forms of rifamycins	*Nocardia mediterranei*

Inoculum Development

The process adopted to produce a culture volume sufficient for inoculation in the fermentation medium is called inoculum development. In order to achieve maximal yield of product via fermentation, the culture inoculum should have the following characteristics: it should be a) metabolically highly active, b) easy to prepare in large volume, c) of suitable morphological form and d) free from microbial contamination.

Ideally, the size of inoculum varies from 2-10% (v/v) of the fermentation medium. Normally for bacterial fermentations inoculum size varies from 2-5% and for fungal fermentations it varies from 3-10%. In industry, the size of fermentation medium can be very high e.g. 100,000 litres, which means that the minimal inoculum size will be 2000 litres. To prepare an inoculum of this magnitude is not an easy task. Starting from a stock culture, which may be in lyophilized form or on a slant-agar, the inoculum is built up in a number of stages. In the first stage, a small amount of culture is inoculated in a shake-flask and incubated. Once the culture has grown, it is transferred to a larger flask and incubated. The inoculum thus prepared is then transferred to a small laboratory fermenter. The size of culture inoculum is further increased by transferring this culture into a pilot scale fermenter. Usually, the chemical composition of seed culture medium and fermentation medium is quite similar so as to minimize lag phase of culture growth.

The inoculum developed using unicellular bacteria or yeast is of uniform consistency. However, filamentous organisms, like actinomyces and especially fungi, the growth of vegetative inoculum is either pellet-type which consists of a compact discreet mass of hyphae or filamentous-type in which fungal hyphae form a uniform suspension in the medium. Filamentous-type of growth results in the formation of viscous medium which is difficult to aerate whereas pellet-type growth give rise to less homogenous type of growth and the cells located in the center of pellet are not metabolically very active. These morphological forms of fungal growth have a great influence on the yield of bioproduct. Filamentous form of inoculum is preferred for penicillin production by *Penicillium chrysogenum* and pellet form is preferred for citric acid production by *Aspergillus niger*.

Downstream Processing

The extraction and purification of a biological product from the fermentation broth is called as down stream processing (DSP) or product recovery. DSP is quite complex and variable depending upon the type of the product. Broadly it can be divided into five stages:
   i)   Cell harvesting
   ii)   Lyses/breakage of cells
   iii)   Concentration
   iv)   Purification
   v)   Formulation

Cell harvesting

Once the fermentation is complete, the solid phase (i.e. cell bio-mass) is separated from the liquid phase by any of the following methods: Flotation, Flocculation, Filtration and Centrifugation.

Flotation: A gas is passed through the fermentation broth. This gas forms tiny bubbles to which cells get adsorbed. These bubbles rise to the surface of the broth and form a foam. Some times along with the gas, collector substances (fatty acids) are added which facilitates foam fermentation. The foam containing the cells is removed.

Flocculation: At high cell density some cells (yeast cells) aggregate and thus settle down at the bottom of the fermenters. This process can be accelerated by the addition of flocculating agent like salts, organic polyelectrolyte and mineral hydrocolloid.

Filtration: It is the most common type of cell separation technique. Many types of filters are used: *Depth filters:* These are composed of filamentous materials e.g. glass wools, asbestos or cellulose papers. Cells get entrapped in the matrix and clear cell free material passed out. This technique is used for the separation of filamentous fungi. *Asbestos filters:* These filters have specific porosity which can retain cells and let go liquid material. This is most suited for separation of bacteria. *Rotary drum vacuum filters:* This is usually applied for the separation of yeast cells. This unit consists of a rotary drum which is partially immersed in a fermentation broth. Rotating drum adsorbs cellular bio-mass. Once the biomass is out of fermentation broth, it is partially dried and then removed from the drum.

Centrifugation: This is a process of separating cells from the liquid based on the differences in their density. The cell culture is spun at a high speed in an instrument called centrifuge. This results in settling of denser material i.e. cells at the bottom or sides of the holder. In industry, continuous flow centrifuges are used in which culture is continuously supplied to the centrifuge. The cells get collected in the centrifuges and cell free supernatant flows out of the centrifuge.

Lyses/breakage of cells

If the desired product is located inside the cell, then the cells are fist recovered from the fermenter by any of the methods explained earlier in this chapter. These cells are suspended in buffer and their cell walls are disintegrated, so as to break open the cells by physical or chemical means in order to release intracellular products in the medium.

Physical methods: *a) Ultrasonication:* The cells are disrupted by passing ultra-waves through samples. This technique is ideal in laboratory where sample size is small. *b) Osmotic shock:* The cells are suspended in a viscous solution like 20% (w/v) sucrose or glucose. The cell suspension is then transferred to the cold water (4°C) which results in cell lyses. *c) Heat shock (Thermolysis):* The cells are exposed to heat which results in disintegration of the cells. It is an economical method but the product has to be heat stable. *d) High pressure homogenization:* The cell suspension is forced to pass through a narrow pore at a high pressure which results in breakage of cells. *e) Grinding with glass beads:* The cell suspension containing glass beads is subjected to a very high speed in a vessel. The cells break as they are forced against the walls of the vessel by the beads.

Chemical method: Various chemicals can destroy the cell walls/cell membrane of the cells. These chemicals can be alkali (sodium hydroxide, water miscible organic solvents like alcohol and detergents (sodium lauryl sulphate, triton X-100, cetyl trimethyl ammonium-bromide).

Enzymatic lysis: The cells are broken with the help of enzymes. Bacterial cells are lysed by the addition of lysozymes. This enzyme hydrolyses β-1,4 glycosidic linkage of peptidoglycan layer of bacterial cell wall. Fungal cells are lysed by the addition of chitnases, cellulases and mannases.

Concentration

More than 90% of the cell free supernatant is water and the amount of desired product is very less. Evaporation process concentrates the requisite product. Water is evaporated by applying heat to the supernatant with/without vacuum. The amount of heat applied is decided by the heat stability of the product. Various forms of evaporators are used e.g. plate evaporator, falling film evaporator, forced film evaporator and centrifugal forced film evaporator.

Liquid-liquid extraction: A desired product (solute) can be concentrated by the transfer of the solute from one liquid to another liquid. This process also results in partial purification of the product. Liquid-liquid extraction is basically of two types: (a) extraction of low molecular weight products (b) extraction of high molecular weight products.

Extraction of low molecular weight products: This is primarily applied for extraction of lipid soluble compounds. This process can be carried by the following techniques: a) Physical extraction: The aqueous solution containing the product is mixed with an organic solvent. The amount of product getting into organic solvent depends on the partial co-efficient of the product. b) Reactive extraction: It is like physical extraction procedure except that the product is first reacted with a carrier compound (e.g. phosphorus compound, aliphatic amine) and then mixed with organic solvent.

Extraction of high molecular weight: This technique is applied to compounds like proteins, which get inactivated in the presence of organic solvents like ethanol, methanol, chloroform, hexane etc. These compounds are extracted by using an aqueous two phase system (ATPS). In this technique a polymer (e.g. polyethylene glycol) and salt solution (e.g. ammonium sulphate) are added to the aqueous solution containing the product to be concentrated. The particulate matter i.e. cells remain in one phase and the proteins (the desired matter) are transferred to other phase. The solubility of the desired product is dependent on various properties of the protein e.g. ionic character, hydrophobicity, size etc.

Membrane filtration: This technique involves the use of semi-permeable membrane. Many kinds of membranes are available based on the size of the pores and their chemical nature (polyether sulfone or polyvinyl difluoride). Unfortunately, these filters cannot be sterilized. Now membranes made up of ceramics and steel are being introduced which can be easily autoclaved. Membrane filtration techniques like macro filtration, ultra-filtration and hyper filtration are described in Table 22.

Table 22: Membrane filtration processes

Type	Pore size limit (um)	Compounds separated
Microfiltration	0.1-10.0	Cells and viruses
Ultrafiltration	0.001-0.1	Compounds with molecular weight > 1000 daltons.
Hyperfiltration (Reverse osmosis)	0.0001-0.001	Compounds with molecular weight < 1000 daltons.

Membrane adsorber: The membrane contains charged groups or ligands to which a desired product can combine specifically once the aqueous solvent, containing the product, is passed through this. The adsorbed material is then eluted using various buffers and salts.

Precipitation: This is the most commonly used procedure for concentration of compounds especially proteins and polysaccharides. The agents commonly used in the process of precipitation are neutral salts (ammonium sulphate), organic solvents (ethanol, acetone, propanol), non-ionic polymers (PEG) and ionic polymers (polyacrylic acid, polyethylene amine).

Purification by chromatography

Chromatography is a procedure for separating molecules based on their sizes, charge, hydrophobicity and specific binding to ligands. The chromatography techniques used are as follows:

Gel Filtration chromatography (size exclusive chromatography): The matrix is made up of tiny beads having many pores in them. Many types of beads are available having different porosity. Small molecules enter the beads whereas large molecules cannot enter and therefore come out of the column first. By this technique protein of variable sizes can be purified.

Ion exchange chromatography: Most of the proteins have a net positive or negative charge. This property of the proteins is exploited for the purification of proteins by passing protein solutions through columns of charged resins. Two types of resins are used in the industry. Cation exchange resins and anion exchange resins. Cation exchangers (carboximethyl cellulose) have negative charged groups whereas anion exchangers (diethyl aminoethyl) have positive charged groups. Proteins carrying net positive charge bind to cation exchangers where as proteins carrying net negative charge bind to anion exchangers.

Affinity chromatography: In this procedure, the proteins are separated based on their affinity for a product compound i.e. ligand (Table 23). Once the protein is bound to the affinity matrix, it is eluted by changing the pH of the eluting buffer or alteration of ionic strength etc.

Table 23: Affinity chromatography ligands

Ligand	Protein
Antibody	Antigen
Hapten	Antibody
Lectins	Glycoproteins
Metal ions	Metallo-proteins
Receptor	Hormone

Commercial Production of Penicillin *(Sub Merged Fermentation)*

Penicillin is one of the first antibiotics to be discovered. There are many types of penicillins, but the important ones are penicillin V and penicillin G. Some of the penicillins are naturally

occurring and others are man made. Naturally occurring penicillins have a basic structure composed of 6-aminopenicillanic acid (6-APA) which is made up of β-lactam ring fused to thiazolidine ring (Fig. 17). The 6 amino position of β-lactam ring carries a variety of acyl moieties.
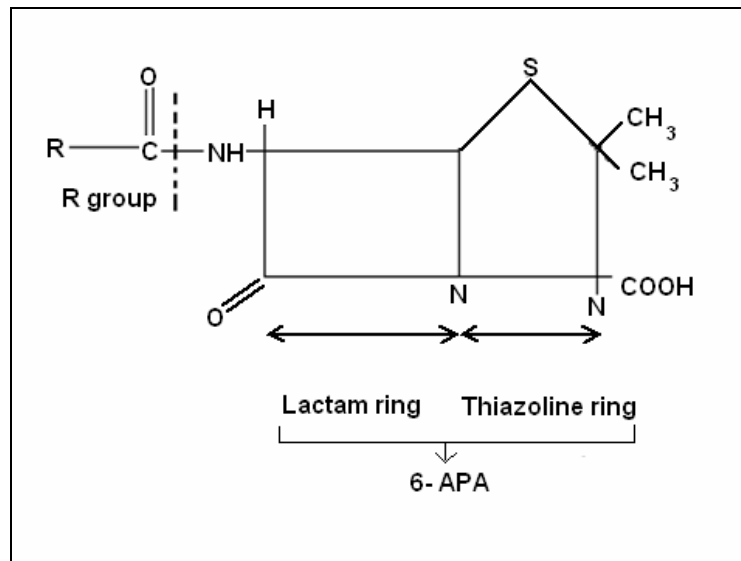
6-APA: 6-Aminopenicillanic acid
Fig. 17: Structure of β–lactam antibiotic, penicillin

Penicillins are bactericidal in nature and are highly effective against Gram positive bacteria like *Staphylococcus, Neisseria* etc. The major producer of penicillins are fungi especially *Penicillium chrysogenum*. Penicillin exhibits the property of a typical secondary metabolite being produced at the end of log phase. The yield of penicillin has been increased tremendously by the use of classical mutations, genetic engineering and media optimization studies. At industrial level yields of 50g/litre of growth medium have been achieved.
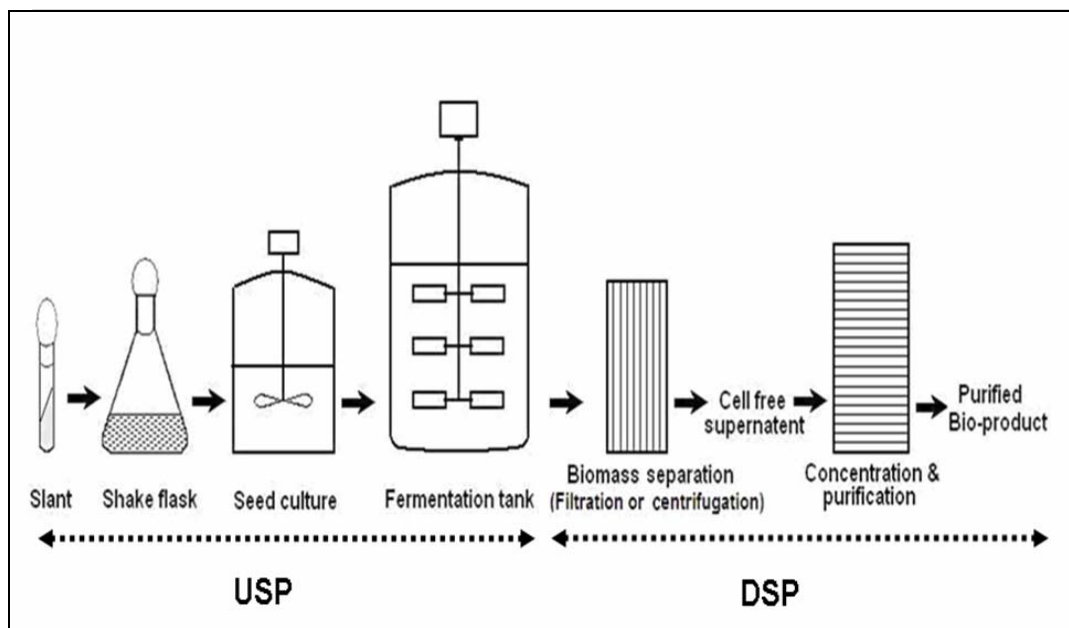
USP: Up stream processing;   DSP: Down stream processing
Fig. 18: Upstream and downstream processing in penicillin fermentation

Commercial production of penicillin

Fig. 18 shows a birds eye view of the production, purification and recovery of penicillin. In general, commercial penicillin is produced by fed-batch fermentation process using bioreactors of 30K-250K capacity. The fermentation temperature is maintained around 25-27°C, pH 6.5-7.0 and a constant supply of oxygen (25-60mmoles/l/hr) depending upon the type of *P.chrysogenum* used. The fermentation medium is composed of a carbon source like lactose and a nitrogen source like corn steep liquor. Additional supplements like yeast extract, soy meal, ammonium salts are also added. In addition, side chain precursors like phenyl acetic acid or phenoxyacetic acid are also added to the medium. The fermentation process involves a vegetative growth phase (around 40 hrs) followed by penicillin production phase (150-180 hrs, Fig. 19).
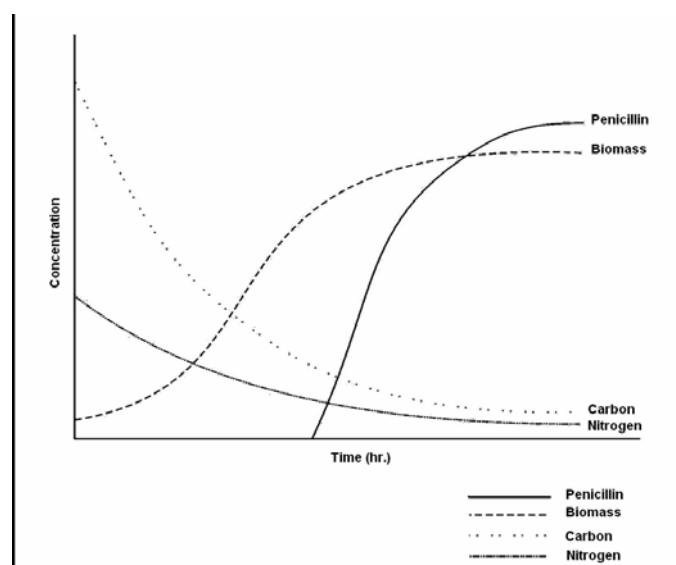

Fig. 19: Kinetics of penicillin production by *P.chrysogenum*

The culture inoculum is prepared by transferring spore suspension of *P.chrysogenum* into a 100-200ml liquid medium. The vegetative inoculum thus prepared is transferred to 1-2 litres fermenters. The mycelium is then sub-cultured for one or two stages with increasing the volume of fermentation till sufficient inoculum is ready for penicillin production. Care is taken to prepare the fungal inoculum in loose form rather compact pellet. The latter condition of mycelium is indicative of metabolically poor fungal biomass.

Recovery and purification of penicillin

The fermentation broth is passed through filters to remove fungal mycelium. The pH of the broth is adjusted around 3.5 and is mixed with organic solvent (amyl acetate or butyl acetate or methyl ketone) which extracts the penicillin from aqueous phase under cold conditions. The penicillin rich solvent is passed through activated charcoal to remove impurities and pigments. Subsequent addition of sodium or potassium acetate results in crystallization of penicillin as sodium or potassium penicillin. The crystals are then recovered by rotary vacuum filtration. By this procedure penicillin is more than 90% pure.

Commercial Production of Citric Acid *(solid state fermentation)*

Citric acid is a component of TCA cycle, also called Krebs cycle. It has immense application in the industrial sector. It is widely used as an acidulant and flavouring agent in food and beverage industry. In chemical industry it is used as an antifoam agent and maintaining metals in solution for electroplating. In pharma sector a salt of citric acid, trisodium citrate is used as blood preservative. Citric acid is also used as an alternative to polyphosphates in detergent industry. The most commonly used strain for citric acid production is an aerobic fungus, *Aspergillus niger.*

Citric acid biosynthesis

Citric acid is a primary metabolite product and is produced by the involvement of Embden-Meyerhof-Parnas (EMP) pathway and TCA cycle. Glucose is first converted to pyruvate. The latter metabolite is decarboxylated to oxaloacetate, which then combines with acetyl CoA to give rise to citric acid. In strains used for citric acid production, TCA cycle is kept active by the involvement of anaplerotic reactions.

Production medium

Easily degradable carbon sources like glucose or sucrose are ideal for citric acid production. For this reason, cane molasses or beet molasses are used. Starch is another source of carbon for citric acid production. For nitrogen source, ammonium ions or urea is added to the medium. Certain trace metals like manganese, iron and zinc are ideal for the growth of *A. niger.* Oxygen is an integral component as *A.niger* is an aerobic organism. The pH of the medium should be less than 3.0 for maximal production of citric acid.

Commercial production

There are two processes by which citric acid is produced in the industry: surface process and submerged process.

Surface process: This process is the very ancient and is in use in developing countries because it is simple and easy to use. In this process the fungus is grown on the surface of the growth medium, which may be solid or liquid.

Solid surface process: The wheat bran or sweet potato waste adjusted to 70-80% moisture content and pH around 4.0 is sterilized in shallow pans and then sprayed with spores of *A.niger.* Incubation temperature is around 28°C. Sterile air is passed through the incubation chamber for adequate supply of oxygen. Optimal yields of citric acid are produced after 70-100 hrs of incubation.

Liquid surface process: The liquid fermentation medium basically comprises of beet molasses supplemented with salts and pH adjusted to 4.0 and sterilized. This medium is transferred to a sterile shallow aluminum or steel pans and inoculated with spores of *A.niger* and incubation temperature of 28°C is maintained. Within 24-48 hrs a layer of vegetative mycelium is formed on the surface of medium. Optimal yields of citric acid are produced after a week to 10 days of incubation.

Submerged process: Nearly 80% of citric acid production is carried out by submerged process. Here, two types of bioreactors are used: stirred tank and air lift bioreactors. The fermentation vessel is made up of high quality stainless steel as citric acid is very corrosive in nature. The media composition is essentially the same as mentioned in liquid surface process. However, vegetative mycelium and not spore suspension is used for the inoculation of the medium. The vegetative inoculum is prepared by inoculating spores in a small liquid medium. After incubation, the inoculum is sub cultured into a fresh and large volume medium. This process is repeated till desired amount of inoculum in the form of fungal pellets is achieved. Care is also taken to produce pellets which should be small in size (less than 1mm diameter) and have fluffy centers.

Recovery of citric acid

The process for recovery and purification of citric acid is quite similar for surface and submerged fermentations. Initially the solid biomass is filtered out of the aqueous medium. The medium is acidified to pH 3.0 with lime (CaO) to precipitate undesirable co-product, oxalic acid. The acidic pH of broth is neutralized to pH 7.0 and heated up to 70-90°C. This step results in precipitation of citric acid. The precipitates are recovered and treated with dilute sulphuric acid solution. This results in formation of citric acid and precipitation of calcium sulphate (gypsum). After filtration, the dilute solution of citric acid is passed through columns of activated charcoal and ion exchangers so as to remove colours and other impurities. Purified crystals of citric acid are obtained after evaporation of water.

Microbial Biomass Production: Bakers yeast

In most of the industrial fermentations the aim is to produce maximal bioproduct from the microbial biomass generated during fermentation. However, in some cases this biomass is the bioproduct e.g. a) seed culture for fermentations, b) silage production, c) biopesticdes, d) animal fodder, f) inoculants for bioleaching of minerals and g) source of protein for humans. The fermentation process involved for microbial biomass production is quite similar to classical fermentations used for production of a bioproduct with slight variations. This aspect will be explained by understanding the production of baker's yeast (*Saccharomyces cerevisae*) used for preparation of bread and in some cases inoculum for alcoholic beverages especially beer.

The culture from slant is transferred to a small liquid medium contained in a glass conical flask. After incubation (28-30°C, shaking, 24-36 hrs), whole of the culture is transferred to a bigger culture flask and incubated. In this way 6-8 sub culturings, increasing the volume of culture medium nearly 10 folds at each step are done till enough inoculum is prepared. Last two or three sub culturings are carried out in small to mid size fermenters. Fed batch mode of fermentation is preferred for production of yeast biomass. The fermentation medium comprises of molasses supplemented with nitrogen source (ammonium salts or urea), minerals and vitamins. The pH of the medium is adjusted to 4.0-4.5. The fermentation is carried out in highly aerobic conditions (130-150mmol/L/hr). Fermentation process is considered complete when the nutrients get exhausted. The aerobic fermentation is continued for 30-40 min. This period is called yeast ripening phase in which storage carbohydrate (trehalose) content increase and protein and RNA content decreases. Yeast ripening also increases the storage life of the yeast as well. Decrease in RNA content is highly desirable as it is not easily degraded by humans and results in the accumulation of uric acid in the blood . High levels of uric acid cause gouts in humans.

Yeast cells are centrifuged to separate them from culture supernatant. Yeast biomass thus obtained is thoroughly washed with sterile water and chilled to 2-4°C. Final step involves the removal of moisture from the yeast cells, to increase their shelf life, by use of vacuum dehydrators. Adjusting biomass water content to (a) 70-73% (w/w) results in the formation of thick paste of biomass which is cut into one kg bricks and stored at 2-4°C or b) 5-10% (w/w) to form dried yeast which can be stored without refrigeration.

Enzyme / Cell Immobiliztion

In the course of the last few years, enzymes have gradually gained preference over their chemical counterparts, especially in the food and pharmaceutical industries. This may be attributed to the high stereo specificity of enzymes which in turn contributes to a decrease in the proportion of undesirable side products. Moreover, the existing biodiversity more than ensures the possibility of obtaining an enzyme for a particular reaction. However, there exist significant challenges in the use of enzymes on a commercial basis. Very often, the conditions employed in the bioprocess, lead to the denaturation of the enzyme. Moreover, the high cost of enzymes can render a process financially nonviable. This is where the concept of immobilization has stepped in and provided a means to combat some of the challenges faced by the industry in the use of enzymes on a commercial scale.

Immobilization essentially involves, the stable association of an enzyme with an insoluble and inert support, so as to render the enzyme stable and facilitate its reuse. Moreover, the product obtained is not contaminated with the enzyme, thus leading to a significant reduction in the costs involved in the downstream processing. In case of immobilization, there exists the possibility of using either isolated enzymes or even whole cells. The latter affords the convenience of doing away with the lengthy procedures involved in the purification of enzymes and also ensures the functionality of the enzyme since its natural environment remains unaltered. However, unwanted side reactions may occur, in which case, immobilization of the pure enzyme may be preferred.

Support material

The choice of the support matrix is a very important consideration, the parameters for which are defined, based on the chemical nature of the enzyme, the substrate, the type of reaction, as well as the intended use of the product. Some of the criteria used in the selection of an appropriate support include physical parameters like, the strength of the matrix, the available surface area, the degree of porosity, and the flow rate as well as the chemical properties such as its hydrophillicity, inertness and the available functional groups. Moreover, there are other important considerations which include, the susceptibility of the support to microbial attacks or disruption due to the chemicals, pH, temperature or the action of proteases, as well as its availability and cost, thus determining the feasibility of its usage during scale up. A very important point to be taken care of is that, the matrix should not render the product toxic, especially if the product is meant for consumption.

Principles used in immobilization

Adsorption: This is one of the simplest means of immobilization, and involves electrostatic forces such as vanderwaal forces, ionic bridges and hydrogen bonds, which are instrumental

in establishing the reversible surface interactions between the support and the enzyme/cells (Fig. 20). Hydrophobic interactions, though weak, are enough in number to ensure sufficiently reasonable binding. The procedure basically involves mixing of the biological component with the matrix for a fixed period of time at an optimum pH, followed by washing, in order to get rid of the non biological components. Here, the catalytic properties of the enzyme remain unaltered, since no change is brought about in the chemical nature of either the enzyme or the supporting matrix. Moreover it serves as a rapid and economical means of immobilization, allowing the regeneration of matrix, since it is a reversible process. However, a major limitation of this method lies in the possibility of the enzyme leaking from the support owing to changes in the reaction conditions.

□ , enzyme;   - - - -, electrostatic forces; ∿ , matrix.

Fig. 20: Enzyme immobilization by Adsorption technique

Entrapment: Herein, the enzyme molecules are free in solution but their movement is restricted owing to the lattices in the gel (Fig. 21). The degree of porosity is set so as to prevent leakiness of the enzyme but allows unrestricted diffusion of the substrates as well as the products.
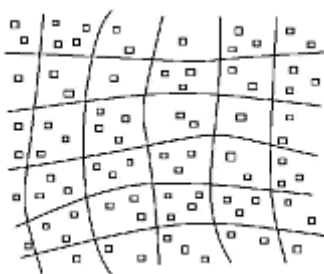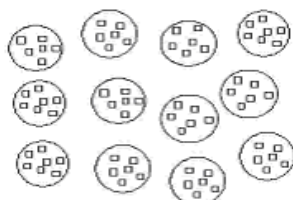

□, enzyme molecules;   , matrix.

Fig. 21: Enzyme immobilization by Entrapment technique

Entrapment may be achieved by a number of means which include the following:
  i)   Ionotropic gelation of macromolecules with multivalent cations: This involves mixing of the polyionic polymer with the enzyme, followed by cross linking of the polymer with multivalent cations, in an ion exchange reaction, to form the required lattice structure.
  ii) Temperature induced gelation: This is brought about by adding the enzyme to a 1-2 % preparation of agarose or gelatin kept at 45°C, and then bringing down the temperature.
  iii) Organic polymerization by chemical/photochemical reaction: Here, the enzyme is mixed with chemical monomers which are polymerized to form a cross linked network, trapping the enzyme.

Encapsulation: The basic concept underlying this technique is that, large molecules such as enzymes cannot pass through semi permeable membranes, while substrates and products can, owing to their smaller size (Fig. 22). Hence, materials such as cellulose nitrate and nylon are employed to construct microcapsules having diameters in the range of 10-100μ. Moreover, biological cells such as the red blood corpuscles (RBC) may also be used as capsules.

□, enzyme molecules ;   ○, matrix.

Fig. 22: Enzyme immobilization by encapsulation technique

Cross linking: This technique involves joining of cells/enzymes to each other, giving rise to a three dimensional complex structure. This is achieved by the formation of covalent bonds, which in turn, is brought about by means of reagents like glutaraldehyde and toluene diisocyanate (Fig. 23). However, owing to the toxicity of these reagents, this method is not generally used when viable cells are involved.
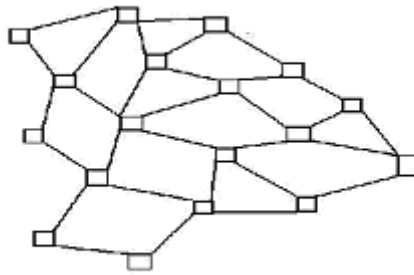

□, enzyme molecules ; —— , covalent bond ; 〜〜 matrix.

Fig. 23: Enzyme immobilization by cross linking technique

Covalent binding: This method involves the formation of covalent interactions between the functional groups present on the support surface and   those present on the amino acid residues on the enzyme surface (Fig.. 24).. While there are a number of reaction procedures to achieve this (e.g. formation of an isourea linkage or a diazo linkage, a peptide bond or an alkylation reaction), it is important to select a method which will not inactivate the enzyme by means of a reaction with one of its active site residues. The first step involves the activation of the functional group on the support surface (essentially making it strongly electrophilic), followed by the coupling of the enzyme to the support. For example, in case of carbodiamide activation, the carboxyl group of the support is joined to the enzyme via a peptide linkage. Moreover, the range of a matrix may be increased by chemical modifications (e.g. pure cellulose has only a hydroxyl group but its range can be increased by its chemical conversion to carboxymethyl cellulose or diethylaminoethyl cellulose).

□, enzyme molecules;   ——— , covalent bonds

Fig. 24: Enzyme immobilization by covalent bonding technique

Applications of immobilization technique

Immobilization of enzymes/cells has found varied applications in industry. Some of the most important applications of these in the food industry have been the conversion of glucose to high concentration fructose syrups by the enzyme glucose isomerase, the immobilized invertase systems and the production of lactose hydrolyzed milk by use of β-galactosidase. In the case of the pharmaceutical industry, the production of 6-amino penicillanic acid or 7-amino deacetoxy cephalosporanic acid by means of immobilized systems is seen as a major achievement. Immobilized proteases offer a great deal of applications in the field of synthesis and modification of peptides as well as in the leather industry. Moreover, they have been used as biosensors. Immobilized glucose oxidase and peroxidase enzymes have been used as detection systems for serum glucose levels. Uricase has been used in a similar fashion for detection of uric acid levels while cholesterol esterase and cholesterol oxidase have been used for detection of serum cholesterol. However, while a number of exciting possibilities do exist in this field, for an immobilized system to succeed on an industrial scale, there are a number of considerations such as, the costs involved, the feasibility of using a particular matrix, as well as the purity of the end product.

Suggested Readings

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