FOOD AND INDUSTRIAL MICROBIOLOGY

Production of Metabolites, Industrial enzymes, Amino acid, Organic acids, Antibiotics, Vitamins and Single Cell Proteins

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Metabolite, industrial enzyme, amylase, protease, cellulose, amino acid, glutamic acid, lysine, organic acid, antibiotics, pencillin,streptomycin,vitamins, Single Cell Proteins.

Microorganisms play a prominent role in generating variety of products having applications in different areas, such as energy, food, chemicals, diagnostics, pharmaceuticals etc. They are the source of enzymes, organic acids, amino acids, antibiotics, vitamins, single cell proteins and other commodity chemicals having commercial importance. The advances in microbial fermentation techniques have lead to the development of ecofriendly processes replacing the some of the conventional chemical processes. The microbial enzymes are being used for catalyzing reactions which otherwise require very harsh chemical and physical conditions besides generating undesired byproducts and toxic effluents. The enzymes are mainly used in food processing, chemical transformations, therapeutics and molecular biology techniques. The production of organic acid (citric acid, lactic acid) through microbial fermentations using less expensive raw material is also one of the major achievements of industrial microbiology. Antibiotics are secondary metabolites of microbes which are extensively used for control of diseases caused by microorganisms. Vitamins produced by microbial fermentations are widely used in pharmaceutical formulation. Microbial cells are rich in protein and can be easily grown on cheaper substrates and have application as food and feed additives. Some of these microbial products will be discussed in this chapter to understand and realize the vast potential of microorganisms in food and industrial sector.

Principles of overproduction of primary and secondary metabolites

Fermentation is enzymatically controlled transformation of an organic compound. The fermentation product may be a metabolite or biomass. Metabolites are the intermediates and products of metabolism. The term metabolite is generally restricted to small molecules. The microorganisms during growth phase (trophophase) synthesize a range of metabolites that are essential for normal growth, development and reproduction and collectively called primary metabolites. Amino acids, organic acids, alcohol, nucleotides and enzymes are some of products of primary metabolism that have industrial significance. After growth phase the microbe enters in stationary phase (idiophase) due to exhaustion of nutrients in the medium. In this phase, active growth of the microorganism ceased and some microorganisms produce metabolite, which are not essential for growth and reproduction. These metabolites are referred as secondary metabolites. Secondary metabolism produces diverse, often species-specific end-products like alkaloids, antibiotics, toxins and some pigments that have commercial value.

The microbial metabolites are produced through fermentation and their overproduction is of prime importance to shorten the production time and space and at the same time reducing the product cost. Overproduction of metabolites depends on the genetic makeup of microbial strain and the environmental conditions under which the fermentation is carried out. To increase the production of fermentation product, different approaches applied are as follows.

Optimization of cultural conditions

The physiological behaviour of the microorganism varies during growth and it largely depends on the composition of media. The physiological state of the inoculum greatly effects the metabolite production especially in the case of antibiotics. The overproduction of a particular metabolite is influenced by fermentation process parameters e.g. aeration, medium pH, temperature, nutrients (types and their concentration), metal ions, inducers/inhibitors for a

particular strain and there are many examples which describe the complex interaction of these parameters effecting the metabolite biosynthesis.

For example, in citric acid synthesis iron concentration in the production media has to be reduced to lower the aconitase activity that is responsible for further progression of citric acid cycle and thus overproduction of citric acid occurs. Medium pH below 3.0 prevents the formation of oxalic aid and gluconic acid. In case of industrial enzyme production, the fermentation media are formulated with the specific inducers (starch in case of amylase, cellulosic material in case of cellulases and proteins in case of proteases) for overproduction.

In lactic acid fermentation, calcium carbonate is added to maintain the pH between 5.5 and 6.5 and to neutralize lactic acid which is otherwise toxic to the bacteria. The fermentation process yields 80-90% lactic acid, when compared with theoretical yield.

Genetic approaches for overproduction of metabolites

Generally wild strains are not suitable for industrial fermentation because of low yield of the metabolite. In such cases, genetic improvement of strain becomes essential for overproduction of a particular metabolite. Genetic approaches for augmenting production of metabolites by microorganisms include the following:

Mutation

Mutant generation of the existing wild strains is the most practiced strategy for enhancing the yield of primary and secondary metabolites. For overproduction of primary metabolites, feed back inhibition by the end product of a particular pathway is suppressed by generation of auxotrophs i.e. mutation to cause accumulation of metabolite of interest.

Mutants resistant to anti metabolites through modification of enzyme structure at allosteric site or modification of operator or regulator gene to express the enzyme constitutively is another method employed for the over production of primary metabolites. Overproduction of secondary metabolites is regulated by the structural genes directly participating in their biosynthesis, regulatory genes, antibiotic resistance gene immunizing responsible for their own metabolites and genes involved in primary metabolism affecting the biosynthesis of secondary metabolites. Mutant generation has improved the yield of certain antibiotics by 15-400 times in comparison to wild strains. Greater insight of the biochemical pathways, fluxes of intermediates inside and outside of cells, enzymes' role, are being now studied and applied to in metabolic engineering.

Recombinant DNA technology

Recombinant DNA technology is used for modification of biochemical pathways which ultimately overproduce a particular metabolite and better utilization of media components. The bioinformatics tools now being used in metabolic engineering for closer understanding of the gene networks in biochemical pathways to determine the hotspots that could be modified to increase the metabolite yield (Shimizu, 2002). Metabolic engineering involves directed improvement of cellular properties through the modification of specific biochemical reactions or the introduction of new genes using recombinant DNA technology with the goal to increase

process productivity, such as the production of antibiotics, biosynthetic precursors or polymers. Examples of metabolic engineering include the microbial production of indigo (Genencor) and propylene glycol (DuPont).

Industrial enzymes – Amylase, Protease, Cellulase

The enzymes are biocatalysts, which act in relatively mild conditions of temperature, pH, and pressure. The importance of enzymes in industrial processes can be assessed by the fact that they perform very specific reactions/modification of the substrate without formation of unwanted by-products in comparison to the chemical catalyst. Processes based on enzymes are eco-friendly, require less energy input and do not require reaction vessels made up of expensive corrosion resistant material. In enzymatic processes, the need for extensive refining of the target product is greatly reduced since enzymes catalyse both stereo- and regio-selective modification of substrate in comparison to the chemical catalysts in which side reactions are very common. There are some enzymes which operate in non-aqueous organic media, particularly hydrophobic solvents, more efficiently, especially in conditions where the substrates have limited water solubility. The enzymes are used in different processes (Fig.1) for specific purposes and to reduce the energy and cost inputs to a great extent.

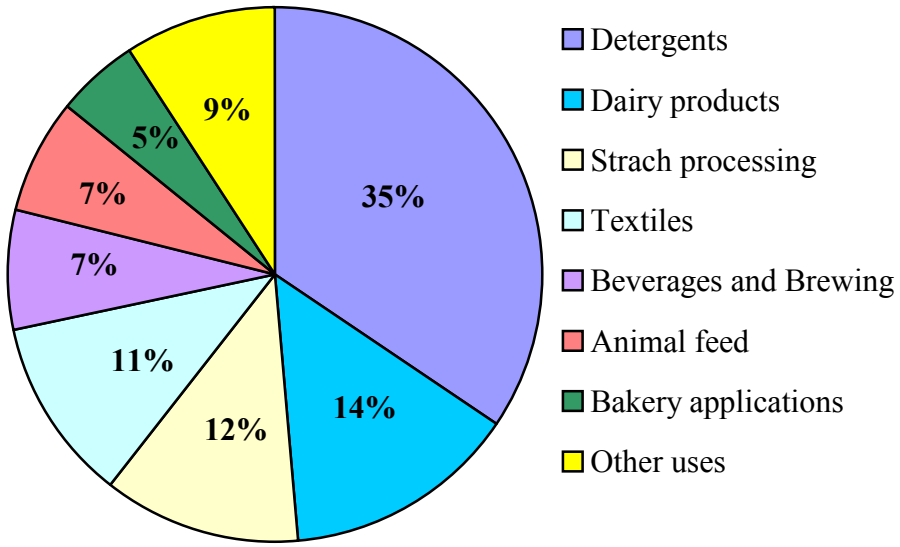


Fig. 1: Applications of enzymes vis-à-vis their market shares in various sectors

Jhokichi Takamine, in 1894, first time started the industrial production of digestive enzyme (amylases) preparation by wheat bran koji culture of *Aspergillus oryzae* for treating digestive disorders. The supplementation of detergents with the enzymes for effective washing of fabrics was also one of the initial applications of enzymes. The present market of the enzymes and the companies producing them is increasing day by day and the **Business Communications Company, Inc.** (www.bccresearch.com) estimated a $2 billion of industrial enzymes market in

2004. The industrial enzymes find application in many areas mainly three segments: technical enzymes, food enzymes, and animal feed enzymes. Among the specific types of industrial enzymes, protease and amylase lead the market.

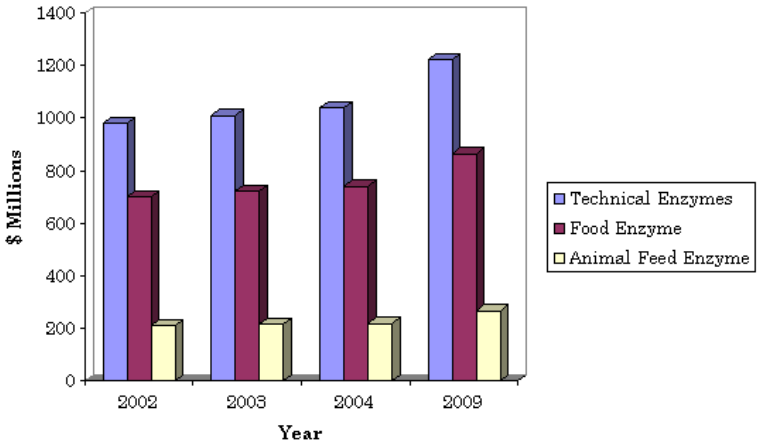


Fig. 2: Global market of industrial enzymes in different application sectors by 2009
(Source BCC Inc.)

The enzymes are present in all organisms; however, the microbes are excellent source of enzymes because of their rapid growth, easy cultivation in large fermenters and genetic manipulation. In the proceeding sections the sources and applications of amylases, proteases, and cellulases will be described.

Amylases

Higher plants store carbohydrates in the form of starch (granules) which is composed of 20-30% amylose (linear polymer of 500-20,000 α-1,4 linked D-glucose units) and 70-80% amylopectin (branched polymer formed by joining of linear polymer of 24-30 α-1,4 linked D-glucose units by α-1,6 glycosidic bond). Starch hydrolyzing enzymes are referred as **amylases**, and are mainly used in the production of sweeteners for the food industry. Enzymatic hydrolysis of starch first produces short-chain polymers of glucose called dextrins, then the disaccharide maltose, and finally glucose. Starch saccharification process involves use of α-amylases, β-amylases, glucoamylases, pullulanases and isoamylases glucose isomerases.

The commercially important amylases of microbial origin that split α-1, 4 and/or α-1, 6 bonds in starch molecule have been classified into six groups as shown in Table 1 and the specific glycosidic bond hydrolysed by amylases is depicted in Fig. 3 .

α-Amylases

α-Amylases (1,4- α-glucan-glucanohydrolases) are extracellular enzymes which hydrolyze α-1,4-glycosidic bonds present in the interior of starch and thus are endoacting enzymes. α-Amylases are produced by many bacteria and fungi and are classified on the basis of their starch-liquefying and/or saccharogenic effect, pH optimum, temperature range, and stability. Saccharogenic

amylases produce free sugars upon starch hydrolysis, whereas starch-liquefying amylases breakdown the starch polymer but do not produce free sugars. Bacillus subtilis Marburg, B. subtilis var. amylosaccharaticus, and B. natto produces saccharogenic α-amylase, where as B. amyloliquefaciens produces liquefying α-amylase. α-Amylases contain a large proportion of tyrosine and tryptophan in enzyme protein and most of them require calcium as a stabilizer. This enzyme is extensively used in different industry (Table 2).

Table 1: Classification of amylases on the basis of glycosidic bond hydrolysis
(Source : Fogarty and Kelly, 1979)

Class	Glycosidic bonds hydrolyzing properties	Examples
1	Hydrolyse α-1,4 bond and bypass α-1,6 linkages	α-Amylase (endoacting amylases)
2	Hydrolyse α-1,4 bond and cannot bypass α-1,6 linkages	β-Amylase (exoacting amylases producing maltose as a major end product)
3	Hydrolyse α-1,4 and α-1,6 linkages	Amyloglucosidase (glucoamylase) and exoacting amylase
4	Hydrolyze only α-1,6 linkages	Pullulanase and other debranching enzymes
5	Hydrolyse preferentially α -1,4 linkages in short chain oligosaccharides produced after hydrolysis of amylose and amylopectin by other amylases	α-Glucosidases
6	Hydrolyse starch to a series of nonreducing cyclic D-glucosyl polymers (cyclodextrins or Sachardinger dextrins)	*Bacillus macerans* amylase (cyclodextrin producing enzyme)

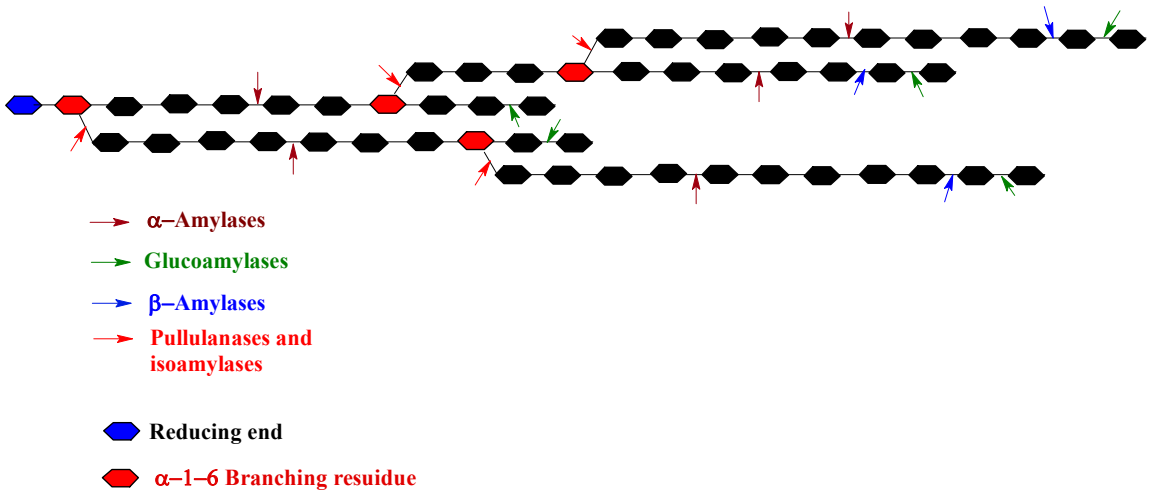


Fig 3: Molecular structure of amylopectin showing specific bonds hydrolysed by various amylases

Table 2: Uses of α-amylase in different industries

Industry	Application
Starch Processing	Liquefaction of starch in the production of sugar syrup
Milling	Modification of α-amylase-deficient strains
Baking	Generation of fermentable sugars in flour, and improvement of crust colour
Brewing	Starch hydrolysis during wort preparation from barley
Paper	Liquefaction of starch without sugar production for sizing of paper
Textile	Continuous desizing at high temperatures
Feed	Treatment of barley for poultry and calf
Biological detergents	Starch removal from food stains
Sugar industry	Breakdown of starch from cane juice to improve filterability

α-Amylase producing bacteria

Ba*cillus subtilis, B. subtilis var. amylosaccharaticus, B. natto, B. cereus, B. amyloliquefaciens, B. coagulans, B. polymyxa, B. stearothermophilus, B. cladolyticus, B. acidocaldarius, B. subtilis var. amylosaccharticus, B. licheniformis, Lactobacillus, Micrococcus, Pseudomonas, Arthrobacter, Escherichia, Proteus, Thermonospora,* and *Serratia* are some α-amylase producing bacteria. However, *Bacillus amyloliquefaciens* and *B. licheniformis* are mainly produced for the industrial production of α-amylase.

α-Amylase producing fungi

Aspergillus, *Penicillium*, *Cephalosporium*, *Mucor*, *Candida*, *Neurospora* and *Rhizopus* are some α-amylase producing molds and *Aspergillus oryzae* is one of the mold used as source for the industrial production of α-amylase.

Production of α-Amylase

Bacterial α-amylase

The bacterial α-amylase is inducible and repressible by glucose produced by the hydrolysis of starch. It is assumed that a basal level of the enzyme is produced constitutively, which hydrolyses the starch generating the low molecular weight inducers. The production media for amylase includes 5% starch, 0.5% NH_4NO_3, 0.28% sodium citrate, 0.13% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.5% peptone. 0.2% yeast extract and the fermentation is carried out either in batch or in fed-batch manner around pH 6.8. Mostly thermophilic strains are being used for the production of the amylase for applications at elevated temperatures. The temperature of the fermentation process depends on the strain used. In the fermentation process, amylase is produced during the growth of the bacteria and formation of spores.

Fungal α-amylase

Fungi generally produce amylases constitutively but experience repressive effects of some regulators. The fermentation media used for the production of amylase by Aspergillus oryzae contains 8% starch, 1.2% NaNO3, 0.1% MgSO4•7H2O, 0.05% KCl, 0.003% FeSO4, 0.08% Mg(NO3)2, 0.005% Mg(H2PO4)2, and 2.0% malt extract. The fermentation is carried out at 28-30ºC for 3-4 days.

β-Amylases

β-Amylases (α-1,4-glucan-maltohydrolases) are the exoacting enzymes hydrolyzing the α-1,4-glycosidic bonds from the non-reducing ends producing maltose and limit dextrin as the major product and are unable to hydrolyse the α-1,6 branching present in amylopectin. This enzyme is mainly present in plants but some microbes are known to produce this enzyme which includes: *Bacillus polymyxa*, *B. cereus*, *B. megatarium*, *Streptomyces* sp., *Pseudomonas* sp. and *Rhizopus japonicus*. β-Amylase has been produced on starch waste by a hyper-amylolytic strain of *B. megaterium* B6 mutant UN12 in Submerged fermentation (SmF) and Solid state fermentation (SSF) (Ray *et al.* 1997). The starchy wastes from arrowroot, arum, maize, potato, pulse, rice, rice husk, tamarind kernel, cassava, water chestnut, wheat and wheat bran are used as substrate. This enzyme is mainly used in the production of maltose syrup and also digestion of barley starch during beer production.

Glucoamylases

Glucoamylases (α-1,4-glucan-glucohydrolases) hydrolyse starch from the non reducing end producing glucose, maltose, and limit dextrins. *Aspergillus niger*, *A. oryzae*, *A. awamori*, *Rhizopus niveus*, *R. delemar*, *R. formosaensis*, and *R. javanicus* are the strains employed for the production of glucoamylases. This enzyme is mainly used for the production of fructose syrup and its production is carried out in submerged fermenter. Starch or dextrin induces the production of glucoamylases, therefore, starch is generally added in the production media. The production of the enzyme is carried at 28-30 ºC for 3-5 days depending on the strain. Glucoamylase also catabolite repressible by glucose, glutamic acid and lactose.

1, 6-Glycosidic bond hydrolyzing enzymes

The amylopectin is a branched polymer of glucose chain linked by α-1,6 glycosidic bonds. Pullulanases and isoamylases hydrolyse this bond resulting production of straight chain maltodextrin. Pullulan is obtained from *Pullularia pullulans* which is neutral glucan polymer consisting of α-1,4 linked maltotriose unit joined to each other by α-1,6 glycosidic bond. Pullulanases are the enzymes that hydrolyse the α-1,6 glycosidic bond of the pullulan and amylopectins but the isoamylases only hydrolyse the α-1,6 glycosidic bond of amylopectin. *Aerobactor aerogens*, *Bacillus acidopullulyticus*, *B. polymyxa*, *Pseudomonas* s*accharophila*, *Streptococcus* sp., *Strptomyces* sp., are some of the strains used for pullulanase production. Isoamylases are obtained from *Agrobacterium, Erwinia*, *Staphylococcus*, *Serratia*, *Nocardia*, *Bacillus*, *Pediococcus*, *Lactobacillus*, and *Leuconostoc*. Pullulanases and isoamylases are used in the starch hydrolysis process.

Application of amylases

Amylases were the first enzymes to be produced industrially and share 20% of the present enzyme market. Amylases are used in food, feed, textile, and pharmaceutical industries. In the food sector they are mainly used for liquefaction of starch, manufacture of maltose, high fructose containing syrups, oligosaccharide mixture, maltotetraose syrup, and high molecular weight branched dextrins. These products are used for various food preparations (cake, candies, etc) adding characteristic high or low sweetness and maintain texture. The use of amylase has replaced the chemical hydrolysis of the starch as the latter used to yield undesirable by-products and is also uneconomical. Amylase is also used for removal of starch sizer from textile (desizing) making the fabric ready for scouring and dyeing.

Ethanol production from starchy substrates has been improved by using amylases or coculturing the amylolytic strains with ethanol producing microbes in starch based media. This technique has also been used to digest the starch in the brewing process. Amylases are also used for processing waste containing starch generated from food processing plants ultimately reducing the pollution load of effluent and producing microbial biomass protein. Alkaline amylases are used in detergents and dish bar for removing the starch stains on cloths and utensils respectively. Amylases are also ingredient of the digestive syrups used for treating digestive disorders. Amylase treated flour is used for preparing animal feed and have improved digestibility.

Proteases

Proteases are the enzymes which catalyse the hydrolysis of peptide bonds of the proteins. The amino acid composition of proteins is very diverse so the proteases responsible for their hydrolysis are also diverse. Microbes have both intracellular and extracellular proteases, the intracellular proteases are responsible for the maintenance of amino acid pool inside the cell by degrading the unwanted proteins and the extracellular proteases hydrolyse proteins outside the cells into peptides and amino acid required by the cells for their growth. Proteases are classified into two major groups: the exopeptidases (peptidases) and the endopeptidases (proteinases). The peptidases hydrolyse the protein from C- or N-terminus releasing single amino acid and the endopeptidases as the name suggests hydrolyses the peptide bond in the middle of the amino acid chain. Further the proteases are also classified into alkaline, acid and neutral proteases based on their pH optima of activity. On the basis of the functional group present at the catalytic site these proteases are classified as serine proteases, cysteine proteases, aspartic proteases, threonine proteases, glutamic acid proteases and metalloproteases. The classification of proteases has been summarized in Fig 4.

The proteases represent one of the three largest groups of industrial enzymes others being the amylases and lipases. The proteases find their application in detergents, leather, food, pharmaceutical industries and bioremediation processes. The major uses of microbial proteases have been listed in Table 4.

Both bacterial and fungal proteases are produced commercially and their production conditions are very different from each other. Some organisms are thermophilic especially grown to obtain thermostable proteases for use in detergents. In the proceeding sections alkaline, neutral and acid proteases have been described along with their major producers and important applications.

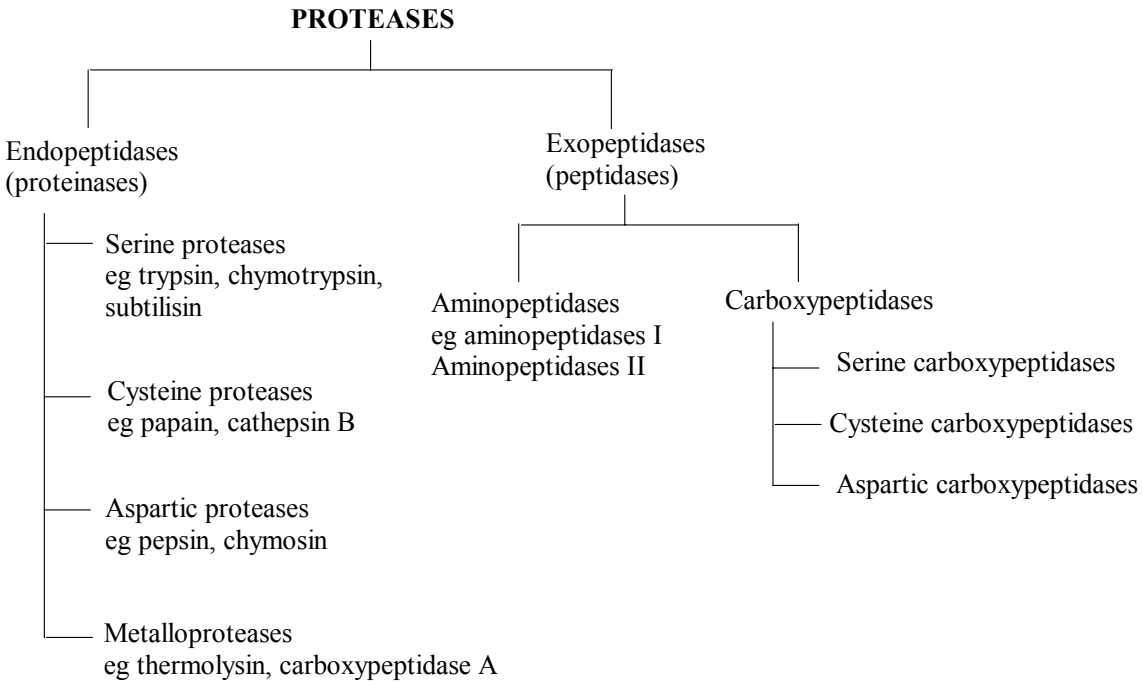


Fig 4: Classification of proteases *(Source : Rao et al., 1998)*

Table 4: Industrial uses of proteases

Industry	**Protease**	**Application**
Baking	Neutral protease	Dough conditioner
Dairy	Fungal acid proteases	Cheese preparation
Detergent	Alkaline protease from *Bacillus* sp	Laundry detergent for protein stain removal
Food processing	Several proteases	Modification of protein rich material (soy protein or gluten), meat tenderization
Leather	Neutral proteases	Bating of leather, dehairing of hides
Brewery	Neutral proteases	Hydrolyse cereal mash to release peptides and amino acids for utilization by yeast
Photographic	Alkaline protease	Digestion of gelatin for recovery of silver from films
Biopharmaceutical	Several proteases	Digestive syrup, contact lens cleaner, necrotic tissue removal, blood clot digestion
Peptide synthesis	Thermolysin, α-chymosin and several other protease	Reverse reaction to synthesise aspartame and other peptides of pharmaceutical importance

Alkaline proteases

Strains of *Bacillus*, Steptomyces*, Aspergillus* are the major producers of alkaline proteases. Proteases from *Bacillus* (Bacillopeptidases) are mainly used in detergents. Subtilisin carlsberg (protease from *B. licheniformis*) and Subtilisin Novo (protease from *B. amyloliquefaciens*) are the best know proteases used in detergents. These proteases have serine at the active site, and are not inhibited by EDTA (ethylenediaminetetraacetic acid), but are inhibited by DFP (diisopropyl flurophosphate). These proteases are stable at high temperature, active in alkaline pH (9-11) and stable in presence of chelating and perborates, which is an important characteristic of these enzyme for use in detergents.

Screening of organism producing alkaline proteases is done using strongly basic media and the colonies are tested on protein agar plates (pH 10). The wild strains have been improved by altering the amino acid sequences of the proteases by genetic engineering tools, changing their substrate specificity, pH optimum, and stability to the bleaching agents. Extensive genetic manipulations have also been carried out to improve the yields of the proteases during the fermentation.

Proteases to be used as detergent additive should be stable and active in the presence of surfactants, bleaching agents, bleach activators, fillers, fabric softeners and various other formulation of a typical detergent. In textile industry, proteases may also be used to remove the stiff and dull gum layer of sericine from the raw silk fibre to achieve improved luster and softness. Protease treatment also modifies the surface of wool and silk fibers to provide unique finishes. The alkaline proteases also have potential application in removal of gelatin from the used photographic films vis-à-vis recovery of silver from them.

Production process

The alkaline proteases are produced at large scale using 40-100 m^3 fermenters. Fermentation process is operated in fed-batch manner to maintain low levels of ammonium and free amino acids, since at high concentrations they repress the protease production. Continuous process for protease production has been developed but it is not practiced at industrial scale. Oxygen is supplied at the rate of 1vvm to get the optimal protease titers. The fermentation lasts for 48-72 h depending on the organism used. The proteases once produced are converted to particulate form by encapsulation so that the persons involved in the production or use of enzyme could avoid allergic reactions due to inhalation of dry enzyme powder. The encapsulation is done by mixing the wet paste of enzyme at 50-70ºC with hydrophobic substances (polyethylene glycol), and then making tinny particles/globules.

Neutral protease

Neutral proteases are obtained from plants e.g. papain (from *Carica papaya*), bromelain (from *Ananas comorus*) and ficin (from *Ficus* spp.), which are cysteine proteases. Neutral proteases are produced by bacteria (*Clostridium histolyticum*, *Streptococcus* spp., *Bacillus subtilis*, *B. cereus*, *B. megaterium*, *B. stearothermophilus*, *B. thuringiensis*, *B. pumilus*, *B. polymyxa*, *B. licheniformis*, *B. amyloliquefaciens*. *B. stearothermophilus*, *Pseudomonas aeruginosa*, *Streptomyces griseus*) and fungi (*Aspergillus oryzae*, *A. sojae*, *Penicillium* spp., *Pericularia oryzae*). These microbial neutral proteases are either cysteine or metalloproteases. Neutral

metalloproteases have specificity towards peptide linkages that contain hydrophobic amino acids to the amino side. The neutral proteases are unstable and require calcium, sodium, and chloride ions for their stability. Not only the pH range for these proteases is small, but they also get inactivated at elevated temperatures. Commercial fungal neutral proteases are used in baking, food processing, protein modification, and in leather, animal feeds and pharmaceutical industries.

Acid proteases

Rennins from calf stomach, pepsin of humans are the well known examples of acid proteases catalysing hydrolysis of protein around pH 2-4. Some of the fungi also produce acid proteases which are rennin-like and used mainly in cheese production. Acid proteases are also used in the preparation of digestive syrup, soy protein digestion during sauce preparation, hydrolyzing the gluten from wheat dough used for preparing biscuits in bakery making them crispy. Silver from the film roll is recovered by digesting the gelatin by acid proteases. *Alcaligens*, *Bacillus*, *Corynebacterium*, *Lactobacillus*, *Pseudomonas*, *Serratia*, *Streptococcus*, and *Streptomyces* are the bacteria, and *Aspergillus*, *Candida*, *Coriolus*, *Endothia*, *Endomophthora*, *Irpex*, *Mucor*, *Penicillium*¸*Rhizopus*, *Sclerotium*, and *Torulopsis* are the some of the fungi producing rennin like proteases which find applications in cheese processing. There are three strains being used for acid protease production and divided into two groups on the basis of culture conditions (Fig 5)

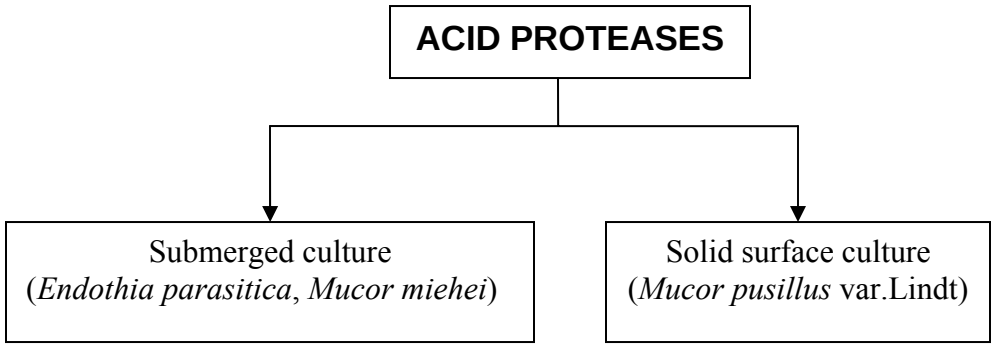


Fig 5: Strains and the methods for producing acid proteases

The rennin produced by *Endothia parasitica* was the first rennin of microbial origin marketed in the year 1967. Media containing 3 % soy meal, 1 % glucose, 1 % skim milk, 0.3 % $NaNO_3$, 0.005 % K_2HPO_4, 0.025 % $MgSO_4.7H_2O$; pH 6.8 is used for the production of protease by the above strain. The fermentation is completed in 48 h at 28 ºC, after which the mycelium is removed and enzyme is concentrated and precipitated by evaporation process. This protease has a molecular weight of 34-37.5 kDa and is stable in the pH range of 4.0-5.5 and temperature up to 50ºC. Rennin-protease from *Mucor pusillus* is produced by solid surface process. The production of rennin-protease by *Mucor miehei* is carried out in medium containing 4% potato starch, 3% soy meal, 10% ground barley, 0.5% $CaCO_3$, at 40˚C for 5-6 days. The microbial rennins are stable at high temperature, and cause harmful proteolysis. The calf rennin has been successfully

cloned into *E. coli* for production and use of rennin enzyme for avoiding the problems encountered with microbial rennin.

Cellulases

Cellulose is the most abundant organic macromolecule on earth and mainly constitutes the cell wall of plant cells. This macromolecule is a linear polymer of glucose residues linked by β-1,4 glycosidic linkage and utilized as carbon source by many microorganisms present in soil and guts of ruminants. These organisms produce cellulases, the enzymes that hydrolyze the β-1,4 glycosidic bond of cellulose. There are three different types of cellulases; endo-1,4-β-D-glucanase (EC-3.2.1.4), exo-1,4-β-glucanase (EC-3.2.1.91) and β-D-glucosidase (EC-3.2.1.21) having specific hydrolyzing properties (Table 5). The cellulase has three types of functionally different domains, (a) catalytically active core, (b) cellulose binding domain, and (c) a linker domain which is flexible connecting both the domains.

Table 5: Types of cellulases and their activities *(Source : Lynd et al. 2002)*

Type	**Specific name**	**Mode of action**
Endoglucanases (EG)	1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4)	Hydrolyses amorphous cellulose randomly at internal sites generating oligosaccharides of various length generating new ends
Exoglucanases or cellobiohydrolases (CBH)	1,4- β-D-glucan glucanohydrolases (cellodextrinases) (EC 3.2.1.74) and 1,4- β-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91).	Hydrolyse cellulose polysaccharide in a sequential manner from reducing or nonreducing ends, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products
β–Glucosidases or cellobiase	β-Glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21)	β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose

Industrial production of cellulase started in early 1980s for treating animal feed along with other enzymes to degrade non-starch polysaccharide to improve nutritive values, later applied in food industry for juice extraction and vegetable processing. Further cellulases were used in the textile, laundry as well as in the pulp and paper industries. The use of cellulases, along with hemicellulases and pectinases has increased considerably sharing approximately 20% of the world enzyme market. The market of cellulase alone is about 190 million US $ in a year. Presently cellulase is being produced mainly by the mutant strains of *Trichoderma reesei*.

Cellulase producing microorganisms

Bacteria

Aerobic thermophilic

Acidothermus cellulolyticus, Caldibacillus cellovorans, Cellulomonas flavigena, Rhodothermus marinus, Thermobifida fusca and *Thermomyces lanuginosus.*

Aerobic mesophilic

Bacillus pumilis, Cellulomonas uda, C. flavigena, Cellvibrio fulvus, C. gilvus, Cytophaga hutchinsonii, Erwinia carotovora, Micromonospora chalcae, Pseudomonas fluorescens var. cellulosa, Sporocytophaga myxococcoides and *Streptomyces reticuli.*

Anaerobic thermophilic

Anaerocellum thermophilum, Caldicellulosiruptor saccharolyticum, Clostridium thermocellum, C. cellulolyticum, Fervidobacterium islandicum, Spirochaeta thermophila and *Thermotoga neapolitana.*

Anaerobic mesophilic
Acetivibrio cellulolyticus, Butyrivibrio fibrisolvens, Eubacterium cellulosolvens, Fibrobacter succinogenes, Halocella cellulolytica, Ruminococcus albus and *R. flavefaciens*

Fungi

Acremonium cellulolyticus, Aspergillus acculeatus, A. fumigatus, A. niger, Humicola insolens, Fusarium solani, Irpex lacteus, Penicillium funmiculosum, Phanerochaete chrysosporium, Schizophyllum commune, Sclerotium rolfsii, Sporotrichum cellulophilum, Talaromyces emersonii, Thielavia terrestris, Trichoderma koningii, T. reesei and *T. viride.*

Cellulase production

Cellulose is the best substrate for the production of cellulase by various organisms (bacteria, actinomycetes and fungi). The purified cellulose is very expensive to be used for the cellulase production so different media based on cheaper cellulosic materials (sugarcane bagasse, corn cob and straw, cattle dung, municipal and agricultural waste, sawdust, etc.) are employed for cellulase production. These complex substrates require preliminary mechanical, chemical or biological treatment to expose the crystalline cellulose to be acted upon by the cellulase excreted by the microbe. Production conditions (temperature, pH, aeration, etc.) vary from organism to organism. The co-culture of two species (*Trichoderma reesei* and *Aspergillus wenti*, *T. reesei* and *A. phaenicis*, and *Chaetomium cellulolyticum* and *Sporotrichum pulveruleutum*) has better utilization of cellulosic biomass and cellulase production in respect of the pure cultures. In comparison to the submerged or solid state fermentation, combined (submerged and solid state) fermentation has higher production of cellulase and cell biomass. *Trichoderma reesei* strains used for the production of cellulase secrete up to 40 g/L of protein in the production media under optimized condition of which cellulases are the major component. The proportion of CBH I is about 60%, CBH II 25% and endoglucanases 15%.

In bacteria, cellulase is constitutively produced, whereas fungi produce cellulase only in the presence of cellulose as substrate. The induction in fungi is believed to be triggered by the soluble cellooligosacharides formed during cellulose hydrolysis by a basal level of constitutive cellulase. Cellobiose (β-1-4, linked disaccharide of glucose), sophorose (β-1-2, linked disaccharide of glucose) and gentibiose ((β-1-6, linked disaccharide of glucose) are some of the natural inducers for cellulase expression. These are easily metabolized, thus some stable analogues (thiocellobiose, thiogentobiose) have been successfully used for high titer of cellulase. The bottle neck in industrial use of these analogues is their complicated synthesis procedure.

Application of cellulases

Saccharification of cellulosic waste materials

The cellulosic waste has complex structure formed by cellulose, hemicellulose, lignin, and ash. The saccharification of cellulosic waste requires pre-treatment with alkali to remove the lignin and other inhibitors, which increases the crystalline nature of cellulose thus enhancing its reactivity with the cellulase. Mutant strains of *Trichoderma reesei* QM-9414 producing cellulases are used for saccharification of cellulosic waste materials.

Large quantities of free enzyme is produced in plug flow reactor in comparison to batch, continuous stirred tank reactor (CSTR) and exceeds the saccharification rate by 80% at a high saccharification concentration. The cellulase is recovered and reused for saccharification by centrifugation and ultrafiltration techniques. Reverse osmosis is employed to concentrate the sugars obtained by the saccharification process. This saccharified product is used for supplementing fermentation media for ethanol production.

Extraction of fruit/vegetable juices and olive oil

Cellulases along with other macerating enzymes (pectinases and hemicellulases) are employed for extraction and clarification of fruit and vegetable juices. The use of enzymes increase juice yield without additional capital investment. The macerating enzymes are responsible for partial or complete liquefaction of fruit/vegetable pulp, which enhances the juice yield, reduces the processing time and to some extent cause the release of valuable fruit components in to the juices.

The macerating property of the cellulase, pectinases and hemicellulases are now being commercially utilized for the extraction of olive oil extraction. This has improved the yield of olive oil (1-2 kg per 10 kg olives), increased anti-oxidants and vitamin E content of oil, and also reduces oil content in the waste water.

Beer and wine industry

During malting of barley, the seed germinates and produces α- and β-amylases, carboxypeptidases and β-glucanses which hydrolyse the seed reserve for further fermentation by yeast. Sometimes the malting is not proper due to poor quality of barley resulting in gel formation, poor filtration, and haze development in the final product. Gel formation in wort is due to the presence of β-glucan. β-glucanases, endoglucanses, cellobiohydrolase II are used for

digesting β-glucan and reducing the wort viscosity. The enzyme improves the quality of beer produced from poor quality of barley.

Commercial macerating enzymes (cellulases, pectinases and hemicellulases) are used for extraction of grape juice during wine making. The enzymatic treatment improves the filtration rate, wine stability, colour extraction, and reduces the must viscosity.

Feed industry

The treatment of feed materials (grains, vegetable, forage) with cellulases and other hydrolytic enzymes is practiced to improve the nutritive values. Better digestibility, weight gain, and milk production have been observed in the animals fed on enzymatically treated feed. Attempts are also there to clone the cellulase and xylanase in animals for improving their digestion efficiency.

Textile and laundry industry

The thermophilic cellulase find application in textile industry as they are used to give stone washed look to denim jeans (biostoning) and increasing the brightness (biopolishing). Earlier pumice stone were used to give aged and faded look to the jeans, making them more comfortable to wear. The use of stones had many drawbacks, including, damage to the washing machines, loss of durability of garment, manual removal of stone pieces from the garment, and also environmental pollution. The cellulases are commonly used to create stone wash look in jeans and make them softer afterwards. Thermophilic cellulases easily erode dye, which is used for colouring jeans, and maintain the durability of garment as compared to mechanical processes. Cellulosic fibre are greatly used in the manufacture of fabrics which shows 'fuzz' formation and 'pilling' effects, reducing the commercial value of the fabric. Cellulases have been successfully used for removing the short fibres from the fabric surface making it smooth and glossy, and increase the brightness and moisture absorbance. This application is referred as biopolishing.

The thermophilic alkalophilic cellulases are added in the laundry detergents to decrease the discolouration and fuzzing effects caused by many washes. This maintains the smoothness and softness of the fabric. Cellulases digest the cellulose microfibrils helping in removal the dirt entrapped in the microfibrils of cotton and cotton blend fabrics.

Paper Processing

The cellulases along with hemicellulases find application in the pulping process of the woody raw material referred as biopulping, reduces the energy consumption and also improves in the paper strength. The cellulase treatment of pulp improves the betability and drainage property which increases the speed of paper mill. The cellulases are commercially used for deinking of xerographic and laser inks from paper which are otherwise difficult to remove by chemical methods. The de-inking of paper helps in recycling of paper without reducing the fibre strength. All these enzymatic process have greatly reduced the energy consumption and chemical usage in paper manufacture.

Production of ethanol from cellulosic (Bio ethanol)

The conversion of cellulosic waste to fermentable sugars with the use of efficient cellulases has promising process technology fermentation processes for the production of ethanol. This ethanol

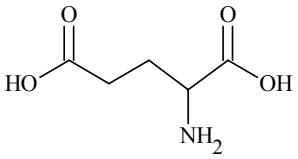
is referred as bio ethanol and is a great achievement in the area of non-conventional sources of energy. The production process involves pretreatment of the cellulosic waste (agricultural and municipal wastes) by steam explosion process to expose the cellulose to be acted by the cellulases and other enzymes producing C6 and C5 sugars. Finally fermentative microorganisms convert both C6 and C5 sugars into ethanol. The use of the bio ethanol decreases the 90% CO_2 emmision in comparison to gasoline.

Amino acid production: L-Glutamic acid and L-Lysine

Amino acids are extensively used in food industry, as feed additives, in medicine and cosmetics and as starting materials for the synthesis of myriads of compounds in chemical industry. In the last 20 years, the demand for amino acid has been growing with a rate of 5-7 % annually. The amino acids - L-lysine, L-methionine, L-threonine, and L-tryptophan used as feed additives have the largest share (56%) of the total amino acid market (US $4.5 billion) estimated in 2004. The three amino acid – L-glutamic acid (monosodium glutamate, MSG) as flavor enhancer, and L-aspartic acid and L-phenylalanine for synthesizing peptide sweetener L-aspartyl - L-phenylalanyl methyl ester (Aspartame) are widely used in food industries. Total worldwide consumption of amino acids is estimated to be more than 2 million tons per year. Monosodium glutamate, L-lysine hydrochloride, L-methionine and L-threonine are the major ones.

The production of amino acids may involve any of these processes: microbial fermentation, extraction from animal or plant protein hydrolysates, chemical synthesis, and enzymatic transformation. Fermentation processes mainly employ stains of *Corynebacterium glutamicum* and *Escherichia coli* to produce L-glutamic acid (monosodium glutamate, MSG), L-aspartic acid, L-phenylalanine, L-lysine, L-methionine, L-threonine, and L-tryptophan, from sugar sources such as molasses, sucrose, or glucose. These amino acids serve as building blocks for active ingredients that are applied as pharmaceuticals, cosmetics, and agricultural products (Leuchtenberger *et al*., 2005). In the future the demand of amino acids in food, feed, pharmaceuticals, cosmetics, agriculture, will lead to further exploration of the potential of microorganisms, plants and enzymes to develop more efficient processes for amino acid production. The industrial production of L-glutamic acid and L-lysine will be discussed in the proceeding sections.

L-Glutamic acid



Kyowa Hakko, Japan was the first company to start the production of L-glutamic acid by fermentation using *Corynebacterium glutamicum* (syn. *Micrococcus glutamicus*) in the year 1957 after its discovery in the spent medium. *Breviacterium*, *Microbacterium*, *Arthrobacter* are some other glutamic acid producing bacteria. These bacteria are collectively referred as ‘glutamic acid bacteria’ and have some common morphological and physiological characterstics. These bacteria are Gram positive, nonsporulating, nonmotile, require biotin, lack or have little α-ketoglutarate dehydrogenase activity and show high glutamate dehydrogenase activity.

Biosynthesis of L-glutamic acid

Isocitrate dehyderogenase catalyses the decarboxylation and dehydrogenation of isocitrate to α-ketoglutarate in tricarboxylic acid (TCA) cycle. This α-ketoglutarate is the key precursor, which is converted into L-glutamic acid via reductive amination with free NH_4^+ ions, catalysed by glutamate dehydrogenase (Fig 6).

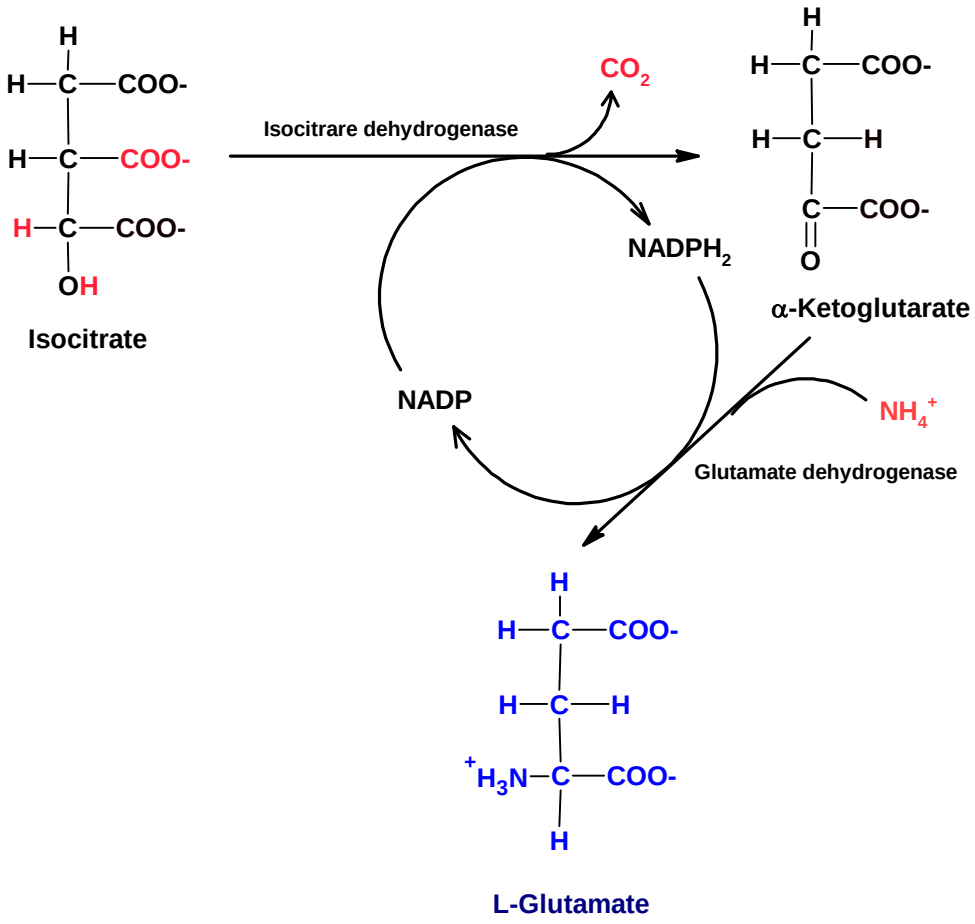


Fig. 6: Enzymes involved in the biosynthesis of L-glutamate

In normal TCA cycle α-ketoglutarate is converted to succinyl-CoA by the α-ketoglutarate dehydrogenase complex. The strains used for commercial production of glutamic acid lack or have very low α-ketoglutarate dehydrogenase activity. However, the interruption in the TCA cycle is compensated by some anaplerotic reactions to synthesize oxaloacetate which combines with acetyl-CoA to produce isocitrate which is used for the synthesis of glutamic acid as shown in Fig 7.

The glutamic acid bacteria in normal growth conditions do not secrete glutamate out of the cell because of the rigid cell wall. The permeability of the bacterial cells can be enhanced by: i) restricting the formation of normal phospholipid biosynthesis using biotin deficient media, ii) limiting oleic acid in oleic acid auxotrophs, iii) limiting glycerol in glycerol auxotrophs, iv)

addition of surfactants (e.g. Tween 60) or by adding C_{16} - C_{18} saturated fatty acids and v) addition of penicillin in case of biotin rich media to weaken the cell wall thus rendering high permeability for secreting glutamic acid in the culture broth.

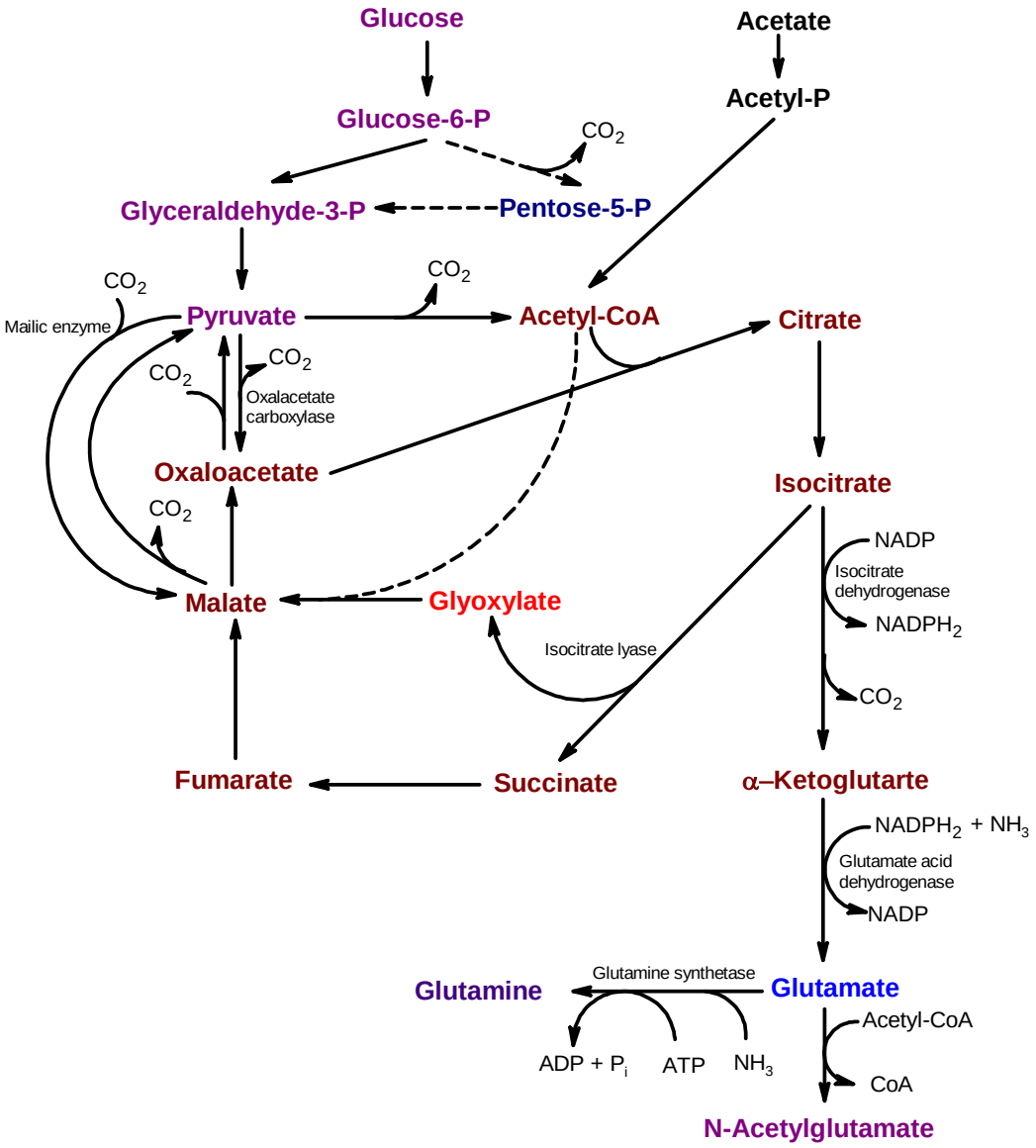


Fig. 7: Biosynthesis of L-glutamic acid from glucose and acetate as the carbon source. The anaplerotic cycle completing the citric acid cycle is also shown

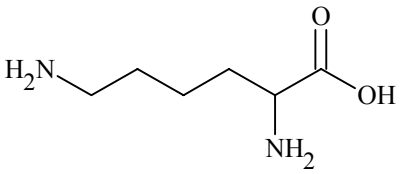
Industrial production of L-glutamic acid

Glutamic acid bacteria convert 50-60% of the added carbon source to L-glutamic acid under the optimal culture conditions. Stirred tank reactor up to 450 m^3 is used for industrial production of glutamic acid. The fermentation is carried out aerobically at 30-37˚C, depending on the

microorganisms used. Glucose, fructose, sugar cane and sugar beet molasses, and starch hydrolysates are some carbon sources used in production of L-glutamic acid. Penicillin or fatty acid derivatives (e.g. Tween 60) are added in the sugar cane or sugar beet molasses based medium upsetting the cell wall synthesis of these bacteria as these carbon sources contain high biotin (0.02-0.12 mg/Kg) content favoring the formation of cell membrane with high lipid content. Acetate, methanol, ethanol, acetaldehyde, or n-alkane have also been employed as carbon source in the production of of L-glutamic acid by bacteria, but still cane sugar molasses or starch hydrolysates are the main carbon sources. Ammonium salts or ammonia are generally used as nitrogen source. In case of glutamic acid bacteria having high urease activity, urea can also be used as nitrogen source in the medium.

The optimal biotin concentration in the production medium depends on the carbon source used, i.e., 5μg/L biotin for media with glucose and 0.2 – 1.0 μg/L in case of media containing acetate. L-Cysteine as an additional growth factor is required by some strains and media based on n-alkane require thiamine supplementation. Oxygen supply is necessary for glutamic acid production and under oxygen deficiency, excretion of lactate and succinate occurs, whereas excess oxygen results in ammonium ion deficiency, ceasing the growth and production of α-ketoglutarate, thus lowering the L-glutamic acid yield in both cases. Medium pH during fermentation is maintained at 7-8 by the addition of alkali/ammonia. L-glutamic acid starts accumulating from the mid way of the fermentation process which normally lasts for 30-35 h and finally L-glutamic acid level reaches to 100g/L in the fermentation broth in case of *Brevibacterium divaricatum* (NRRL-B-231) (Miescher, 1975). In acidic pH with excess ammonia, glutamine is produced instead of L-glutamic acid. L-Glutamic acid is recovered from the fermentation broth by separating the cells from the culture medium and its crystallization is done by lowering the pH to 3.2 (isoelectric point) of the cell free broth using HCl. Crystals are then filtered, washed and monosodium glutamate (MSG) is prepared by adding sodium hydroxide to the crystalline L-glutamic acid followed by recrystallization.

L-Lysine



L-Lysine is an essential amino acid that is not synthesized by humans and other mammals and it has to be obtained from the diet. Cereals and vegetables have low lysine content, thus to increase the nutritive value of food products and animal feed derived from plant sources, these are supplemented with lysine. Lysine was first produced by combination of *Escherichia coli* (ATCC 13002), which is a lysine-histidine double auxotrophic mutant and *Aerobacter aerogenes* (ATCC 12409). *E. coli* (ATCC 13002) is grown in molasses based medium to produce diaminopimelic acid (DAP) with an yield of 19-24 g/L. The entire fermentation broth is then incubated with *Aerobacter aerogenes* (ATCC 12409) at 35 ºC, which decarboxylates the DAP to L-lysine.

Homoserine auxotroph of *Corynebacterium* (*Micrococcus*) *glutamicum* developed by UV radiation which accumulates large amount of L-lysine in the culture broth was for the first time used for the direct production of L-lysine from carbohydrate (Kinoshita *et al*., 1958, Nakayama *et al*., 1961). Non-carbohydrates based fermentation using homoserine auxotrophic and S-(2-aminoethyl)-L-cysteine resistant mutants of thermophilic *Bacillus methanolicus* accumulates nearly 40 g/L of L-lysine in a fed-batch fermentation with methanol (0.5 %v/v) as sole carbon source have also been developed (Flickinger, 2004).

Biosynthesis of L-lysine

Bacteria, actinomycetes, cyanobacteria, some phycomycetes, and protozoa sythesise lysine via diaminopimelic acid (DAP) pathway whereas some phycomycetes, all ascomycetes, basidiomycetes, and eukaryotic algae use the aminodipic acid pathway for lysine biosynthesis. The biosynthetic pathway via DAP is shown in Fig 8. The regulation of this pathway involves feedback inhibition of various enzymes of this pathway. In case of *C. glutamicum*, aspartokinase the first key enzyme catalyses the phosphorylation of aspartate to asparatyl phosphate is regulated by feed back inhibition by lysine and threonine, the end products of this branched pathway. Homoserine dehydrogenase suffers feedback inhibition by threonine and repression by methionine. Dihydropicolinate synthatase, the first enzyme following the branch point for lysine biosynthesis is not inhibited by lysine accumulation which is quite unusual.

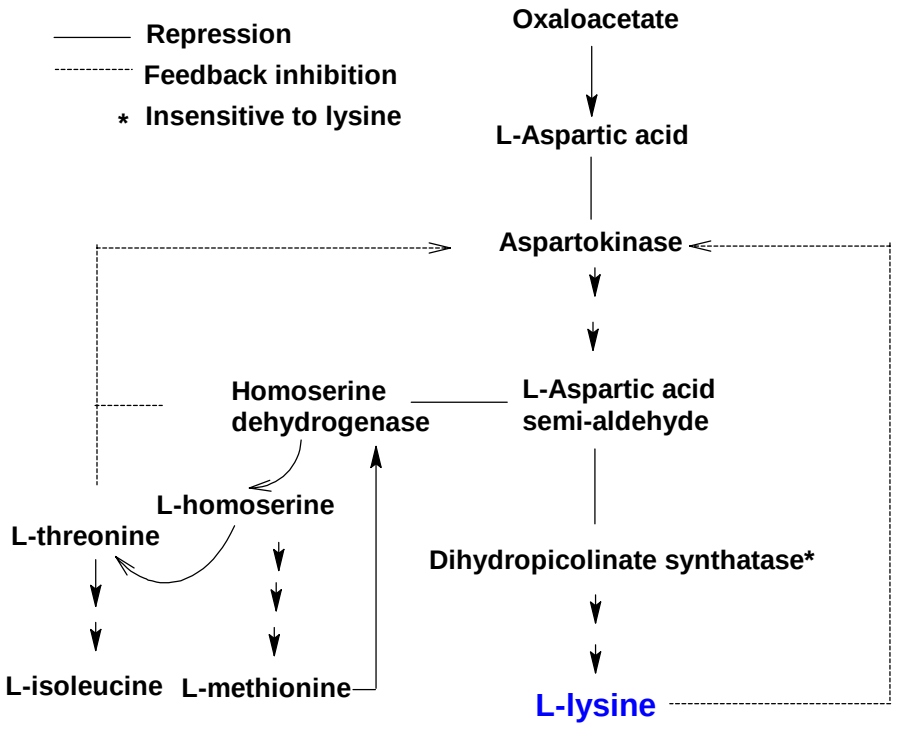


Fig 8 Regulation of L-lysine biosynthesis in *Corynebacterium glutamicum*

A defect in the feedback control mechanism is observed in the strains of *C. glutamicum* which overproduce lysine. Homoserine dehydrogenase activity is lacking in these strains, as a result the flux of aspartate semi-aldehyde to threonine is reduced and it also prevents the feed back inhibition of the aspartokinase due to threonine. Mutants having hyper sensitive homoserine dehydrogenase to feed back inhibition through threonine also efficiently convert all aspartate semialdehyde to lysine. In mutants resistant to S-(β-aminoethyl)-L-cysteine (AEC), an antimetabolite of lysine, the aspartokinase is also insensitive to multivalent feedback inhibition. The excess aspartate semi-aldehyde flows into lysine biosynthesis while its conversion to L-threonine is prevented by feedback inhibition to homoserine dehydrogenase. Lysine is secreted in the medium through active transport by these microorganisms.

Industrial production of lysine

The mutants of glutamic-acid-producing bacteria, Co*rynebacterium* and *Brevibacterium* which are homoserine auxotrophs or methionine-threonine double auxotrophs are the industrial strains used for the production of L-lysine. Industrial production of lysine is carried out as batch process in aerated stirred tank rectors. Sugarcane molasses is generally used as carbon source, however, acetate, ethanol or alkanes supplemented with soybean hydrolysates can also be used as carbon sources. Gaseous ammonia or ammonium salts are used as nitrogen source and urea can also be used since the organism has fairly high activity of urease. Growth factors like L-homoserine or L-threonine and L-methionine are added in suboptimal concentration into the production media. The biotin content in the medium should be >30 μg/L for optimal lysine production and its concentration <30 μg/L results in the accumulation of L-glutamate instead of L-lysine. During production temperature is maintained at 28 ºC and pH 7 by feeding ammonia or urea. Large size of inoculum i.e. 10% v/v is used to shorten the lag phase during fermentation. Lysine production starts in the early log phase of growth and continues through the stationary phase and a maximum of production 40-45 g/L lysine is achieved in 60h.

Lysine is recovered by acidifying the cell free fermentation broth to pH 2.0 with hydrochloric acid followed by cation exchange column where L-lysine gets adsorbed in the ammonium form. Bound L-lysine is then eluted from column with dilute ammonia solution. The elute is reacidified and the product is finally crystallized as L-lysine hydrochloride.

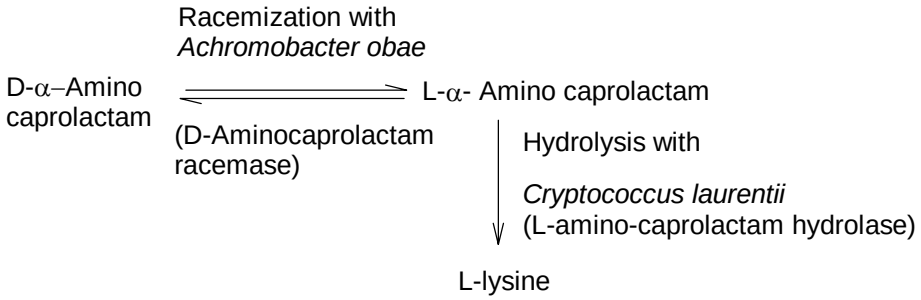


Fig. 9: Enzymatic transformation of DL-α-amino caprolactam to L-lysine

Enzymatic transformation of DL- amino caprolactam into L-lysine is also carried out by Torary Company, Japan with the production of 400 tonnes /year. In this process 10% DL-amino

caprolactam solution (pH 8.0) is added to 0.1 % (w/v) acetone dried cells of *Cryptococcus laurentii* and of *Achromobacter obae* (Fig 9). A conversion efficiency of 99.8% is obtained at 40 ºC after 24 h.

Organic acids – Citric acid and Lactic acid

The organic acids find their wide application in the food, pharmaceutical and chemical industries. Some organic acids like citric acid, lactic acid, fumaric acid, propionic acid, malic acid, α-ketoglutaric acid, 5- ketoglutaric acid, 2- ketoglutaric acid, gluconic acid, acetic acid, kojic acid, itaconic acid are produced through fermentation. Advances in the fermentation technology have helped to manufacture organic acid at industrial scale. The chemical synthesis of these organic acids requires very harsh conditions and involves many steps which make their large scale production impractical. The microbial fermentation is a very simple method to synthesize these organic acids in very pure form. The fermentation methods require less energy input and are cost effective due to simple media formulations in many cases. In the proceeding section citric acid and lactic acid production will be discussed.

Citric acid

Citric acid (2-hydroxypropane-1,2,3-tricarboxylic acid) is one of the world's major fermentation products with the annual production of over 550,000 tonnes, and its demand is increasing at the rate of 2-3% every year. It was first isolated in 1784 from lemon juice and crystallized by Scheele. Until the 1920s, citric acid was extracted from the lemon juice and referred as 'natural citric acid'. Whehmer first time in 1923 described that citric acid is a metabolic product of *Penicillium* and *Mucor*. In 1923, Pfizer became the first industry to produce citric acid through fermentation based process in USA, by culturing *Aspergillus niger* in surface culture in a medium containing sucrose and mineral salts. As on today most of the citric acid is produced by fermentation, and the major producers are located in Western Europe, USA and China. Citric acid has GRAS (generally regarded as safe) status and its major applications are summarized in Table 6.

Table 6: Uses of citric acid in different industries

Industry	**Application**
Food and beverage products	Acidulant, pH regulator, flavour enhancer, preservative and antioxidant synergist
Bakery	Leavening agent
Chemical industry	Antifoam, softener, electroplating, pickling agent, alkyd resins and plastics
Detergents	To replace polyphosphate
Steam boiler	Removal of scales formed due to salt deposition
Oil wells	Removing the iron clogged in the pores of the sand
Medical and cosmetics	Effervescent type denture cleansers, and in shampoos and cosmetics

Microorganisms

Citric acid is a primary metabolite and excreted in traces by *Aspergillus wentii, A. clavatus, Penicillium luteum, P. citrium, Mucor piriformis, Paecilomyces divaricatum, Citromyces pfefferianus, Candida guilliermondii, Saccharomycopsis lipolytica, Trichoderma viride, Arthrobacter paraffineus,* and *corynebacterium* sp. Many microorganisms including filamentus fungi, yeasts and bacteria could be used to produce citric acid, however, the mutants of A. niger are generally used for commercial production.

Citric acid biosynthesis

Glucose is metabolished to pyruvate in glycolysis, which is further decarboxylated to produce acetyl CoA (Fig. 10). Citrate synthase catalyses the first step of aldol condensation of acetyl CoA and oxaloacetate to form citric acid in the tricarboxylic acid cycle (TCA). In citric acid producing microbes, the enzymes of the TCA cycle are present throughout the trophophase and during the idiophase, all enzymes of this pathway are produced but the activity of α-ketoglutarate dehydrogenase sharply declines as compared to trophophase. Oxaloacetate formed during TCA cycle is consumed by citrate synthase for the formation of citric acid as the enzymes involved in further metabolism of citric acid are not produced, the regeneration of oxaloacetate ceases and ultimately may cease the citrate synthesis. To overcome this problem citric acid producing organisms have anaplerotic pathways for replenishing the TCA cycle intermediates and citric acid synthesis continues. One such pathway catalyzed by pyruvate decarboxylase converts pyruvate and CO_2 into oxaloacetate, utilizing one ATP, another such pathway involves phosphoenol pyruvate (PEP) carboxykinase converting PEP and CO_2 into oxaloacetate. In absence of glucose (n-alkane C_9-C_{23} as carbon source), glyoxylate cycle operates where isocitrate lyase and malate synthase are involved. Isocitrate lyase breaks down isocitrate into glyoxylate and succinate (intermediate of TCA cycle). Glyoxylate is combined with acetyl CoA by malate synthase to form malate.

Citric acid production media

The commonly used carbon sources in the citric acid production media include starch from potatoes, starch hydrolysates, glucose syrup from saccharified starch, sucrose, sugarcane syrup, sugarcane molasses, sugar beet molasses. Starch used during fermentation is hydrolysed either by amylases secreted by the growing fungus or by extraneous amylases. Trace elements Cu, Mg, Mn, Fe, Zn, and Mo are necessary in ppm range for optimal growth of the fungus. These may exert inhibitory effects if the optimal concentration exceeds.

Iron is one of the very important trace element required for the production of citric acid by *A. niger*, because the optimal growth of this fungus requires high iron concentration (since iron is co-factor of aconitase), but only 0.05-0.5 ppm is needed to lower aconitase activity which leads to maximizing citric acid production. The pH of medium also influences citric acid production. The fermentation starts at pH 5 and in first 48 h during trophophase, the pH falls below 3.0 as a result of utilization of ammonium ions. The pH below 3.0 during idiophase prevents the formation of oxalic acid and gluconic acid this also reduces the risk of contamination.

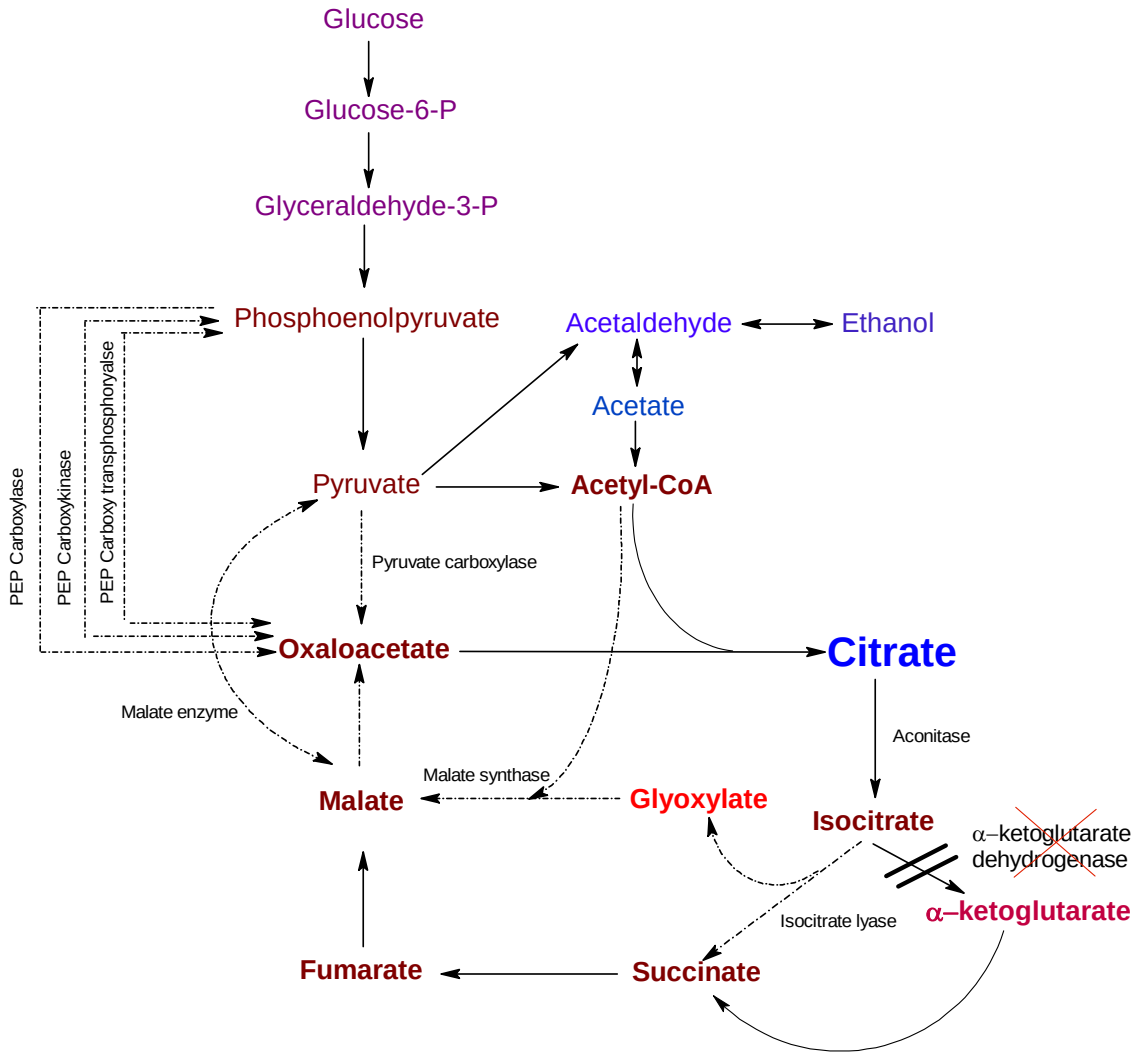


Fig. 10 : Citric acid synthesis in citric acid producing microorganism. The enzyme α-ketoglutarate dehydrogenase is absent in such microorganism inhibiting the α-ketoglutarate synthesis. The anaplerotic pathways (-----) replenish the intermediates of the citric acid cycle

Fermentation process used in citric acid production

Citric acid is produced industrially by both surface and submerged processes. In surface processes either solid or liquid media is used and the fungus is allowed to grow on the surface of the medium while in the submerged process, the stirred tank reactors or air lift fermenters are used.

Inoculum preparation

The spore suspension is used as inoculum and the spores are produced by growing the fungus on the solid substrates at 25 ºC in glass bottles for 10-14 days. Theses spores are induced to

germinate at 32 ºC in medium containing 15% sugar to form pellets of mycelium (0.2-0.5 mm diameter). These pellets are used for inoculating the production medium. Aspergilli are genetically unstable, so minimum number of preliminary steps is performed to produce the final inoculum and in some cases spores are also directly used to inoculate the production medium.

Surface and solid-substrate fermentations

The liquid surface methods are the oldest production method and about 20 % of the world's supply of citric acid is met by these processes. The bioreactors used in these processes are shallow aluminum or stainless steel trays (5-20 cm deep). It is a labour intensive process than the deep vat fermentations, due to manual cleaning of pipes, trays, and walls of the system. Media containing beet molasses (320-400 g/L), NH_4NO_3 (1.6-3.2 g/L), CaH_2PO_4 (0.3-1.0 g/L), $MrSO_4.7H_2O$ (0.2-0.5 g/L), $ZnSO_4$ (0.01-0.1 g/L) and calcium hexacyanoferrarte (0.4-2.0 g/L) is sterilized continuously and pumped onto the trays. The fermentation is initiated by inoculating the medium by spraying dry spores or spore suspension. Sterile air is blown over the surface to control the incubation temperature (30 ºC) and to lower the CO_2 level produced during the fermentation. The spores germinate within 24 h and as the growth proceeds, the pH of the medium declines to 1.5-2.0 due to the uptake of the ammonium ions, and citric acid production begins. The fermentation is continued for 8-12 days and a production of about 1.0 kg of citric acid monohydrate /m^3 per day is achieved.

Solid-state fermentation processes are performed at small scale using steam sterilized wheat bran or sweet potato waste. The pH of the medium is adjusted to 4.5 and inoculated with spores of *A. niger*, and spread over trays to a depth of 3-5 cm. Sterile air is circulated and temperature is maintained around 28 ºC. The fermentation is allowed to proceed for 5- days after which the citric acid from the medium is extacted using hot water.

Submerged fermentation processes

The submerged fermentation is carried out in stirred tanks of 40-200 m^3 capacity or in larger airlift fermenters of 200-900 m^3 size, about 80% of the citric acid is produced through these processes. It is very necessary to use stainless steel fermenter vessel or alternatively special glass or plastic lining is done to prevent the leaching of heavy metals because of the lowering of the pH during fermentation.

The structure of the mycelium in the trophophase influences the productivity of citric acid during fermentation. Small compact pellets (1 mm) with fluffy centers and smooth surface consisting of short forked bulbous hyphae characteristically have optimal citric acid production rates. High manganese level in the medium is responsible for development of long unbranched hyphae with loose texture which produce lesser citric acid. This necessitates the pretreatment of raw materials to reduce manganese concentration to below 0.02 mM. The low manganese concentration also limits the pentose phosphate pathway and shifts the glucose flux to glycolysis and citric acid production. Addition of copper ions to the medium prevents the uptake of magnesium by the fungus which also diminishes the aconitase activity involved in further metabolism of citrate. The level of iron in the medium is also low in order to inhibit the activity of aconitase enzyme.

The sugar concentration of 140 g/L in the production medium is necessary for promoting activity of both glycolytic enzymes and pyruvate carboxylase. Ammonium salts at 0.1-0.4 g/L restrict the increase in biomass and also activates citric acid production by counteracting the inhibitory effect of citrate on phosphofructokinase. The fermentation is carried out at 30 ºC with a high aeration rate of 0.2-1 vvm during the idiophase. The initial pH of medium is 5-7 which decreases during the idiophase to 2 or below inhibiting the glucose oxidase activity, responsible for gluconic acid production. The fermentation is carried for 8 days and yield of 0.7-0.9 g citrate per gram glucose is achieved. The overall yield of citrate is around 18.0 kg/m^3 per day.

Citric acid recovery

The citric acid is recovered by separation of fungal mycelium from the fermentation broth by rotary filtration or centrifugation. The filtrate is treated with lime ($Ca(OH)_2$) to precipitate out oxalic acid formed in sub-optimal production conditions in the form of calcium oxalate. The precipitates of calcium oxalate is separated by filtration and the pH of the filtrate is raised to 7.2 ± 0.2 and heated to 70-90 ºC with lime forming calcium citrate precipitates. These precipitates are separated by filtration by means of rotatory filters and treated with sulphuric acid to release citric acid. This is again filtered to separate citric acid in solution from the calcium sulphate precipitates. The citric acid solution thus obtained is diluted and passed through activated carbon for decolourising. Finally the solution is evaporated to form citric acid crystals. To avoid the use of lime and sulphuric acid, other methods of purification like solvent extraction, ion-pair extraction and electrodialysis are used.

Lactic acid

Lactic acid (2-hydroxypropanoic acid) was discovered and isolated in 1780 by the Swedish chemist Scheele from sour milk and the first organic acid produced microbiologically in 1881 by Charles E. Avery at Littleton, Massachusetts, USA. It is classified as GRAS (generally regarded as safe) by Food and Drug Authority (FDA) in the USA and its annual consumption is estimated to be 30 000 tonnes. Lactic acid is used in various industries for different applications (Table7).

Table 7: Applications of lactic acid in different industries

Industry	**Application**
Food	Preservative, acidulant, buffering agent, pickling agent and dough conditioner
Meat	Prolongs the shelf life of poultry and fish during packaging
Textile	Finishing, antimony lactate as a mordant during dyeing
Metal	Electroplating bath, plasticizer and corrosion inhibitor
Leather	Acidulant
Pharmaceutical	Ointments, lotions, anti acne solutions, humectants, and parenteral solutions, calcium lactate (anti caries agent) and biodegradable polymers for sutures in medicine
Medical	Orthopedic implants, controlled drug release
Chemical	Ethyl/butyl lactate are used as green solvents

Lactic acid bacteria

There are two groups of lactic acid bacteria, one is heterofermentative and other is homofermentative. The heterofermentative (e.g. *Luconostoc mesenteroides*) lactic acid bacteria produce many byproducts other than lactic acid and are not suitable for commercial processes. In case of homofermentative bacteria (*Lactobacillus* sp.), very little substrate is used for producing cell mass and other metabolites and majority of the carbon source is converted to lactic acid, and here the percent conversion of sugars to lactic acid is virtually equivalent to the theoretical yield of two moles of lactic acid per mole of hexose sugar utilized. In Table 8 some *Lactobacillus* species and their preferred carbon sources are listed.

Table 8: Some homofermentative lactic acid bacteria and their preferred carbon sources

Organism	**Carbon source**
Lactobacillus delbrueckii	Glucose
Lactobacillus leichmannnii	Glucose
Lactobacillus bulgaricus	Lactose
Lactobacillus helviticus	Lactose and galactose
Lactobacillus amylophyllus	Starch
Lactobacillus amylovirus	Starch
Lactobacillus lactis	Glucose, sucrose and galactose
Lactobacillus pentosus	pentoses of sulfite waste liquor

The homofermentative lactic acid bacteria are facultative anaerobes which can be grown in low oxygen concentration and are used for industrial production of lactic acid.

Lactic acid biosynthesis

The lactic acid is an anaerobic fermentation end product of glycolysis i.e. pyruvate is reduced to lactic acid by lactate dehydrogenase (EC 1.1.1.27) generating NAD for glycolysis (Fig 11). The lactic acid bacteria are able to produce either D(-)-lactic acid, L(+)-lactic acid or the racemic mixture of both D and L isomers (Fig 12). Two stereospecific lactate dehydrogenases (LDH) are present in organisms synthesizing the L(+) or D(-)-lactic acid. In some *Lactobacilli*, accumulation of L(+)-lactic acid induces racemase which converts it into D(-)-lactic acid until equilibrium is obtained. D-Lactic acid is levorotatory and L-form is dextrorotary whereas the salts of D- and L- lactic acid have reversed optical rotations.

Fermentation process

Semirefined corn sugar (dextrose), molasses, or whey are some commonly used carbon sources for the industrial production of lactic acid. The typical medium employed in the fermentation process consists of 10-15 % dextrose, 10 % calcium carbonate, and small amounts of nitrogenous

substances such as malt sprouts, $(NH_4)_2HPO_4$ (0.25 %). The presence of calcium carbonate neutralizes the free lactic acid, which is toxic to the bacteria and maintains the pH of the medium between 5.5 and 6.5. The medium is also supplemented with B vitamins for proper growth of the organism. Lactic acid fermentation with *Lactobacillus delbrueckii* carried out at 45-50 ºC. No aeration is required during fermentation as lactic acid fermentation is anaerobic one but for proper mixing the calcium carbonate to react with lactic acid produced, agitation is essential. The fermentation is continued for six days or less till complete utilizastion of sugar in the medium. Lactic acid yield is 80-90 % and it is very near to the theoretical value. The temperature during fermentation in case of *Lactobacillus pentoses*, *Lactobacillus casei*, or *Streptococcus lactis* is kept relatively low around 30 ºC.

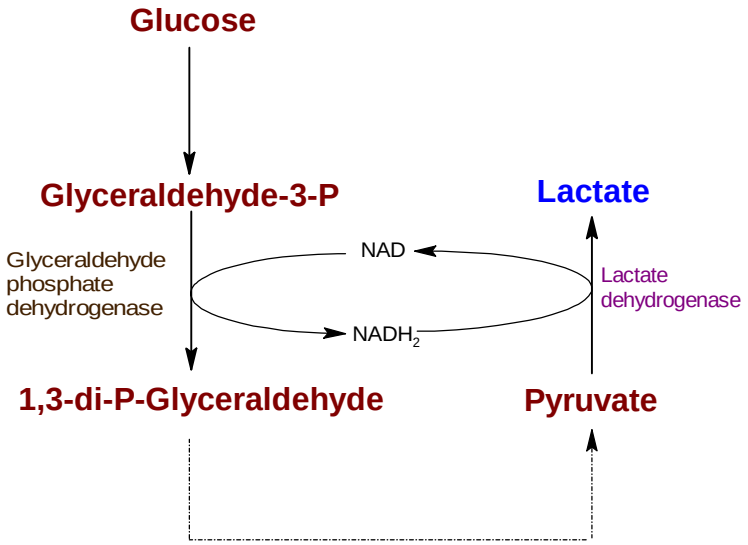


Fig. 11: Lactic acid biosynthesis from glucose

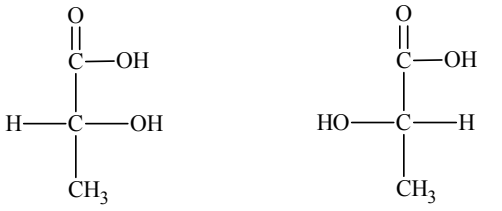


Fig. 12: D and L enantiomers of lactic acid

Undissociated lactic acid inhibits its formation as the fermentation progresses. Extractive lactic acid fermentation technique has been applied to overcome this inhibitory effect and lactic acid yield of 0.99g/l and productivity of 1.67 g/l/h has been achieved in comparison to the

conventional batch reactor yielding 0.83 g/l of lactic acid and of 0.31 g/l/h productivity (Srivastava *et al*., 1992). High yield of lactic acid is obtained either by continuous cell recycle fermentation process or by fed batch fermentation. Inhibitory effect of lactic acid has also been overcome by electrodialysis fermentation method which continuously remove lactic acid from the fermentation broth resulting in continuation of fermentation activity and 82.2 g/liter lactic acid which is about 5.5 times greater than that produced in non-pH controlled fermentation (Hongo *et al*. 1986). Using membrane cell recycle bioreactor higher volumetric productivity of 117 g/l/h has been obtained but high product concentration does not occur. Membrane Cell Recycle Bioreactors (MCRB) in series having lactic acid productivity of 5.7 g/l/h, and 92 g/l lactic acid concentration has also been reported (Kwon et al. 2001). Continuous fermentation processes using immobilized cells for the production of lactic acid have also been developed.

Recovery of lactic acid

A number of methods can be applied for the separation of lactate salt from fermented medium which involve extraction by solvents or separation by ion-exchange, adsorption, vacuum distillation and membrane filtration (Eyal et al. 2001). The medium after completion of fermentation consists of either pure lactic acid or its salt or the mixture of the two. Earlier method used addition of excess of calcium carbonate to the medium at the end of the fermentation and pH adjusted to 10, heated and filtered. In this procedure all the lactic acid is converted in to calcium lactate, the bacteria are killed and protein in the medium gets coagulated. A number of other methods have been developed to recover and purify lactic acid. In one such method, the filtrate is concentrated to crystallize calcium lactate following which sulphuric acid is added to precipitate calcium as calcium sulphate and removed by filtration. Lactic acid is again recrystallized as calcium lactate and passed through activated carbon to remove coloured impurities. Lactic acid is also extracted with isopropyl ether directly from the heated and filtered fermentation broth, by counter-current continuous extraction method, and lactic acid is further recovered from the solvent by counter-current washing with water. A preferred process for the lactic acid recovery from the mixture containing free lactic acid and the dissolved lactate salt involves first lowering down of the pH of fermented broth to 3.0-4.2 and then hydrophilic membrane and the volatile amine weak base (VAWB) are used to separate lactic acid from the fermented broth. The lactic acid is finally regenerated from salts of weak amine base by selectively vaporizing the volatile amine base (Eyal et al. 2001).

Production of Antibiotics: Penicillin and Streptomycin

Penicillin was the first antibiotic discovered by a classical observation of the inhibition of *Staphylococcus aureus* growth on agar plates due to a contaminating mold later identified as *Penicillium notatum* by Alexander Fleming in 1929. The demand of chemotherapeutic agents for preventing the bacterial infection encountered by the soldiers in World War II led to the development of industrial scale production of penicillin. At that time many of the bacterial infections were fatal since no antibiotics were available for their prevention. Antibiotics are the secondary metabolites produced by one organism which inhibits the growth of other organism even at a very small concentration. Till date a huge number of antibiotics have been discovered mainly produced by fungi, bacteria, and actinomycetes. Actinomycetes produce largest number of antibiotics of various structures, especially the genus *Streptomyces*.

Penicillin

Penicillin a broad-spectrum antibiotic belongs to β-lactam group and is active against many Gram positive bacteria including actinomycetes. This antibiotic was first discovered by Alexander Fleming in 1929 and the producing organism associated with its production was *Penicillium notatum*. His findings remained unnoticed until 1940 when Sir Howard Florey and E B Chain at Oxford University studied Fleming's observations systematically, and used this antibiotic to treat mice injected with lethal dose of *Streptococcus*. A patient named Albert Alexander suffering from septicaemia caused by streptococcal and staphylococcal infection was the first person treated with this antibiotic in 1941. In the beginning the response was good but later he died because of insufficient amount of penicillin required for the treatment. Both of the researchers went to US in 1941, where the scaling up of the production process started. During that time US entered in the World War II, and the penicillin production programme was declared 'top secret' by the War Production Board of US as this antibiotic had great potential for treating the bacterial infection occurring to the war injured soldiers. After end of the World War II penicillin production started in other countries, and Fleming, Florey and Chain were awarded with Noble Prize for Medicine and Physiology in 1945 for this great scientific discovery. Till date penicillins and their derivatives share the major antibiotic market.

The basic structure of the penicillin is 6-amonpenicillinic acid (6-APA) which consists of a thiazolidine ring with a condensed β-lactam ring (Fig 13). Depending upon the acyl moiety at position 6 there are various penicillins. The acyl group to be attached to the ring is either added in the growth medium producing biosynthetic penicillins or it can be attached chemically to 6-APA obtained by deacetylation of penicillin through acylases forming semisynthetic penicillins. The penicillin produced without addition of any side-chain precursors are referred as natural penicillins. Penicillin G, penicillin V are the important natural penicillins.

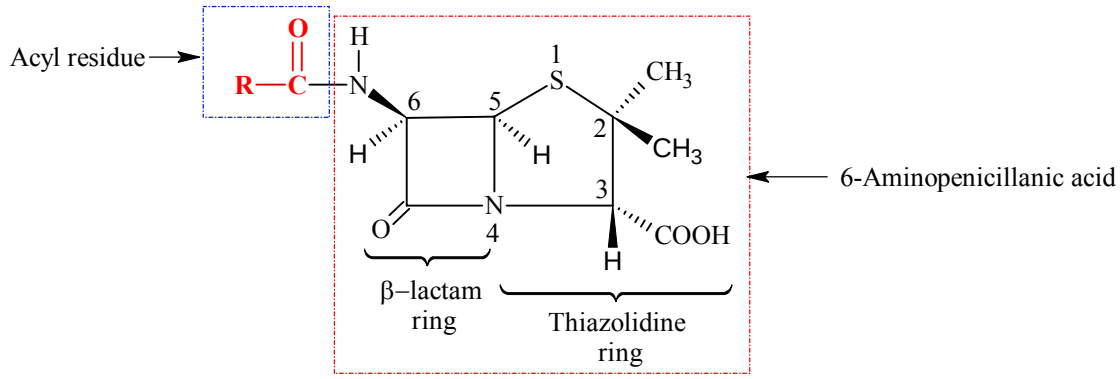


Fig. 13 : Chemical structure of penicillin

Biosynthesis of penicillin

Three amino acids namely L-α-aminoadipic acid, L-cysteine, L-valine are involved in the synthesis of penicillin (Fig 14). These three amino acids are converted into tripeptide L-α-aminoadipoyl-L-cysteinyl-L-valine (ACV) through nonribosomal process catalysed by ACV synthetase (ACVS). ACV is then cyclized to form a bicyclic isopenicillin N (IPN) catalysed by non-haem iron (II) dependant oxidase isopenicillin N synthase (IPNS). The penicillin

transacylase converts α-aminoadipoyl side chain to one of many other alternatives, giving rise to myriad of natural and non-natural penicillins.

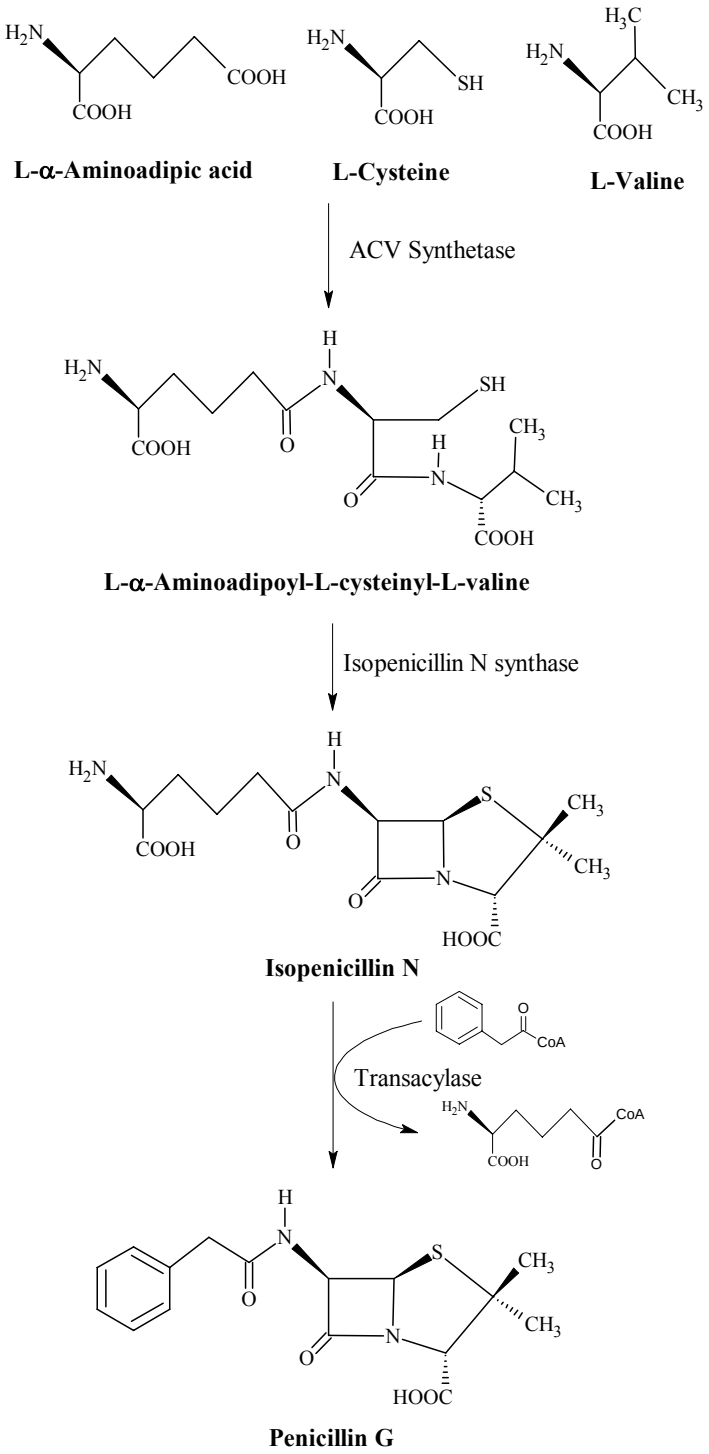


Fig. 14: Biosynthesis of penicillin

Mechanism of action

Penicillin inhibits the cell wall synthesis in growing bacterial cells by combining with the so-called penicillin-binding protein (PBP) of the bacterial cell wall involved in the transpeptidation reaction (cross linking) of the peptide side chains of the adjacent peptidoglycan in the bacterial cell wall. Due to the defective cell wall, the bacteria are unable to withstand the osmotic shocks and are ultimately get lysed.

Strains and production of penicillin

The strain of *Penicillium notatum* discovered by Fleming had low productivity (2 IU/ml). Later in the year 1945 Raper and Alexander found a new strain *Penicillium chrysogenum* having higher yield of penicillin. Further improvement was achieved by inducing mutations via UV irridaition and strain Wis Q 176 was isolated. Presently the strains have been improved much to produce 15000 IU/ml of penicillin during fermentation.

The media used for the production of penicillin contains cornsteep liquor solids 3.5%, lactose 3.5%, glucose 1%, calcium carbonate 1%, potassium dihydrogen phosphate 0.4%, edible oil 0.25% and penicillin precursors. Submerged fermentation processes in 40,000-200,000 liter fermenters with high aeration and agitation is carried out. The pH of the media is maintained around 5.5-6.0 and temperature about 25-27 ºC. Lyophilized spores are used as inoculum to a concentration of 5×10^3/ml medium and development of loose mycelial pellets are required for better production of penicillin. The penicillin fermentation process can be divided into two phases i.e. first is the **growth phase** and the second is the **penicillin production phase**. The growth phase lasts for about 40 h during which the cell mass increases very rapidly (doubling time 6 h) and oxygen demand is very high. After this phase the penicillin production phase starts in which the biomas production is greatly reduced (specific growth rate, $\mu = 0.01$) and rate of penicillin production increases. Various media component are fed during this phase to extend the penicillin production for 120-180 h.

Recovery of penicillin

In the downstream processing of penicillin G, the fermentation broth is first chilled and passed through rotary filter to remove biomass. The filtrate is then acidified and the penicillin is extracted with butyl acetate. Penicillin is extracted from solvent (butyl acetate) into aqueous buffer with pH 7.0. The aqueous fraction is acidified to pH 2.0-2.5 with H_2SO_4 and re-extracted into butyl acetate. Potassium acetate is added to the extract which causes the formation of penicillin GK^+ salt crystals via a number of back-extraction and crystallization steps. The salt so formed is washed with acetone by centrifugation and dried under vaccum.

Streptomycin

Streptomycin is a broad spectrum antibiotic belonging to oligosaccharide antibiotic/aminoglycoside family. Streptomycin was discovered by Schatz, Bugie, and Waksman in 1944 from *Streptomyces griseus* isolated from soil. This antibiotic is mainly active against Gram negative bacteria, *Mycobacterium tuberculosis* (causing agent of tuberculosis) and also inhibits the growth of some gram positive bacteria and is mainly used against the pathogenic bacteria resistant of penicillins. Its major use in the anti-TB drug formulations and is available as

sulphate salt. This antibiotic is also active against other diseases, e.g., plague (*Pasteurella pestis*), brucellosis (*Brucella abortus*), tularemia (*Francicella tularieusis*). This antibiotic interferes with the functioning of 7th cranial nerve and can also cause deafness and kidney damage. Streptomycin is also used in the treatment of plant diseases since this antibiotic is systemic (i.e. transported to all parts of the plant through vascular tissue). Streptomycin has three constituents namely; N-methyl L-glucosamine, Streptose and streptidine (Fig. 15).

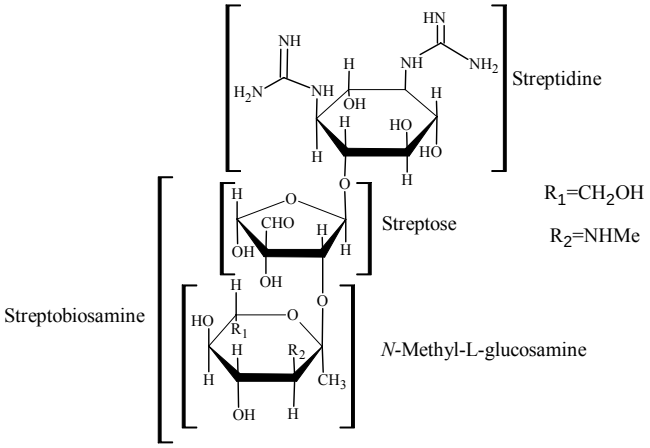


Fig. 15: The streptomycin molecule

Mechanism of action

In sensitive organism the streptomycin binds to the S12 protein of the small subunit of ribosome, which is also the m-RNA binding site during translation. There is competition between the streptomycin and m-RNA for the binding site and thus this antibiotic hinders with the formation of initiation complex as a result the fidelity of the genetic code is not maintained. There is misreading of the genetic code and defective proteins are synthesizing resulting in the death of the microbe.

Production of streptomycin

The present day streptomycin producers are the mutants of *Streptomyces after* screened extensively for higher yields. In the beginning of 1940s, *Streptomyces* strains were poor yielding and only 50 units of streptomycin per ml of broth were produced. New strains developed by mutation and selection yield 25,000 units per ml.

The industrial production of streptomycin is carried out using submerged fermentation processes. The spores of *Streptomyces* mutants are maintained as soil stock or lyophilized form since these strains are genetically unstable. The spores from the stock are used for inoculating sporulation medium, which is then transferred to germinator where biomass is increased for inoculating fermenters. Media consisting of glucose, starch, dextrin, soy meal, corn steep liquor, sodium sulphate are used for the fermentation. The streptomycin fermentation requires high aeration and agitation. The fermentation is carried out at 28-30 ºC and medium pH 7.6-8 for good productivity. The fermentation lasts for 5-7 days with an yield of 1-3 g/L of the fermentation broth.

The streptomycin fermentation proceeds through three phases. In first phase that lasts for 24 h the organism produces proteases which digest the soybean meal releasing ammonia and carbohydrates to be used for increasing the biomass. Glucose utilization is slow and low count ratio of streptomycin is produced during this phase. The medium pH also rises from 6.7 or 6.8 to 7.5 or higher. The next phase is the streptomycin producing phase which ranges from 24 h to 6-7 days. Rapid utilization of ammonia and glucose occurs and substantial production of streptomycin is observed. There is no mycelial growth and pH during this phase remains fairly constant around 7.6 to 8.0. This is the idiophase or the stationary phase during which the streptomycin (secondary metabolite) is produced. In the last phase (death phase) the sugars have been completely depleted in the medium and streptomycin production ceases completely. The ammonia released due to the cell lysis raises medium pH. Fermentation broth is generally harvested before the last phase begins.

Recovery of streptomycin

On completion of fermentation, the mycelium is separated from the broth by filtration and streptomycin is recovered by passing through ion exchange columns where it gets adsorbed. Streptomycin is then eluted from the column as streptomycin sulphate. Further impurities are removed by treating it with sodium hypochlorite, EDTA and activated carbon. The purified streptomycin sulphate solution is concentrated under vacuum and dried aseptically. The ion exchange resin columns have replaced the traditional solvent extraction methods employed for the extraction and purification of streptomycin which significantly reduced the cost of streptomycin production.

Vitamins - Riboflavin, Cyanocobalmin

Vitamins are organic compounds required in small amounts in the diets of animals in order to maintain the normal physiological function of the body. Though vitamins are required in small amounts, the animals are unable to synthesis them. The dietary deficiency of vitamins or its malabsorption results in diseases with characteristic symptoms. The vitamins are the constituent of the coenzymes which are essentially required by the enzymes to catalyze various biochemical reactions. The role of vitamin A in the vision of animals has been clearly demonstrated, vitamin D increases the permeability of the intestinal mucosal cells to calcium ion helping in its absorption. Riboflavin is a precursor of coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) which are hydrogen carriers in biological redox reactions catalyzed by various dehydrogenases. Cyanocobalamin assists the action of methionine synthase and (R)-methylmalonyl-CoA mutase in humans. The vitamins are also essential for proper growth of microorganisms in fermentation processes.

Animal and plant tissues were mainly used for extracting vitamins in the beginning, although dried baker's yeast preparation was also employed as a rich source of B vitamins. Microorganisms have been known to produce vitamins during normal metabolism and are being used for commercial production of various vitamins such as thiamine, riboflavin, folic acid, pyridoxine, cobalamin, biotin, folic acid, L-ascorbic acid, β-carotene, ergosterol and pantothenic acid. Direct fermentation methods are employed for the production of some vitamins, whereas biotransformations or combined chemical and microbiological processes are used for the

production of certain other vitamins. In this section microbial production of riboflavin and cyanocobalamin will be discussed.

Riboflavin

Riboflavin (Vitamin B_2) also known as lactoflavin was first isolated by Kuhn, Gyöygy and Wagner-Jauregg in 1933 from whey. The metabolic importance of riboflavin can be inferred from its role in metabolism, as it is the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) which are essential component of basic cellular metabolism acting as hydrogen carriers in biological redox reactions catalyzed by dehydrogenases. Many microorganisms, plants and fungi are able to synthesize riboflavin. However, vertebrates, including humans lack the biosynthetic pathway for riboflavin and obtain this vitamin from their diet. Dietary riboflavin is present in liver, heart, kidney, egg yolk, milk and meat. The recommended daily requirement of riboflavin is set at 1.3 mg and sufficient amounts of riboflavin need to be ingested regularly as the body is unable to store this water soluble vitamin. The symptoms of riboflavin deficiency (ariboflavinosis) include sore throat, hyperemia, edema of oral and mucous membranes, cheilosis, and glossitis.

The world's annual production of riboflavin is about 2,400 tonnes of which 75% is used as feed additive and the remaining in food and pharmaceutical formulations. This vitamin has intense yellow colour and in small amounts it is used for imparting colour to foods (ice cream, sauces) and as a medical identification aid. The riboflavin is traditionally produced by some chemical processes, but these are being replaced by fermentation based processes using *Eremothecium ashbyii* and *Ashbya gossypii*. Some other potential riboflavin producers of this vitamin are *Candida famata*, *C. flaeri*, *Bacillus subtilis* and *Corynebacterium ammoniagenes*.

Structure of Riboflavin

Ribofalvin (6,7-dimethyl-9-(D-1´-ribityl)-isoalloxazine) is an alloxazine derivative which consists of a pteridine ring condensed to a benzene ring (Fig 16). A side chain of D-ribitol is linked to nitrogen residue at ninth position of the pteridine ring. The nitrogen at 1st and 10th position of isoalloxazine ring participates in the reversible redox system. Oxidized form of flavin is yellow in colour and its reduced form is colourless.

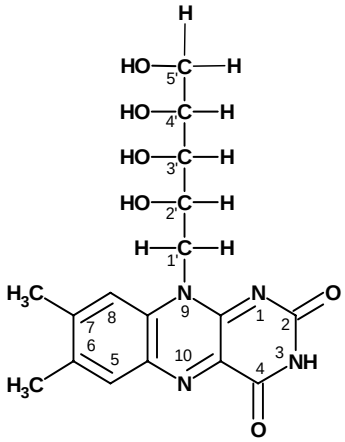


Fig 16: Chemical structure of riboflavin

Biosynthesis of Riboflavin

Riboflavin biosynthesis has been studied in both gram-positive and gram-negative bacteria but it has been worked out in more detail in *B. subtilis* (Perkins and Pero, 2002) and *Escherichia coli* (Bacher *et al.* 1996). It has been well established that the synthesis of this vitamin involves seven enzymes encodes by *rib* operon. Various enzymes and steps involved in the biosynthesis of riboflavin are shown in Fig. 17. The synthesis of one molecule of riboflavin requires one molecule of GTP and two molecule of ribulose-5-phospate. In order to perform its metabolic function, riboflavin is biochemically transformed to the coenzymes FMN and FAD by an essential bifunctional flavokinase/FAD synthetase.

Production Process

Ashbya gossypii NRRL Y-1056 is being currently used for the production of riboflavin. Studies focused on strain improvement and optimization of fermentation process parameters have enhanced the yield to 10-15 g/L. Media containing 2.25% corn steep liquor, 3.5% commercial peptone, 4.5% soy bean oil, supplemented with glycine, distiller's solubles or yeast extract are used for the production of riboflavin. The fermentation is initiated by using small inoculum (0.75-2.0%) of 24-48 h old actively growing culture and the medium pH is maintained around 6.5-7.0. The fermentation is allowed to proceed for 7 days with an aeration of 0.3 vvm at 34-37 ºC.

The fermentation is performed in two stages, in the first stage the organism is allowed to grow and increase its biomass and in second stage riboflavin production is stimulated by restricting the growth or by feeding glucose and inositol along with micronutrients such as iron to obtain high yield of riboflavin. After completion of fermentation, the pH of the fermentation broth is adjusted to 4.5. For feed grade product, the broth is concentrated to about 30% solids and dried on double-drum driers and to obtain crystalline product, the broth is heated for 1 h at 121 ºC to solublize the riboflavin and also release the bound vitamin from the cells. Insoluble matter is removed by centrifugation and the riboflavin so recovered is converted to less soluble form either chemical or by microbiological methods (Riegel and Bissinger, 2003).

Yeasts (*Candida flaeri*, *C. famata*, etc.) and bacteria are also reported to produce riboflavin. Genetically engineered *Bacillus subtilis* (Perkins *et al*., 1999) and *Corynebacterium ammoniagenes* (Koizumi *et al*., 2000) overexpressing genes of the enzymes involved in riboflavin biosynthesis produces 4.5 g/L and 15.3 g/L of riboflavin respectively in the fermentation broth. Some yeasts utilize other carbon sources e.g., *Pichia guilliermondii* (C_{10}-C_{18} aliphatic hydrocarbon), *P. miso* (n-hexadecane, corn steep liquor, urea), *Hansenula polymorpha* (methanol), *Saccharomyces* (acetate) and have exhibited a fairly high potential for the production of this vitamin.

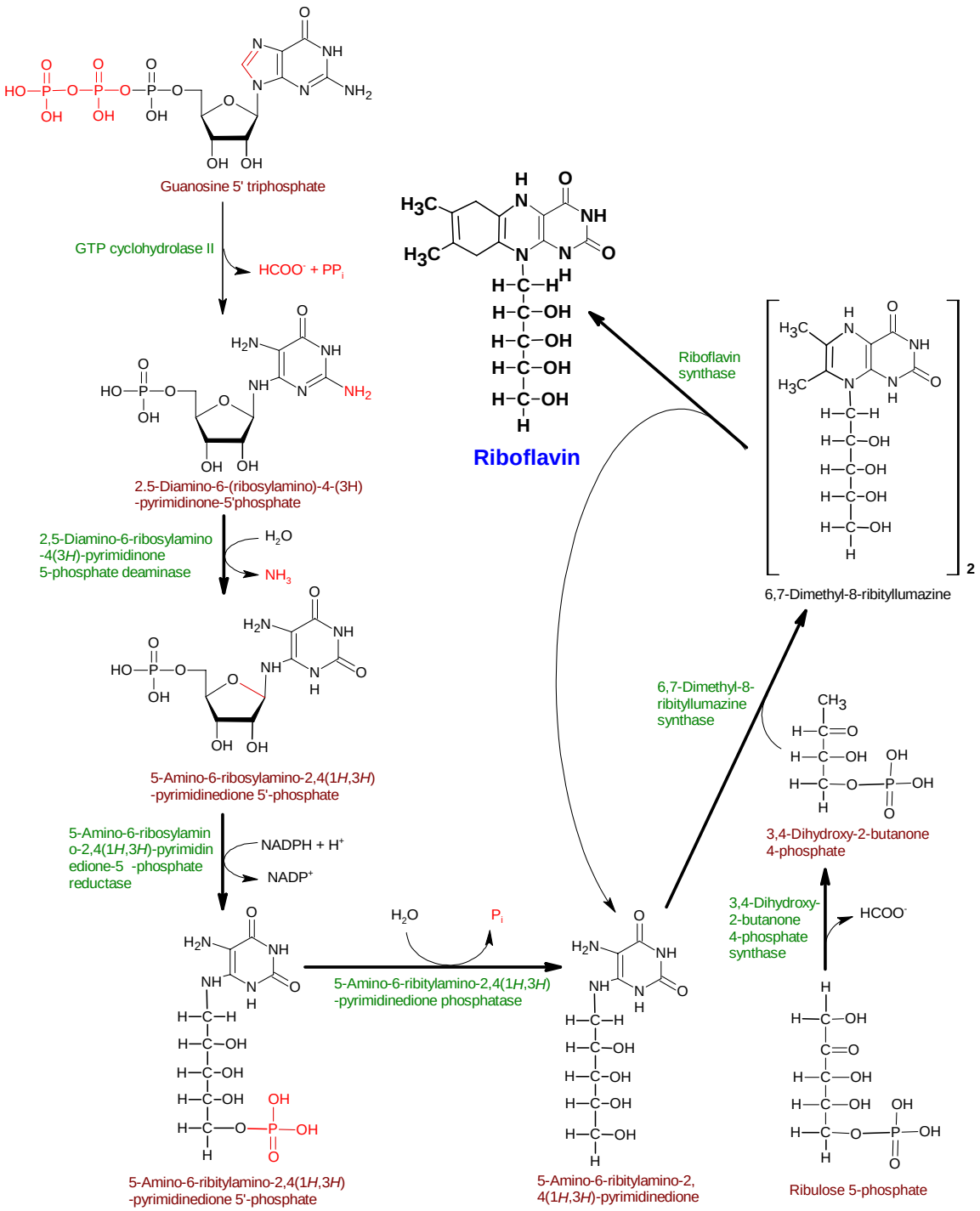


Fig 17: Biosynthesis of riboflavin

Cyanocobalamin

In 1920 G H Whipple was conducting an experiment to induce anemia in dogs by bleeding them and observed that ingestion of raw liver by the dogs cured their anemia. Whipple did not applied his finding to cure human pernicious anemia. Later in 1926, G R Minot and W B Murphy based

on the studies of Whipple used liver extracts to cure human pernicious anemia, and all these three persons shared Nobel Prize in medicine in 1934. The anti pernicious anemia factor from the liver was independently isolated and crystallized by Ricke and coworkers at Merck Laboratories and Smith and Parker at Glaxo Laboratories and named it as cyanocobalamin (vitamin B_{12}). In nature this vitamin is exclusively synthesized by certain prokaryotic microorganisms and it is present at ppm level as adenosyl- or methylcobalamin in every animal tissue (1 ppm in liver).

A specific B_{12} coenzyme synthetase converts vitamin B_{12} (Co^{+2}) to vitamin B_{12} (Co^{+1}) which forms coenzyme of various enzymes catalyzing cleavage of carbon-carbon, carbon-oxygen and carbon-nitrogen bonds and transfer of methyl group. In humans, the vitamin is required in trace amounts (approximately 1 µg/day) to assist the actions of two important enzymes i.e. methionine synthase and (R)-methylmalonyl-CoA mutase. The other biochemical reaction involving vitamin B_{12} occur in bacteria, e.g., methanogenesis. Vitamin B_{12} along with folic acid participates in the shrinking of newly synthesized RBC making them less fragile.

The deficiency of vitamin B_{12} results in low RBC which are larger in size and get damaged quickly. Nerve damage also occurs in the hands and feet and leads to a feeling of pins and needles and numbness. The animals obtain vitamin B_{12} from the food or by absorption of vitamin B_{12} produced by enteric microorganisms. Humans are solely depended on dietary vitamin B_{12} because of non-assimilation of the vitamin B_{12} produced by the microorganism present in large intestinal tract.

Annual production of vitamin B_{12} using fermentation process is more than 10 tonnes from a number of bacterial species. The discovery of vitamin B_{12} in the spent medium of streptomycin and other antibiotic fermentation replaced the use of beef liver for its production. Around 1950 vitamin B_{12} was isolated from antibiotics fermentation broths and dried sewage residue of activated sludge process. Presently this vitamin is being produced by fermentation process using improved bacterial strains. The major use of the vitamin B_{12} is animal feed supplementation in the pharmaceuticals for curing pernicious anemia.

Vitamin B_{12} is chemically related to cobamides consisting of a cobalt porphyrin nucleus to which ribose is linked via phosphate ester bond. In the porphyrin, tetrapyrole ring also referred as corrin ring lacks the methane bridge between rings A and D. Five of the six atoms attached to the cobalt are nitrogen, four of which form the tetrapyrole ring and the fifth one is from the 5,6-dimethylbenzimidazole attached to C-1 of the ribose (Fig 18). The sixth ligand could either be deoxyadenosine, cyanide or a methyl group, forming adenosylcobalamin (coenzyme B_{12}), cyanocobalamin (vitamin B_{12}) and methyl cobalamin respectively.

Biosynthesis

The chronology of the discoveries along the pathway of vitamin B_{12} biosynthesis is reviewed by Scott (2003). Two pathways for the synthesis of vitamin B_{12} have been suggested i.e. one aerobic and another anaerobic which mainly differ in the ring contraction mechanisms converting porphyrin to corrin. The major difference between the two is the timing of cobalt insertion. In anaerobes, cobalt is inserted early in the pathway into precorrin-2 whereas aerobes insert cobalt later in hydrogenobyrinic acid a, c-diamide. All seven methyl groups to the corrin ring are

donated by the alkylating agent S-adenosylmethionine (SAM). Some of the techniques involved in discovering the biosynthetic pathway of vitamin B_{12} included NMR, gene mutation and cloning. The biosynthesis of vitamin B_{12} runs parallel with the biosynthesis of porphyrins and chlorophyll up to the formation of uroporphyrinogen III by succinate-glycine pathway. The uroporphyrinogen III (Urigen III) synthesis starts with the enzyme δ-aminolevulininc acid synthetase (pyridoxal phosphate–dependent) catalyzing the decarboxylation of glycine and then condensing it with succinyl-CoA to produce δ-aminolevulinic acid (ALA). The steps and enzymes involved in the biosynthesis of this vitamin are summarized in Fig. 19.

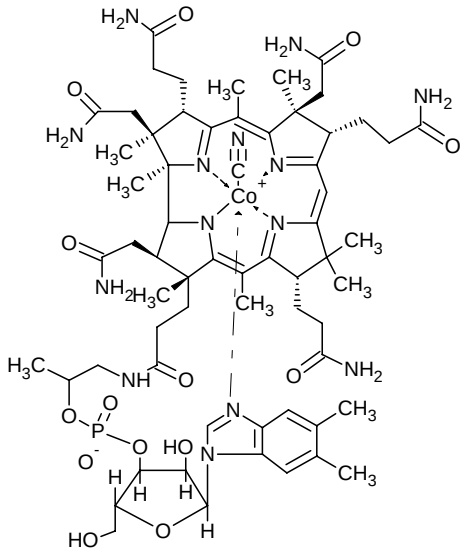


Fig 18: Chemical structure of cyanocobalamin

Production processes

The concentration of vitamin B_{12} is very low in animal tissue and could not be used for commercial production. The activated sludge from the sewage treatment contains 4-10 mg B_{12}/kg has limitations in the separation of various B_{12} froms present in it. The chemical synthesis of this vitamin involves 70 steps which is impractical to apply at commercial scale. Vitamin B_{12} was first commercially produced as by product of various *Streptomyces* fermentations for the production of the antibiotics (streptomycin, chloramphenicol, neomysin) with an yield of about 1 mg/L. The commercial production of vitamin B_{12} is currently based on fermentation processes.

Bacillus megaterium (0.45 mg/L), *Butyribacterium rettgeri* (5 mg/L), *Streptomyces olivaceus* (3.3 mg/L), *Micromonospora* sp. (11.5 mg/L), *Klebsiella pneumoniae* (0.2 mg/L) have been reported to produce vitamin B_{12}. In most of the fermentation processes glucose is used as a carbon source. High yields of vitamin B_{12} from *Propionibacterium freudenreichii* (19 mg/L) and *P. shermanii* (30-40 mg/L) have also been obtained. A strain of *Pseudomonas denitrificans* in a process based on sugarcane molasses yielded 60 mg/L of vitamin B_{12}. *Rhodopseudomonas protamicus* developed by protoplast fusion of *Protaminobacter ruber* and *Rhodopseudomonas spheroides* produces 135 mg/L vitamin B_{12} in the fermentation broth containing glucose as carbon.

A number of new strains able to utilize alcohols and hydrocarbons as carbon source have very good potential to synthesize vitamin B_{12}. Methanol as carbon source has higher yields in comparison to higher alcohol e.g *Methanosarcina barkeri* produces 42 mg/L vitamin B_{12} when methanol is added in fed batch mode.

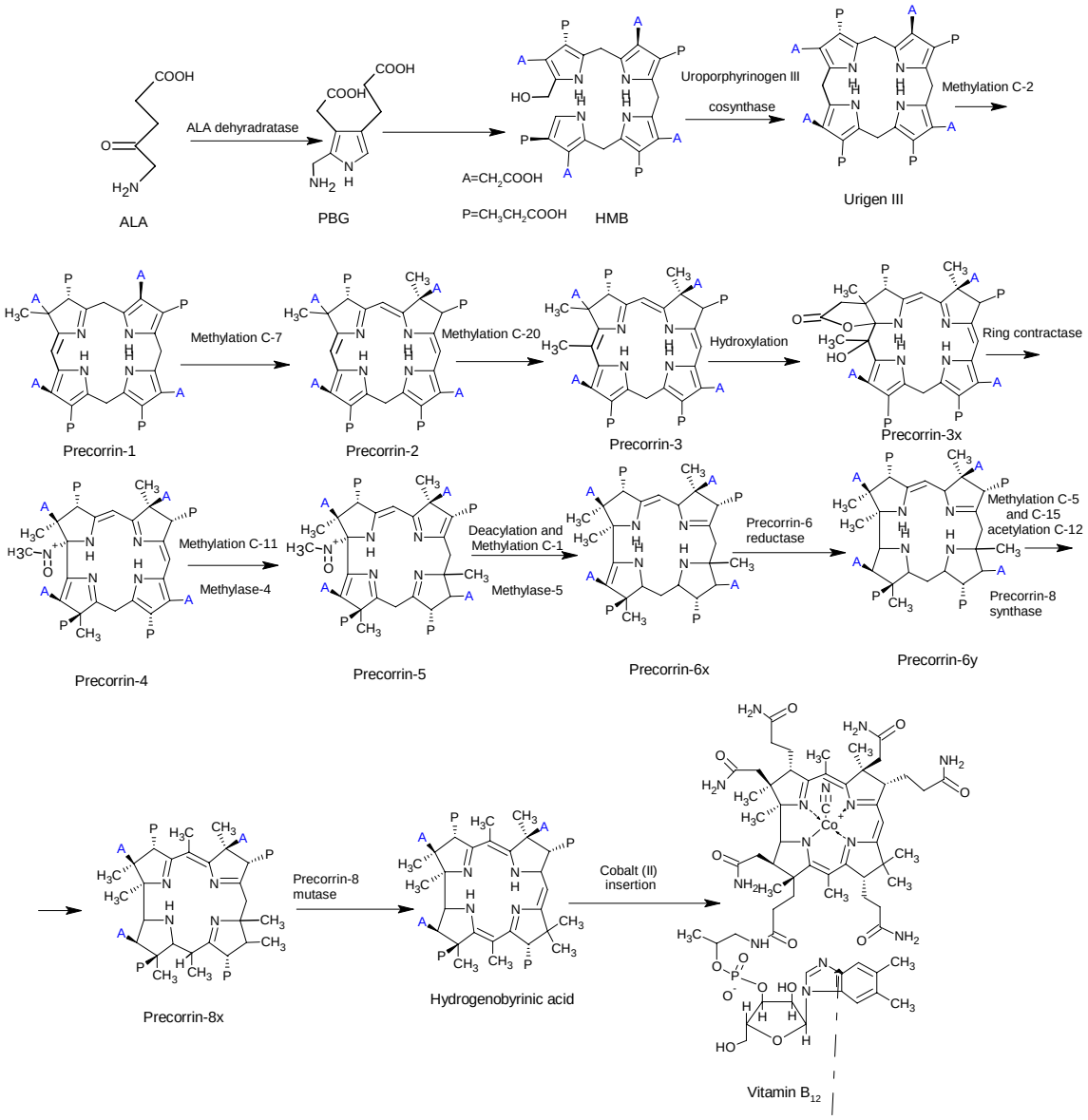


Fig. 19: Pathway for the biosynthesis of Vitamin B_{12} in aerobic bacteria
(Source : Scoot, 2003)

Fermentation using Propionibacterium freudenreichii

Propionibacterium freudenreichii ATCC 6207 and *P. shermanii* ATCC 13673, and some mutants are used for vitamin B_{12} production in a two-stage batch fermentation process to facilitate overproduction. The first stage is the anaerobic one and lasts for 2-4 days during which B_{12} precursor 5′-deoxyadenosylcobinamide accumulates and no vitamin B_{12} is synthesized

preventing its repressive effect on the biosynthesis. The second stage is aerobic one (3-4 days) in which 5,6-dimethylbenimidazole synthesis takes place or it is added to the medium, which finally combines with 5′-deoxyadenosylcobinamide to produce vitamin B_{12} (5′-deoxyadenosylcobalamin). The fermentation medium is supplemented with cobalt (10-100 mg/L). Alternative to batch fermentation, the two stages of vitamin B_{12} fermentations can be performed in a continuous mode with two tanks in a cascade manner.

Fermentation using Pseudomonas denitrificans

Pseudomonas denitrificans is the most productive species among the so far reported pseudomonads producing vitamin B_{12}. The fermentation medium contains 10% sugar beet molasses, 0.2% yeast extract, 0.5% $(NH_4)_2HPO_4$, 0.3% $MgSO_4 \cdot 7H_2O$, 0.02% $MnSO_4 \cdot H_2O$, 0.0188% $Co(NO)_3 \cdot 6H_2O$, 0.0025% 5,6-dimethylbenzimidazole, 0.002% $ZnSO_4 \cdot 7H_2O$, 0.0005% $Na_2MO_4 \cdot 2H_2O$, and fermentation is carried out at 7.4 pH, 420 rpm, 1 vvm aeration and 29 ºC for 90 h (Merck and Co., Inc., 1971). The strain development studies have increased the vitamin B_{12} yield from 0.6 mg/L to 60 mg/L.

Most of the cobalamin synthesized by the microorganism is secreted in the broth but 20% remains bound to cells. The fermentation broth is heated for 10-30 min at 80-120 ºC, pH 6.5-8.5 to solubilize all the vitamin B_{12}. The heated broth is treated with cyanide or thiocyanate to form cyanocobalamin which is separated by cation exchanger (Amberlite IRC 50). It is either extracted with organic solvent (phenol or cresol or in a mixture of benzene, butanol, carbon tetrachloride, or chloroform) or precipitated or crystallized by evaporating the diluents such as cresol or tannic acid containing vitamin B_{12}. In total 98% of the vitamin B_{12} is recovered from the fermentation broth. Vitamin B_{12} having 80% purity is used as feed additive and high purity preparations are used in pharmaceutical formulations.

Single Cell Proteins (SCP)

Single-cell proteins (SCP) are the dried cells of microorganism, which are used as protein supplement in human foods or animal feeds. Microorganisms like algae, fungi, yeast and bacteria, utilize inexpensive feedstocks and wastes as sources of carbon and energy for growth. These waste products can be transformed into biomass, protein concentrate or amino acids by certain microbial proteases.

With increase in population and worldwide protein shortage necessitated the use of microbial biomass as food and feed. These microbial proteins provide an ideal alternative to conventional food source. Although SCP have high nutritive value due to higher protein, vitamin, essential amino acids and lipid content yet these could not replace the conventional protein sources due to their high nucleic acid content and slower in digestibility. These may be considered as foreign material by body, which may subsequently results into allergic reactions. Yeast was the first microorganism whose importance as animal feed supplement was recognized almost a century ago in Institut für Gärungsgewerbe in Berlin. During World War I, Germany replaced half of imported protein sources by yeast. Pruteen was the first commercial single cell protein used as animal feed additive.

Microorganism used for SCP production

Different species of algae, fungi, yeasts and bacteria are used as single cell protein and produced at commercial scale (Table 7). These organism are grown on different carbon sources with some other supplemnts.

Table 7: Microorganism and substrates used for single cell protein production

Microorganism	Substrate
Bacteria	
Aeromonas hydrophylla	Lactose
Achromobacter delvacvate	n-Alkanes
Acinetobactor calcoaceticus	Ethanol
Bacillus megaterium	Non-protein nitrogenous compounds
Bacillus subtilis, Cellulomonas *spp*., Flavobacterium *spp*. Thermomonospora fusca	Celluloses, hemicelluloses
Lactobacillus spp.	Glucose, amylase, maltose
Methylomonas methylotrophus, M. clara	Methanol
Pseudomonas fluorescens	Uric acid and other non-protein nitrogenous compounds
Rhodopseudomonas capsulata	Glucose
Fungi	
Aspergillus fumigatus	Maltose, glucose
Aspergillus niger, *A. oryzae*, *Cephalosporium eichhorniae*, *Chaetomium cellulolyticum*	Celluloses, hemicelluloses
Penicillium cyclopium	Glucose, lactose, glalctose, etc.
Rhizopus chinensis	Glucose, maltose
Scytalidium acidophlium, Trichodrema viridae, T. alba	Cellulose, pentoses
Yeasts	
Amoco torula	Ethanol
Candida tropicalis	Maltose, glucose
Candida utilis	Glucose
Candida novellas	n-Alkanes
Candida intermedia	Lactose
Saccharomyces cerevisiae, S. cerevisiae ellipsoids	Lactose, pentoses, maltose
Algae	
Cholrella pyrenoidosa, C. sorokiana, Chondrous crispus, Scenedesmus *spp.*, Spirulina *spp,* Porphyrium *spp.*	Carbon dioxide through photosynthesis

Process for SCP production using hydrocarbons as substrate

Many genera of yeasts (*Candida tropicalis*, *C. oleophila* and *Saccharomycopsis lipolytica*) and molds utilize aliphatic hydrocarbons (alkanes and alkenes) for growth and for SCP production (Table 8). These microorganisms secrete emulsifying substances during fermentation which increase the solubility of alkanes and alkenes. These substrates are transported by passive diffusion into the cell. The higher lipid content in these microorganisms facilitates the hydrocarbon transport. Alkanes are catabolized by two mechanisms i.e. by terminal oxidation or subterminal oxidation. During terminal oxidation carboxylic acid is produced which is further metabolized either to acetate by β-oxidation or to acetate and succinate by ω-oxidation. The subterminal oxidation can occur interior of the molecule. During catabolism, ketone is first formed which is further converted to acetate and propionate by α-oxidation, subsequent decarboxylation followed by β-oxidation. For large-scale production of SCP, gas oil and C_{10}-C_{17} alkanes were used as substrate. Gas oil fermentation has been carried out in airlift fermenter using *Candida tropicalis* and *C. lipolytica.*

Table 8: Hydrocarbon utilizing microorganisms

Hydrocarbons	**Yeast**	**Filamentous Fungi**
Parafins (alkanes)	*Candida* *Hansenula* *Pichia* *Rhodotorula* *Saccharomyces* *Torulopsis* *Trichosporon*	*Aspergillus* *Cephalosporium* *Fusarium* *Spicaria* *Cunninghamella* *Monilia* *Mucor* *Paecilomyces* *Penicillium* *Rhizopus* *Trichoderma*
Olefins (alkenes)	*Candida* *Debaryomyces* *Hansenula* *Rhodotorula*	*Aspergillus* *Cephalosporium* *Fusarium* *Spicaria*

SCP production from methanol fermentation

Methanol utilizing organisms (bacteria, yeast and fungi) (Table 9) use methanol as an important substrate for SCP production (Fig 20). In methanol fermentation for SCP production, bacteria are preferred over fungi because of their fast growth, high protein content and better yield. Imperial Chemical Industries Company (ICI) first developed commercial continuous methanol fermentation process for SCP production and introduced this product in the market under the trade name **Pruteen**. Fermentation is carried in ICI process using *Pseudomonas methylotrophus* at pH 6.5-6.9 and 34-37 ºC.

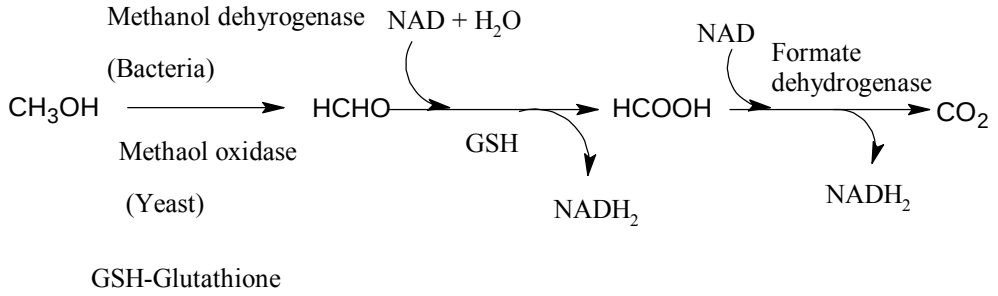


Fig. 20: Methanol oxidation

Table 9: Methanol oxidizing microorganism

Obligate methylotrophic organisms	**Facultative methylotrophic organisms**		
	Bacteria	**Yeast**	**Fungi**
Methylobacter	*Arthrobacter*	*Candida*	*Gliocladium delinquescens*
Methylococcus	*Bacillus*	*Hansenula*	*Paecilomyces varioti*
Methylomonas	*Hyphomicrobium*	*Pichia*	*Trichoderma lignorum*
Methylocystis	*Klebsiella*	*Torulopsis*	
Methylosinus	*Micrococcus*		
	Protaminobacter		
	Pseudomonas		
	Rhodopseudomonas		
	Streptomyces		
	Vibrio		

In downstream processing of SCP, cells are partially lysed by heat and acid treatment and broth is clarified. Cells are spray dried and liquid is recycled into the fermenter. However the cost of production of SCP from methanol in 1984 (600$) was 3 times higher than soymeal (125-190$) and as a result such programs were not carried further.

SCP production from other substrates

Cellulose has emerged as an attractive substrate for SCP production but in nature it is usually found with lignin, hemicellulose, starch, etc. in a complex form. Therefore, if cellulose is to be used as substrate it must be pretreated chemically (acid hydrolysis) or enzymatically (cellulases) to remove cellulose as fermentable sugars. Extracellular cellulases are commercially used in such

process. Cellulasse is a complex of three enzymes endo-β-1, 4-glucanase (also known as endocellulase /carboxymethyl cellulase/ C_x cellulase), exo-β-1, 4-glucanase (also called cellobiohydrolase/ avicelase/ C_1 cellulase) and β-1, 4-glucosidase (cellobiase). The cost of production of these extracellulases is about 0.011$/liter of crude enzyme and 0.11$/liter of purified enzyme. The cost of extraction of fermentable sugar using cellulase is about 0.50$/kg sugar. A number of efficient cellulase producers have been reported but *Trichoderma viride* continued to be well known high cellulase-producing organism. *Chaetomium cellulolyticum* is another cellulolytic fungus which grows faster and forms 80% more biomass-protein than *Trichoderma.* This means that *C. cellulolyticum* is suitable for single-cell protein (SCP) production while *T. viride* is a hyper producer of extracellular cellulases. The amino acid composition of *C. cellulolyticum* is generally better than that of *T. viride* and similar to alfalfa and soya meal protein. Preliminary trials have shown no adverse effects of the SCP produced by *C. cellulolyticum* fermentations.

Mycoprotein were also introduced in market in England in 1986. The fungus *Fusarium graminearum* was grown in a defined medium. The dried mycoprotein resemble chicken, pork and beef meat in its flavour. The cost of production will ultimately determine the fate of SCP in human or animal diet.

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