قسم التقنيات الاحيائية

مادة: التقنيات الصناعية

المرحلة الرابعة

الكورس الثاني

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Lec-1

Industrial Biotechnology

- **An introduction**

Industrial biotechnology, also known as white biotechnology, ***is the modern use and application of biotechnology for the sustainable processing and production of chemicals, materials and fuels from renewable sources, using living cells and/or their enzymes***.

This field is widely regarded as the third wave of biotechnology, distinct from the first two waves (medical or red biotechnology and agricultural or green biotechnology).

Biotechnological processing uses enzymes and microorganisms or plant/animal cells to make products in a wide range of industrial sectors including chemicals, pharmaceuticals, food & feed, detergents, pulp & paper, textiles, energy, materials and polymers.

With the advancement of new technologies and sciences such as enzyme engineering, metabolic engineering, synthetic biology and the expanding "omics" toolbox, that led to much deeper understanding of cell metabolism and material sciences, many new opportunities have been identified and other are continuing to emerge. These advances have provided scientists with toolsets to engineer enzymes and whole cells by expanding the means to identify, understand and make perturbations to the complex machinery within the microorganisms.

Much interest has been generated in industrial biotechnology mainly because of this field is associated with reduced energy consumption, greenhouse gas emissions and waste generation. ***Bioprocess Technology*** is the sub-discipline within industrial biotechnology that combines living matter, in the form of organisms or enzymes, with nutrients under specific optimal conditions to make a desired product. It is responsible for translating discoveries of life sciences into practical and industrial products, processes and techniques that can serve the needs of society. Bioprocess Technology is thus the backbone of the biotechnology that translates the research and development to the industries.

Bioprocess Technology: *make use of microbial, animal and plant cells and components of cells such as enzymes to manufacture new products and destroy harmful wastes.*

Stages of bioprocess

The entire bioprocess can be divided in two stages:

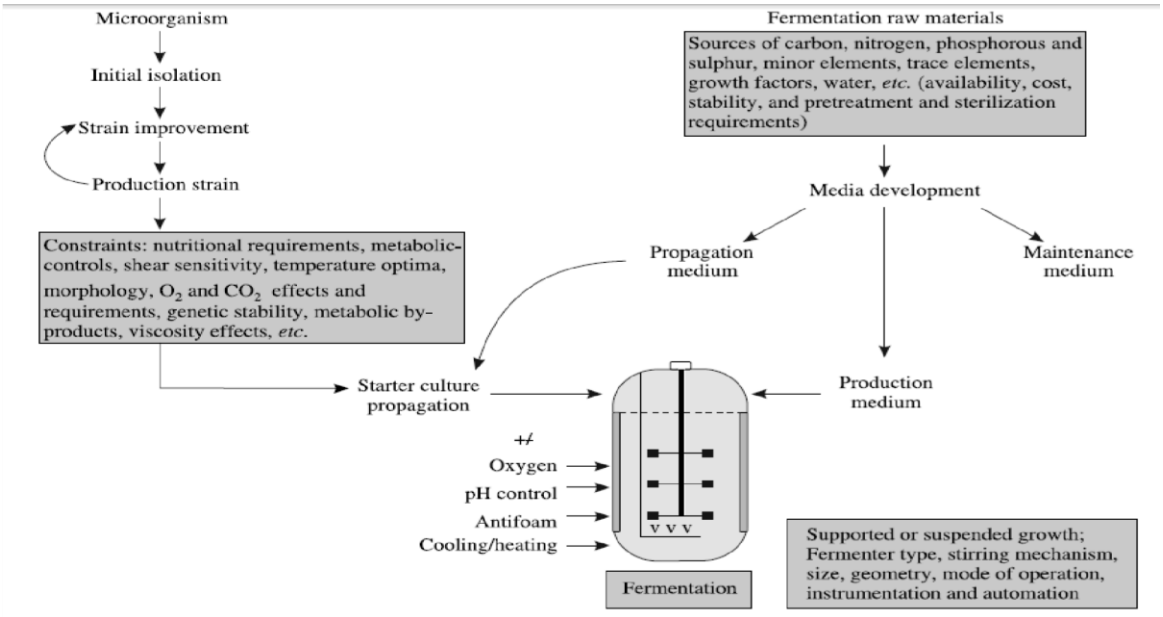
Stage I: Upstream processes (USP):

Refer to the culturing of cells and microorganisms to create the bulk bio-product. This processing is typically done through cell culture or fermentation. This stage includes:

- **Selection of a microbial strain** characterized by the ability to synthesize a specific product having the desired commercial value. This strain then is subjected to improvement protocols to maximize the ability of the strain to synthesize economical amounts of the product.
- **Fermentation** which involves the conversion of substrates to desired product with the help of biological agents such as microorganisms. Fermentation process is carried out in large tanks known as fermenters or bioreactors.

Upstream processing involves Techniques for large-scale production of microbial products. It must both provide an optimum environment for the microbial synthesis of the desired product and be economically feasible on a large scale.

Upstream processing involves all the factors and processes leading to fermentation as well as including the fermentation as shown in the following figure:



Stage II: Downstream processing:

Which involves separation of cells from the fermentation broth, purification and concentration of desired product and waste disposal or recycle.

Downstream processing, the various stages that follow the fermentation process, involves suitable techniques and methods for recovery, purification, and characterization of the desired fermentation product. A vast array of methods for downstream processing, such as centrifugation, filtration, and chromatography, may be applied. These methods vary according to the chemical and physical nature, as well as the desired grade, of the final product.

Improvement of industrial strains

Natural isolates usually produce commercially important products in very low concentrations and therefore huge attempts are made to increase the productivity of the chosen strains via different improvement strategies.

Strain improvement referred to "**the science and technology of manipulating and improving microbial strains in order to enhance their metabolic capacities for biotechnological applications".**

- Purpose of strain improvement:
  1. Increase productivities of the desired metabolite
  2. To change unused co-metabolites
  3. To improve the use of carbon and nitrogen sources
  4. To improve morphology of cells to be a better cells in order to separate the cells and its products.

❖ **Strain improvement may be achieved by**:

1- Optimizing the culture medium and growth 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. 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. However, this approach is limited by the organism's maximum ability to synthesize the product.

Example:

- 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.

2- Genetic modification

Generally wild strains are not suitable for industrial fermentation because of low yield of the metabolite. The potential productivity of the organism is controlled by its genome and, therefore, genetic improvement of the industrial strains becomes essential for overproduction of a particular metabolite.

Genetic modification may be achieved by:

I. Random mutagenesis and screening methods
II. Recombinant DNA technology

I. **Random mutagenesis and screening methods**

This is the most important microbial strain improvement technique. Large numbers of improved strains which are currently available are produced by induced mutations. This process involves subjecting the microorganisms to mutagens and then screening the mutated microbes for increased productivity and finally selecting these microbial strains. Induced mutation does not always produce useful strains so it is vital to select the strains which are of interest.

Mutation can be carried out with physical mutagens like UV-radiation or chemical mutagens like N-methyl-N-nitrosoguanidine or ethyl methanessulphonate. This empirical approach has a long history of success.

✶ **The advantages of mutation/selection** are:

1. Simplicity, since it requires little knowledge of the genetics, biochemistry and physiology of the product biosynthetic pathway.
2. It does not need sophisticated equipment and requires minimal specialized technical manipulation.
3. Effectiveness, since it leads to rapid titer increases.

✶ **Drawbacks of this strategy are**:

1. Labour intensive.
2. The possibility of unwanted mutations.

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Improvement of industrial strains

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II. 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. 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.

❖ **The selection of induced mutants synthesizing improved levels of primary metabolites**

Before considering the methods used for the selection of mutants producing improved levels of primary metabolites, it is necessary to study the mechanisms of control of their biosynthesis. The levels of primary metabolites in micro-organisms are regulated by feedback control systems. The major systems involved are feedback inhibition and feedback repression.

"Feedback inhibition is the situation where the end product of a biochemical pathway inhibits the activity of an enzyme catalysing one of the reactions (normally the first reaction) of the pathway".

Inhibition acts by the end product binding to the enzyme at an allosteric site which results in interference with the attachment of the enzyme to its substrate.

"Feedback repression is the situation where the end product (or a derivative of the end product) of a biochemical pathway prevents the synthesis of an enzyme (or enzymes) catalysing a reaction (or reactions) of the pathway".

Repression occurs at the gene level by a derivative of the end product combining with the genome in such a way as to prevent the transcription of the gene into messenger RNA, thus resulting in the prevention of enzyme synthesis.

For overproduction of primary metabolites, feedback inhibition or repression by the end product of a particular pathway is suppressed by generation of auxotrophs i.e. mutation to cause accumulation of metabolite of interest.

Auxotrophs: "An organism, such as a strain of bacteria, that has lost the ability to synthesize certain substances required for its growth and metabolism as the result of mutational changes"

- The control of pathways giving rise to only one product (**unbranched pathways)** is normally achieved by the first enzyme in the sequence being susceptible to inhibition by the end product and the synthesis of all the enzymes being susceptible to repression by the end product, as shown in Figure 1.

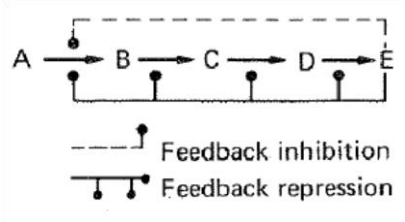

Figure 1: The control of a biosynthetic pathway converting precursor A to end product E via the intermediates B, C and D

- The control of biosynthetic pathways giving rise to a number of end products **(branched pathways)** is more complex than the control of simple, unbranched sequences. The end products of the same, branched biosynthetic pathway are rarely required by the microorganism to the same extent, so that if an end product exerts control over a part of the pathway common to two, or more, end products then the organism may suffer deprivation of the products not participating in the control. Thus, mechanisms have evolved which enable the level of end products of branched pathways to be controlled without depriving the cell of essential intermediates.

  In the following, a description of the main mechanisms that control the level of end products in the branched pathways based on the effect of feedback inhibition, repression or a combination of both systems:

1- Concerted or multivalent feedback control: This control system involves the control of the pathway by more than one end product — the first enzyme of the pathway is inhibited or repressed only when all end products are in excess, (Figure 2).

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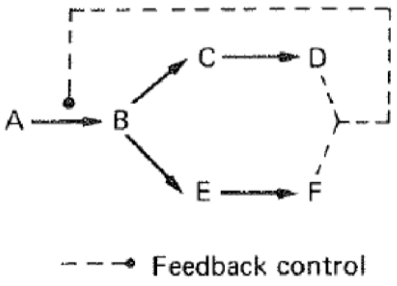
Figure: The control of a biosynthetic pathway by the concerted effects of products D and F on the first enzyme of the pathway.



2- Co-operative feedback control: The system is similar to concerted control except that weak control may be effected by each end product independently. Thus, the presence of all end products in excess results in a synergistic repression or inhibition. The system is illustrated in Figure 3 and it may be seen that for efficient control to occur when one product is in excess there should be a further control operational immediately after the branch point to the excess product. Thus, the reduced flow of intermediates will be diverted to the product which is still required

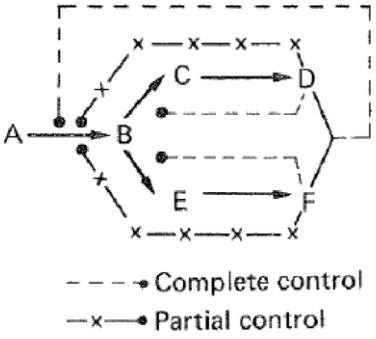


Figure 3: The control of a biosynthetic pathway by the co-operative control by end products D and F.

3- Cumulative feedback control: Each of the end products of the pathway inhibits the first enzyme by a certain percentage independently of the other end products. In Figure 3, both D and F independently reduce the activity of the first enzyme by 50%, resulting in total inhibition when both products are in excess. As in the case of co-operative control, each end product must exert control immediately after the branch point so that the common intermediate, B, is diverted away from the pathway of the product in excess

50% ----<> Inhibition of 50% of the activity of the enzyme

--- - --. Total inhibition of enzyme activity

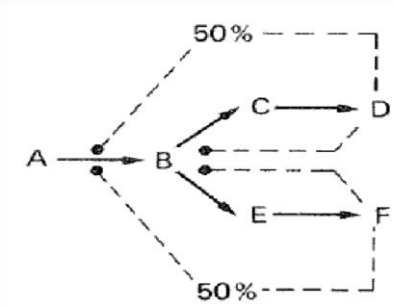
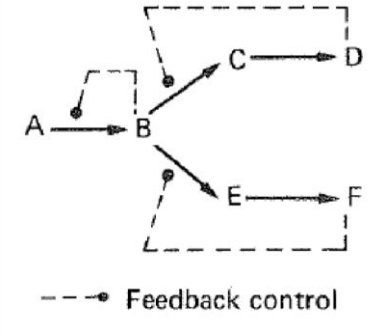
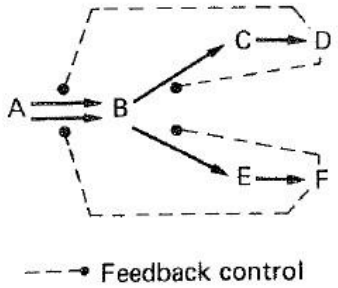


Figure 4: The control of a biosynthetic pathway by the cumulative control of products D and F.

4- Sequential feedback control: Each end product of the pathway controls the enzyme immediately after the branch point to the product. The intermediates which then build up as a result of this control earlier enzymes in the pathway. Thus, in Figure 4, D inhibits the conversion of B to C, and F inhibits the conversion of B to E. The inhibitory action of D, F, or both, would result in an accumulation of B which, in turn, would inhibit the conversion of A to B



5- Isoenzyme control: Isoenzymes are enzymes which catalyse the same reaction but differ in their control characteristics. Thus, if a critical control reaction of a pathway is catalysed by more than one isoenzyme, then the different isoenzymes may be controlled by the different end products. Such a control system should be very efficient, provided that control exists immediately after the branch point so that the reduced flow of intermediates is diverted away from the product in excess. An example of the system is shown in the following Figure (catalysing the conversion of A to B by end products D and F).



The levels of microbial metabolites may be controlled by a variety of mechanisms, such that end products are synthesized in amounts not greater than those required for growth. However, the ideal industrial micro-organism should produce amounts far greater than those required for growth and an understanding of the control of production of a metabolite may enable the construction of the most useful industrial mutant. Such postulated **mutants may be modified in three ways:**

1. The organism may be modified such that the end products which control the key enzymes of the pathway are lost from the cell due to some abnormality in the permeability of the cell membrane.
2. The organism may be modified such that it does not produce the end products which control the key enzymes of the pathway.
3. The organism may be modified such that it does not recognize the presence of inhibiting or repressing levels of the normal control metabolites.

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Improvement of industrial strains

- **The isolation of mutants which do not produce feedback inhibitors**

Mutants which do not produce certain feedback inhibitors or repressors may be useful for the production of intermediates of unbranched pathways, and intermediates and end products of branched pathways. Following are several hypothetical mutants producing intermediates and end products of biosynthetic pathways and these are illustrated in the following Figure.

In Figure 1, the unbranched pathway is normally controlled by feedback inhibition or repression of the first enzyme of the pathway by the end product E. However, the organism represented in the Figure is auxotrophic for E due to the inability to convert C to D so that control of the pathway is lifted and C will be accumulated provided that E is included in the medium at a level sufficient to maintain growth but insufficient to cause inhibition or repression.

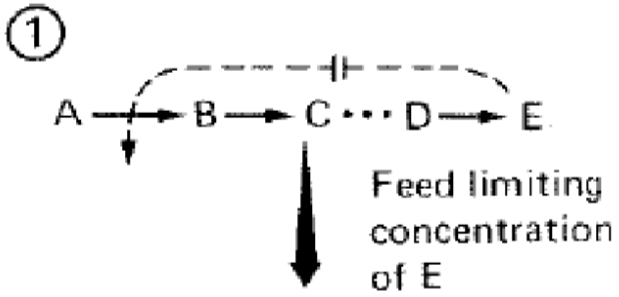
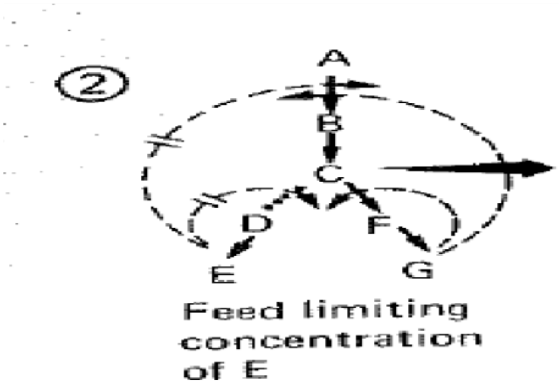


Figure 2

is a branched pathway controlled by the concerted inhibition of the first enzyme in the pathway by the combined effects of E and G. The mutant illustrated is auxotrophic for E due to an inability to convert C to D, resulting in the removal of the concerted control of the first enzyme. Provided that E is included in the medium at a level sufficient to allow growth but insufficient to cause inhibition then C will be accumulated due to the control of the end product G on the conversion of C to F.

Figure 3 is similar to that in Figure 2 except that it is a double auxotroph and requires the feeding of both E and G.



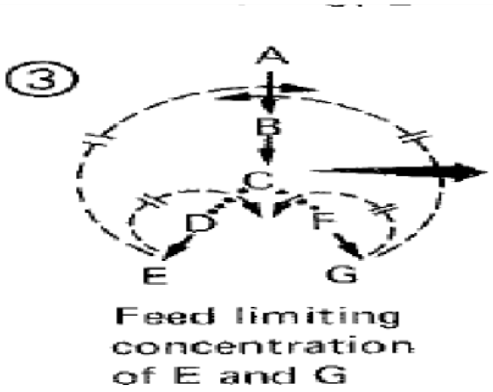


Figure 4 is again, the same pathway and illustrates another double mutant with the deletion for the production of G occurring between F and G, resulting in the accumulation of F.

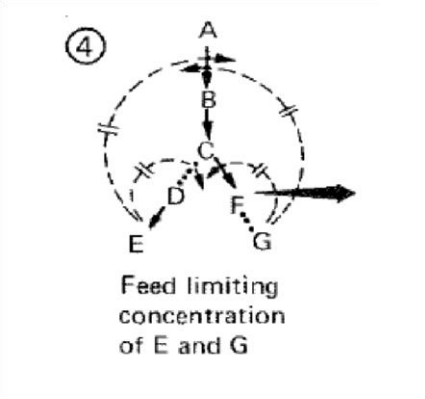
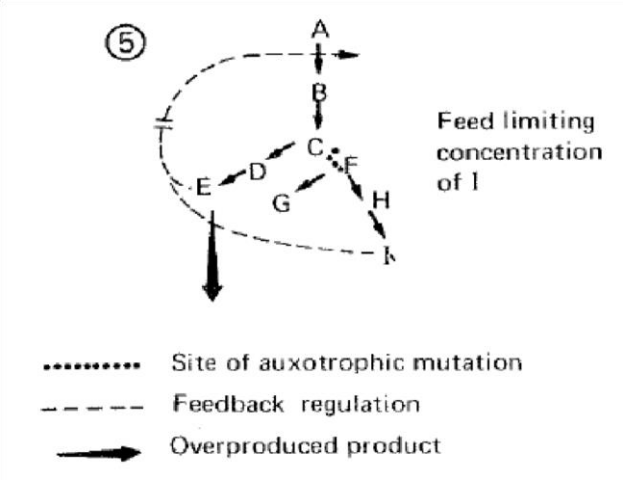


Figure 5 illustrates the accumulation of an end product of a branched pathway which is normally controlled by the feedback inhibition of the first enzyme in the pathway by the concerted effects of E and I. The mutant illustrated is auxotrophic for I and G due to an inability to convert C to F and, thus, provided G and I are supplied in quantities which will satisfy growth requirements without causing inhibition, the end product, E, will be accumulated



The mutants illustrated in the previous Figures do not produce some of the inhibitors or repressors of the pathways considered and, thus, the control of the pathway is lifted, but, because the control factors are also essential for growth, they must be incorporated into the medium at concentrations which will allow growth to proceed but will not evoke the normal control reactions.

All the hypothetical examples discussed above are **auxotrophic mutants** and, under certain circumstances, may accumulate relatively high concentrations of intermediates or end products. Therefore, the isolation of auxotrophic mutants may result in the isolation of high-producing strains, provided that the mutation for auxotrophy occurs at the correct site, e.g. between C and D in Figure (1) and (2).

- **Exercises 1:** consider the following simple pathway:

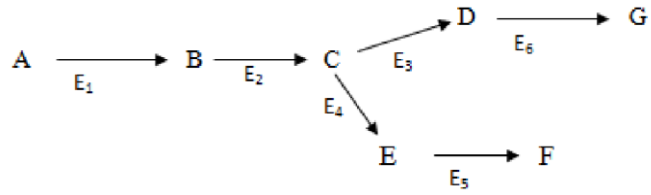
A——— B ——— C _____ D

It was found that:

- No growth or production of D if you supply A or B;
- Growth and production of D if you supply C;

Which step is blocked?

The true answer is: the blocked step is ~~B~~ C



- **Exercise 2:** Assume that the control of the following pathway is a sequential feedback control: Answer **TRUE or FALSE** for each of the following:

1- Synthesis of product G could be stimulated by altering the gene that codes for enzyme 4, so that allosteric site of the enzyme becomes insensitive to interaction with product F.

2- Synthesis of products G and F could be depressed by altering the gene that codes for enzyme 1, so that the allostric site of the enzyme becomes insensitive.

3- Synthesis of products F could be stimulated by adding excess of G to the fermentation medium.

4- Synthesis of product G could be stimulated by altering the gene that codes for enzyme 3, so that the allostric site of the enzyme becomes insensitive.

5- Synthesis of product F could be depressed and synthesis of products G could be stimulated by altering the gene that codes for enzyme 4, so that the active site of the enzyme becomes altered and the enzyme rendered inactive.

True answers: 1. FALSE, 2. FALSE, 3. FALSE, 4. TRUE, 5. FALSE

Lec-4

The recovery of auxotrophs mutants

The most commonly used methods for the recovery of auxotrophic mutants are:

1. The use of enrichment culture.
2. The use of a technique to visually identify the mutants.

…….

1. The use of enrichment culture.

The enrichment processes employed are based on the provision of conditions which adversely affect the prototrophic cells but do not damage the auxotrophs. Such conditions may be achieved by exposing the population, in minimal medium, to an antimicrobial agent which only affects dividing cells which should result in the death of the growing prototrophs but the survival of the non-growing auxotrophs. Several techniques have been developed using different antimicrobials suitable for use with a range of micro-organisms.

An enrichment technique was developed to utilize penicillin as an inhibitory agent. The survivors of a mutation treatment were first cultured in complete medium, harvested by centrifugation, washed and resuspended in minimal medium plus penicillin. Only the growing prototrophic cells were susceptible to the penicillin and the non-growing auxotrophs survived. The cells were harvested by centrifugation, washed (to remove the penicillin and products released from lysed cells) and resuspended in complete medium to allow the growth of the auxotrophs, which could then be purified on solid medium. The nature of the auxotrophs isolated may be determined by the design of the so-called complete medium; if only one addition is made to the minimal medium then mutants auxotrophic for the additive should be isolated.

Example: The use of the penicillin selection method for the isolation of auxotrophic mutants of *Corynebacterium glutamicum*

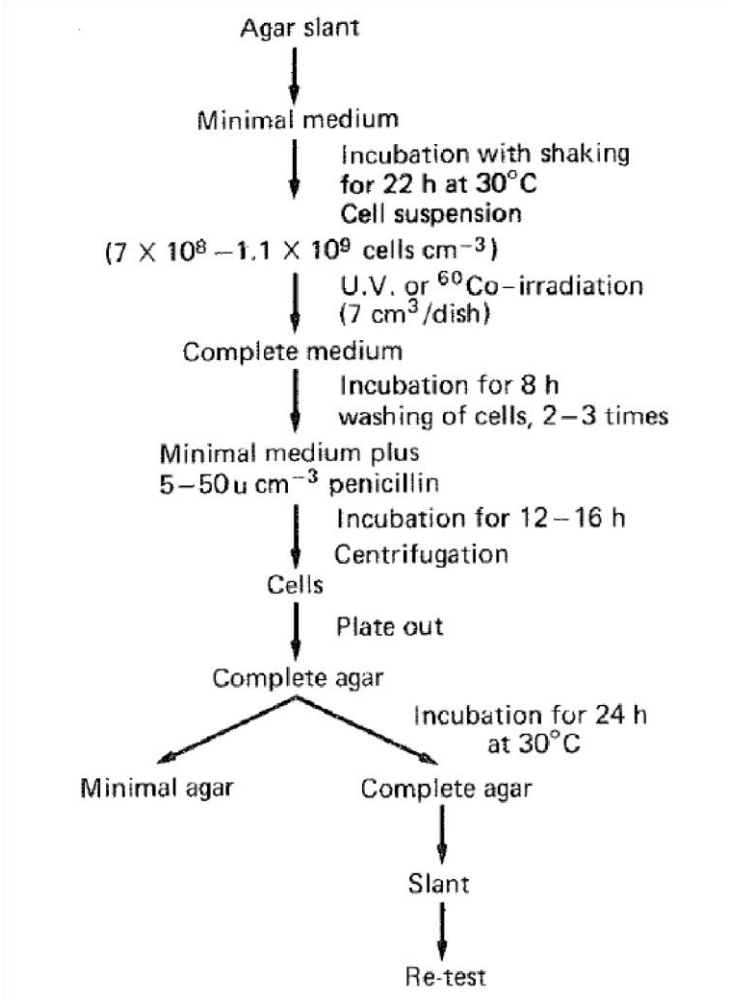


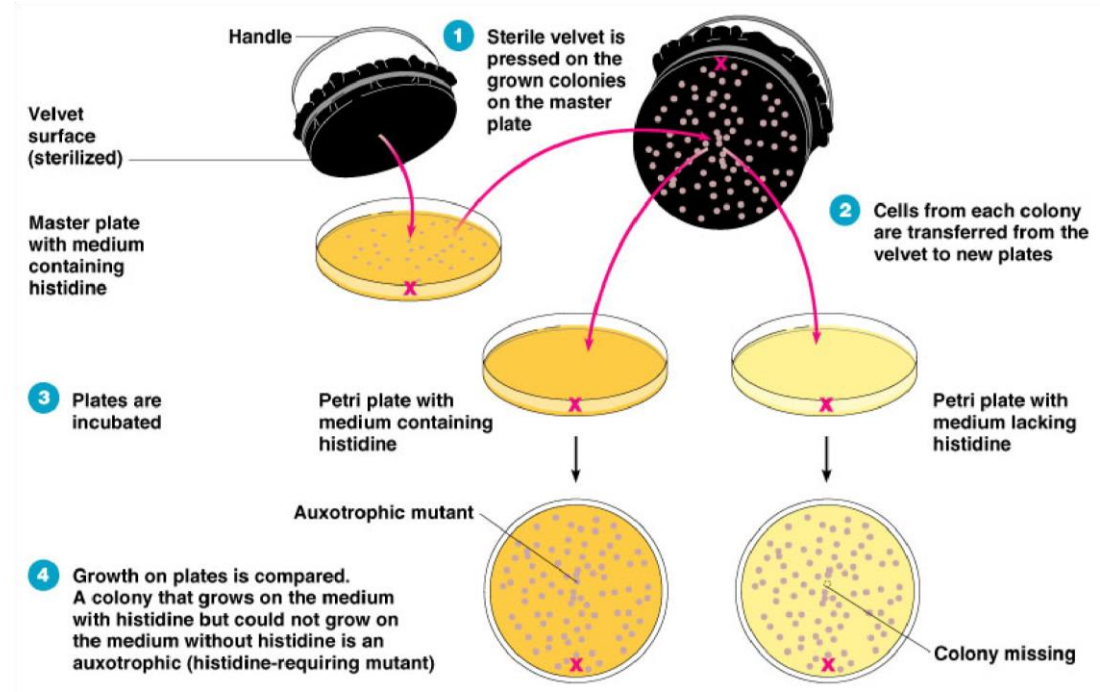
Figure: The use of the penicillin selection method for the isolation of auxotrophic mutants of C. glutamicum

2. The use of a technique to visually identify the mutants.

The visual identification of auxotrophs is based on the alternating exposure of suspected colonies to supplemented and minimal media. Colonies which grow on supplemented media, but not on minimal, are auxotrophic. The alternating exposure of colonies to supplemented and minimal medium has been achieved by replica plating. The technique consists of allowing the survivors of a mutation treatment to develop colonies on petri dishes of supplemented medium and then transferring a portion of each colony to minimal medium. The transfer process may be 'mechanized' by using some form of replicator. For bacteria the replicator is a sterile velvet pad attached to a circular support and replication is achieved by inverting the petri dish on to the pad, thus leaving an imprint of the colonies on the pad which may be used to inoculate new plates by pressing the plates on to the pad. It may be possible to replicate fungal and streptomycete cultures

using a velvet pad, but, if unsatisfactory results are obtained, a steel pin replicator may be more appropriate

Example: Isolation of histidine-auxotrophic mutant



The isolation of induced mutants producing improved yields of secondary metabolites

Microorganisms that produce secondary metabolites exhibit two phases during batch culture, the **trophophase** and **idiophase**. Trophophase is the growth phase of a culture and idiophase is the following period when the secondary metabolites are formed. Production frequently begins when active growth has ceased. The success of any further biosynthesis in the idiophase is dependent on the preceding trophophase.

Secondary metabolism during the idiophase utilizes primary metabolites to produce speciesspecific and chemically diverse end-products that are not essential for growth of the microorganism. Their production involves metabolic pathways which are not used during the

growth phase, at least not during exponential growth. These pathways are often not as well characterized as those for primary metabolism.

Much energy can be expended in the production of secondary metabolites, However, many of these products appear to have no recognizable metabolic function for the producing organism. Though, some compounds may perform specific roles for their producer organism, for example inhibiting competing organisms and others such as gramicidins are associated with the promotion of spore formation. Fortuitously, many secondary metabolites exhibit properties that have proved very useful and have become major industrial microbial products.

It is necessary to study the mechanisms of control of secondary metabolite biosynthesis

Secondary metabolism and its control

The synthesis of secondary metabolites is usually tightly regulated by the cell. Some regulatory mechanisms are common to both primary and secondary metabolism, control of secondary metabolism is provided by the following strategies:

1. Carbon catabolite repression.

Sources that support high growth rates tend to be repressive; for example, glucose suppresses the production of several antibiotics including penicillin and chloramphenicol. **2. Autoregulation**

This may involve small molecules that appear to function as hormone-like autoregulatory compounds that stimulate the formation of secondary metabolites.

Example, factor A which is produced by *Streptomyces* species, controls streptomycin biosynthesis, streptomycin resistance and sporulation, and is effective at concentrations as low as 10^{-9} mol/L.

3. End-product regulation.

As in primary metabolism, end-products may play a role in the feedback inhibition of certain secondary metabolic pathways. For example, the antibiotic chloramphenicol inhibits aryl amine synthatase, an enzyme involved in chloramphenicol biosynthesis.

4. Inducible effects

Production of several antibiotics is stimulated by the addition of inducer compounds to the fermentation medium. Many inducers are primary metabolites; for example, the amino acid methionine induces cephalosporin production in *Acremonium* (*Cephalosporium*) *chrysogenum.* The alkaloid ergoline, which is produced by *Claviceps purpurea*, is stimulated by the addition of l-tryptophan or tryptophan analogues to the culture media during the exponential phase of growth.

5. Nitrogen and phosphate regulation

Readily metabolizable sources of nitrogen, e.g. ammonia, are often seen to inhibit the production of secondary metabolites such as penicillin. Also, formation of candicidin, streptomycin and tetracycline is inhibited by phosphate concentrations in excess of 10 mmol/L. Usually a maximum level of 1 mmol/L phosphate is necessary to ensure that inhibition does not occur. This phenomenon may be associated with changes in EC, which has been shown to be directly influenced by the rate of phosphate flux into some cells. A high rate of flux increases ATP formation and raises the overall EC. In certain cases, the ATP may act as a corepressor in the synthesis of key enzymes involved in the biosynthesis of secondary metabolites.

✶ **Improvement of secondary metabolites:**

In order to obtain high yields of secondary metabolites from industrial fermentations, environmental conditions that elicit these regulatory mechanisms, particularly repression and feedback inhibition, must be avoided. If potentially suppressive nutrients are used, they must be provided at low sub suppressive rates. In addition, overproducing mutant strains are often developed for these processes.

Lec-5

Products of industrial biotechnology

Biotechnology can both replace existing chemical processes and allow the production of new products. Already, there are many products of biotechnology in various areas like food chemicals, pharmaceuticals, detergents, pulp & paper, textiles, energy, materials and polymers, *etc.* The high specificity and mild reaction conditions of enzymes and cellular processes provide products of good quality and have process efficiency advantages. Some of the major biotechnological products that resulted from exploiting enzymes and microbes which have a huge industrial significance will be discussed in the following lectures.

Microbial products

There are five major groups of commercially important microbial products:

1- Microbial biomass: (*e.g.*: the production of yeast to be used in the baking industry and the production of microbial cells to be used as human or animal food

2- Microbial enzymes: (*e.g.*: protease, amylase, lipase ……etc)

3- Microbial metabolites (*e.g.:* include amino acids, nucleotides, proteins, nucleic acids, lipids, carbohydrates, etc as the primary products of metabolism and antibiotics as the secondary compounds of metabolism)

4- Recombinant products: (e.g.*:* Products produced by such genetically engineered organisms include interferon, insulin, human serum albumin, factors VIII and epidermal growth factor, calf chymosin and bovine somatostatin).

5- Transformation processes: (include converting compound into a structurally related, financially more valuable, compound via microbial cells such as the production of high-value compounds including steroids, antibiotics and pros

Microbial biomass production

In many fermentation processes, the conversion of a substrate to biomass is somewhat incidental as the main aim is to produce a useful primary or secondary metabolic product, such as antibiotics, ethanol and organic acids. In such cases, once the optimal amount of target product has been achieved, the organisms produced are often merely waste materials that have to be disposed or simply used as a cheap source of animal feed. However, in dedicated biomass production, the cells

produced during the fermentation process are the products. Consequently, the fermentation is optimized for the production of a maximum concentration of microbial cells.

Microbial biomass is broadly used as a source of protein for human and animal food, because it is often odourless and tasteless, and can therefore be formulated into a wide range of food items.

Single cell protein

The term Single cell protein (SCP) was first used by workers at Massachusetts Institute of technology in 1968 and in most cases, it has been used to describe the production of foods or feedstuffs from unicellular organisms but has also been applied to fungal products.

In more recent times, use of microbial protein has been considered as a potential means of fulfilling the urgent need for low-cost protein in certain parts of the world, which the agriculture in those regions arguably cannot provide. The increased demand is a result of the ever-increasing populations in developing countries. This objective has not been achieved, despite the obvious need and advantages that microbial protein provides over conventional protein sources

SCP is not pure protein, but refers to the whole cells of bacteria, yeasts, filamentous fungi or algae, and also contains carbohydrates, lipids, nucleic acids, mineral salts and vitamins.

- Microorganisms have several advantages as a source for protein over conventional plant and animal protein, include:

1. Rapid growth rate and high productivity.
2. High protein content, 30–80% on a dry weight basis.
3. The ability to utilize a wide range of low-cost carbon sources, including waste materials.
4. Strain selection and further development are relatively straightforward, as these organisms are amenable to genetic modification.
5. The processes occupy little land area.
6. Production is independent of seasonal and climatic variations.
7. Consistent product quality.

The choice of an organism for SCP production

The protein content and quality are largely dependent on the specific microorganism utilized and the fermentation process. Fast-growing aerobic microorganisms are primarily used due to their high yields and high productivity.

Selection of a suitable microbial strain for SCP production must take several characteristics into account, including:

1. Performance (growth rate, productivity and yields) on the specific, preferably lowcost, substrates to be used.
2. Temperature and pH tolerance, Oxygen requirements, heat generation during fermentation and foaming characteristics.
3. Ease of recovery of SCP and requirements for further downstream processing.
4. Structure and composition of the final product, in terms of protein content, amino acid profile, RNA level, flavour, aroma, colour and texture.
5. Safety and acceptability. The organism must be safe during growth, processing and use as a feedstuff.

✶ **SCP- Yeast**

Yeast is an important source of SCP, and have been produced since a long time ago. In World War I, *Candida utilis* (Torula yeast) was produced in Germany and used in soups and sausages. Nowadays, the pet food industry is a major outlet of microbial biomass.. Yeast has some advantages among other SCP sources, such as:

1. Easy to harvest because of their size (larger than bacteria)
2. High level of malic acid content
3. High lysine content
4. Can grow at acidic pH
5. Long history of traditional use

This nutritious microbe unfortunately has few disadvantages that have to be taken as consideration, such as:

1. Lower growth rates compared to bacteria
2. Lower protein content than bacteria (45-65%)
3. Lower methionine content than bacteria, solved by the addition of methionine in the final product.

✶ **SCP- Fungi**

Many filamentous fungi have been reported to produce protein. Therefore, the term SCP is not logical, if an organism produces filaments. The term 'mycoprotein' has been introduced for protein produced on glucose or starch substrates.

During the World War II, attempts were made to use the cultures of *Fusarium* and

Rhizopus grown in fermentation as protein food. The inoculum of *Aspergillus oryzae* or *Rhizopus arrhizus* is chosen because of their non-toxic nature. Saprophytic fungi grow on complex organic compounds and render them into simple forms. As a result of growth, high amount of fungal biomass is produced. Strains of some species, for example, *Aspergillus niger, A. fumigatus, Fusarium graminearum* are very hazardous to human, therefore, use of such fungi should be avoided or toxicological evaluations should be done before recommending to use as SCP.

- **SCP- bacteria**

Many species of bacteria have been investigated for use in single cell protein production. ***Methylophilus Methylotrophus*** has a generation time of about 2 hours and is usually and mainly used in animal feed as bacteria, in general produce a more favorable protein composition than yeast or fungi. Among the characteristics that make bacteria suitable for this application include:

1. Their rapid growth. Their short generation times; most can double their cell mass in 20 minutes to 2 hours.
2. Capable of growing on a variety of raw materials, ranging from carbohydrates such as starch and sugars, to gaseous and liquid hydrocarbons such as methane and petroleum fractions, to petrochemicals such as methanol and ethanol.

A safety aspect that must be considered for all SCP products is nucleic acid content. Most SCP products are currently used as animal feed and not for human consumption.

Q/ why do fungal and bacterial SCP are not suitable for human consumption?

Because these microorganisms have naturally high nucleic acid content and this can be problematic as the digestion of nucleic acids by humans and animals leads to the generation of purine compounds. Their further metabolism results in elevated plasma levels of uric acid, which may crystallize in the joints to give gout-like symptoms or forms kidney stones.

- **Substrate for SCP production**

The major substrates that have been used in commercial SCP production are alcohols, *n*alkanes, molasses, sulphite liquor and whey. However, in choosing a substrate, consideration must be given to:

1. The cost of the substrate.
2. Biomass yield per unit of substrate utilized.
3. Heat produced and the level of fermenter cooling required.
4. Downstream processing costs, including the removal of possible toxic components. ➢ **Single cell protein production processes**

The SCP production processes essentially contain the same basic stages irrespective of the carbon substrate or microorganism used:

1- Medium preparation. The main carbon source may require physical or chemical pretreatment prior to use. Polymeric substrates are often hydrolysed before being incorporated with sources of nitrogen, phosphorus and other essential nutrients.

2- Fermentation. The fermentation may be aseptic or run as a 'clean' operation depending upon the particular objectives. Continuous fermentations are generally used, which are operated at close to the organism's maximum growth rate (mmax), to fully exploit the superior productivity of continuous culture.

3- Separation and downstream processing. The cells are separated from the spent medium by filtration or centrifugation and may be processed in order to reduce the level of nucleic acids. This often involves a thermal shock to inactivate cellular proteases. RNase activity is retained and degrades RNA to nucleotides that diffuse out of the cells. Depending upon the growth medium used, further purification may be required, such as a solvent wash, prior to pasteurization, dehydration and packaging.

Several kinds of processes have been developed for the utilization of different substrate to produce SCP such as:

I. The Symba process

The Symba process was developed in Sweden to produce SCP for animal feed from potato processing wastes. A high proportion of the available substrate is starch, which many microbes cannot directly utilize. To overcome this problem the process was developed with two microorganisms that grow in a symbiotic association. They are the yeasts *Saccharomycopsis fibuligera*, which produces the hydrolytic enzymes necessary for starch degradation, and *Candida utilis*. The process is operated in two stages; in the first stage, *Saccharomycopsis fibuligera* is grown in a small reactor on the sterilized waste, supplemented with a nitrogen source and phosphate. At this point, the starch is hydrolysed. The resulting broth is then pumped into a second larger fermenter where both organisms are present. However, *Candida utilis* comes to dominate the second stage and constitutes up to 90% of the final product. The Symba process operates continuously and after 10 days the lactose content is reduced by 90%. Resultant protein-rich biomass (45% protein) is concentrated by centrifugation and finally sprays or drum dried.

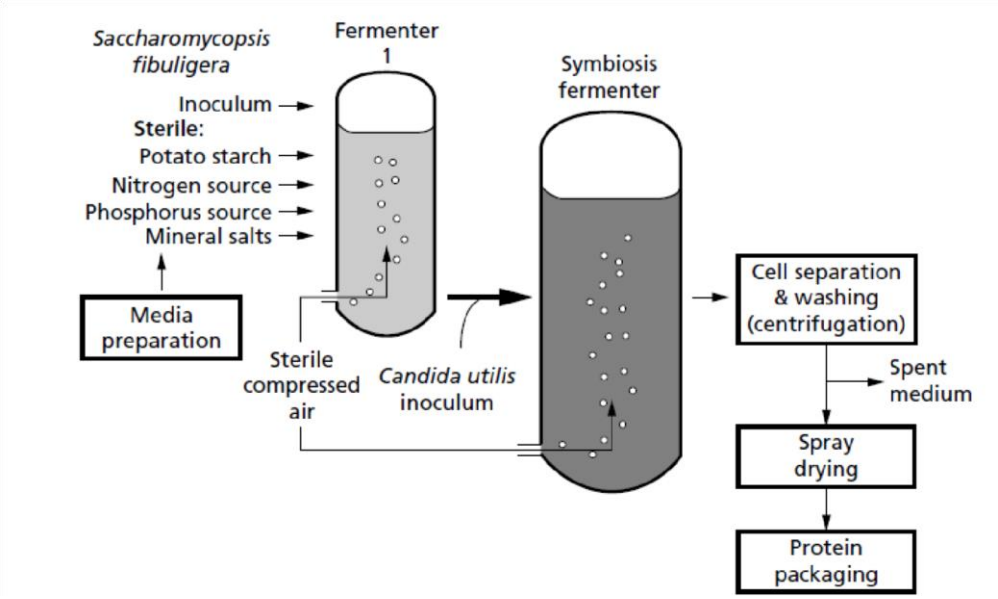


Figure: The Symba process for the production of SCP

II. The Bel process

The Bel process was developed with the aim of reducing the pollution load of dairy industry waste (whey), while simultaneously producing a marketable protein product. It is particularly suitable for the production of SCP using lactose-utilizing yeast. A number of plants are operated using *Kluyveromyces lactis* or *Kluyveromyces marxianus* (formerly *K. fragilis*) to produce a protein, Protibel, which is used for both human and animal consumption.

These processes initially involve whey pasteurization, during which 75% of whey proteins are precipitated. The lactose concentration is adjusted to 34 g/L and mineral salts are also added. This supplemented whey is introduced into a continuous fermenter, maintained at 38°C and pH 3.5. The yeasts utilize the lactose and attain biomass concentrations of 25 g/L, with a biomass yield of 0.45–0.55 g/g lactose. Yeast cells are recovered by centrifugation, and then resuspended in water, recentrifuged and finally roller-dried to 95% solids. Levels of residual sugar remaining in the spent medium are less than 1g/L.

III. The Bioprotein process

The Bioprotein process, developed in the 1990s by Norferm in Norway, uses methanerich natural gas as a sole carbon and energy source for the growth of *Methylococcus capsulatus*. A mixture of heterotrophic bacteria is also present, which helps to stabilize the process. It is highly aerobic continuous fermentation which is performed in a loopfermenter, with medium containing ammonia, minerals and methane, obtained. Biomass is continuously harvested by centrifugation

and ultrafiltration, prior to heat inactivation and spray drying. The final product contains 70% protein and is currently marketed as Pronin. It is approved in the EU for use as a fish and animal feed, but may be used in human foods in the future.

IV. Quorn process

This process is to produce fungal mycelium (mycoprotein) for human consumption using *Fusarium venenatum* and food grade glucose syrup as the carbon source that derived from maize, potato or wheat starch. The product, Quorn, with reduced RNA content, was approved for use as a human food protein in 1985 over 20 years after the start of the project. 1000 tonnes of Quorn are now produced each year in an airlift fermenter that was formerly used as the Pruteen pilot fermenter.

- The fungal biomass generated contains 10% RNA, which is too high for human consumption. RNA levels are subsequently reduced by a thermal shock at 64°C for 30 min, which renders the organism non-viable and activates the organism's RNases. This results in the breakdown of RNA into nucleotides that diffuse out of the cells into the medium. Thus, RNA concentration is reduced to an acceptable level of less than 2% (w/w).

Lec-6

Production of microbial metabolites

A descriptive terminology of the behavior of microbial cells which considered the type of metabolism rather than the kinetics of growth is **trophophase and idiophase**:

- The term 'trophophase' was suggested to describe the log or exponential phase of a culture during which the sole products of metabolism are either essential to growth, such as amino acids, nucleotides, proteins, nucleic acids, lipids, carbohydrates, ***etc.*** or are the by-products of energy-yielding metabolism such as ethanol, acetone and butanol. The metabolites produced during the trophophase are referred to as **primary metabolites**
- The term 'idiophase' was suggested to describe the phase of a culture during which products other than primary metabolites are synthesized, products which do not have an obvious role in cell metabolism. The metabolites produced during the idiophase are referred to as the **secondary metabolites**. Secondary metabolites tend to be synthesized from the intermediates and end-products of primary metabolism.

Industrial microbiological products as primary and secondary metabolites

Products of industrial microorganisms are divided into two broad groups, those which result from primary metabolism and others which derive from secondary metabolism. The line between the two is not always clear cut, but the distinction is useful in discussing industrial products.

- **Products of Primary Metabolism**

Primary metabolism is the inter-related group of reactions within a microorganism which are associated with growth and the maintenance of life. Primary metabolism is essentially the same in all living things and is concerned with the release of energy, and the synthesis of important macromolecules such as proteins, nucleic acids and other cell constituents. When primary metabolism is stopped the organism dies. Products of primary metabolism are associated with growth and their maximum production occurs in the logarithmic phase of growth in a batch culture. Primary catabolic products include ethanol, lactic acid, and butanol while anabolic products include amino-acids, enzymes and nucleic acids. Single-cell proteins and yeasts would also be regarded as primary products.

Table: Some industrial products resulting from primary metabolism

Anabolic Products	*Catabolic Products*
1. Enzymes	1. Ethanol and ethanol-containing products, e.g. wines
2. Amino acids	2. Butanol
3. Vitamins	3. Acetone
4. Polysaccharides	4. Lactic acid
5. Yeast cells	5. Acetic acid (vinegar)
6. Single cell protein	
7. Nucleic acids	
8. Citric acid	

- **Products of Secondary Metabolism**

In contrast to primary metabolism which is associated with the growth of the cell and the continued existence of the organism, secondary metabolism, which was first observed in higher plants, has the following characteristics:

(i) **Secondary metabolism has no apparent function in the organism**. The organism continues to exist if secondary metabolism is blocked by a suitable biochemical means. On the other hand, it would die if primary metabolism were stopped.

(ii) **Secondary metabolites are produced in response to a restriction in nutrients**. They are therefore produced after the growth phase, at the end of the logarithmic phase of growth and in the stationary phase (in a batch culture). They can be more precisely controlled in a continuous culture.

(iii) **Secondary metabolism appears to be restricted to some species of plants and microorganisms (and in a few cases to animals)**. The products of secondary metabolism also appear to be characteristic of the species. Both of these observations could, however, be due to the inadequacy of current methods of recognizing secondary metabolites.

(iv) **Secondary metabolites usually have 'bizarre' and unusual chemical structures and several closely related metabolites may be produced by the same organism in wildtype strains.** This latter observation indicates the existence of a variety of alternate and closely-related pathways.

(v) **The ability to produce a particular secondary metabolite, especially in industrially important strains is easily lost**. This phenomenon is known as strain degeneration. Owing to the ease of the loss of the ability to synthesize secondary

metabolites, particularly when treated with acridine dyes, exposure to high temperature or other treatments known to induce plasmid loss secondary metabolite production is believed to be controlled by plasmids (at least in some cases) rather than by the organism's chromosomes. A confirmation of the possible role of plasmids in the control of secondary metabolites is shown in the case of leupetin, in which the loss of the metabolite following irradiation can be reversed by conjugation with a producing parent.

(vi) **The factors which trigger secondary metabolism, the inducers, also trigger morphological changes (morphogenesis) in the organism.**

The production of microbial metabolites may be achieved in continuous, as well as batch, systems. The chronological separation of trophophase and idiophase in batch culture may be studied in continuous culture in terms of dilution rate. Secondary metabolism will occur at relatively low dilution rates (growth rates) and, therefore, it should be remembered that secondary metabolism is a property of slow growing.

Production of ethanol

Ethanol has been made since ancient times by fermentation of sugar and starch, and is often considered to be one of the first microbial processes used by human. In addition to its role as a beverage, ethanol can serve as a fuel and as a starting material for the manufacture of chemicals such as acetic acid, acetaldehyde, butanol, and ethylene. Also, ethanol is used extensively as a solvent in the manufacture of varnishes and perfumes, as a preservative for biological specimens, in the preparation of essences and flavorings, in many medicines and drugs and as a disinfectant. Ethanol can be used as a fuel for vehicles in its pure form, but it is usually used as a gasoline additive to increase octane and improve vehicle emissions. As a transportation fuel, ethanol has a number of advantages over gasoline. In particular, it burns more cleanly and with higher efficiency.

➢ Biosynthesis of ethanol

Both yeast and bacteria have been used for the production of ethanol. Among the bacteria, the most widely used organism is ***Zymomonas mobilis***. ***Saccharomyces cerevisiae*** is the most commonly used yeast but ***Kluyveromyces fragilis*** has also been employed.

Under aerobic conditions and in the presence of high glucose concentrations, *Saccharomyces cerevisiae* grows well but produce no alcohol. Under anaerobic conditions, however, growth slows

and pyruvate from the glycolytic pathway is split with pyruvate decarboxylase into acetaldehyde and CO_2. Ethanol is then produced from the acetaldehyde by reduction with alcohol dehydrogenase.

Batch systems for ethanol production are started aerobically to obtain maximum biomass, since if anaerobic conditions begin too early, the population density is not high enough to obtain a good conversion rate.

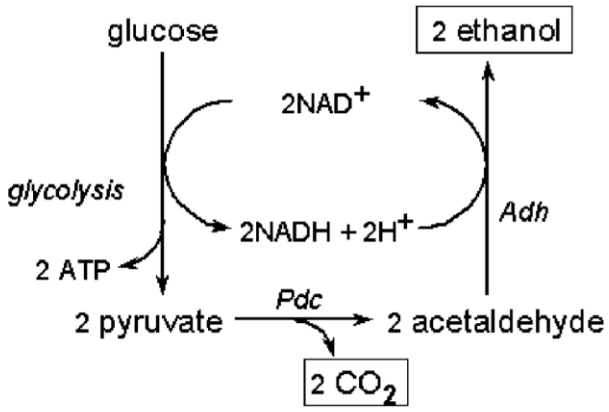


Figure: biosynthesis of ethanol (*Pdc*: Pyruvate decarboxylase; *Adh*: Alcohol dehydrogenase)

An understanding of the arithmetic of ethanol production begins with the equation established by Gay-Lussac in 1810 for the fermentative conversion of glucose to ethanol by yeast:

$$C_6H_{12}O_6 \rightarrow 2C2H5OH + 2CO_2$$

180 g 92 g 88 g

Theoretically, from one gram of glucose, 0.511 grams of ethanol can be obtained. When pure substrates are fermented, the yield is 95% and reduces to 91% when industrial-grade starting materials are used. One hundred grams of pure glucose will yield 48.4 grams of ethanol, 46.6 grams of CO2, 3.3 grams of glycerol, and 1.2gram biomass (yeast cells). If corn starch is used, 100 kg starch (corresponding to 180kg of corn) ferments to 51.5 grams of ethanol.

Lec-7

Ethanol Production process

Ethanol is produced in three stages:

Stage I: From feedstocks to fermentable sugars

In this stage, the carbohydrate-containing raw materials are pretreated in ways that make the sugars they contain readily available to microorganisms. Three types of substrates are used in the production of ethanol by microbial fermentation:

1- **Sugar crops (sugar cane and sugar beets)**

Sugar cane and sugar beets contain up to 20% sucrose by weight. The substrate for fermentation is obtained by extraction of sucrose with water after mechanically crushing the sugar cane or stripping and pulping the sugar beets. Yeast produces the enzyme invertase in both a cytoplasmic and a secreted form, and this enzyme hydrolyzes sucrose to glucose and fructose, which are then fermented by the yeast cells.

2- **Starches**

Corn starch, the major feedstock for the production of fuel alcohol in the United States, consists of a water-soluble fraction, amylose (20%), and a water-insoluble higher molecular weight fraction called amylopectin (80%).

To obtain corn starch, dry corn is milled, water is added, and the slurry (watery suspension) is sent to a cooker. Heating the slurry solubilizes the starch and makes it vulnerable to enzyme hydrolysis. A thermo stable α-amylase is added to liquefy the starch, and in the final prelude to fermentation, glucoamylase is added to catalyze saccharification, hydrolysis of the starch polymers to glucose

3- **lignocellulose**

Cellulose is the most abundant component of lignocellulose. Pretreatment of the lignocellulose makes the cellulose more accessible to hydrolytic enzymes and begins to disrupt, at least in part,

the highly crystalline structure of the cellulose fibers. Small wood chips are charged with steam in a heated pressure vessel to about 500°F and maintained at that temperature for about 20 seconds, at which point the vessel is rapidly decompressed. Pressure in the vessel reaches 600 psi before release. After the explosive decompression, the cellulose in the wood is susceptible to enzyme hydrolysis to produce glucose while the lignin remains insoluble and is subsequently removed by filtration. The glucose solution is then fermented to alcohol.

Stage II: From sugar to alcohol (fermentation)

The second stage utilizes the simple sugars, released from polysaccharides in stage I, as substrates in microbial fermentations to produce alcohol. Many types of yeast, but few bacteria, carry out the conversion of glucose to alcohol. Industrial processes use primarily yeasts in the genus *Saccharomyces*.

Stage III: Alcohol recovery

In this stage, alcohol is recovered by distillation (as a constant-boiling mixture of 95.6% ethanol and 4.4% water, by volume). Before distillation, the cell mass is separated by centrifugation or sedimentation. Further distillation procedures are needed to obtain anhydrous ethanol.

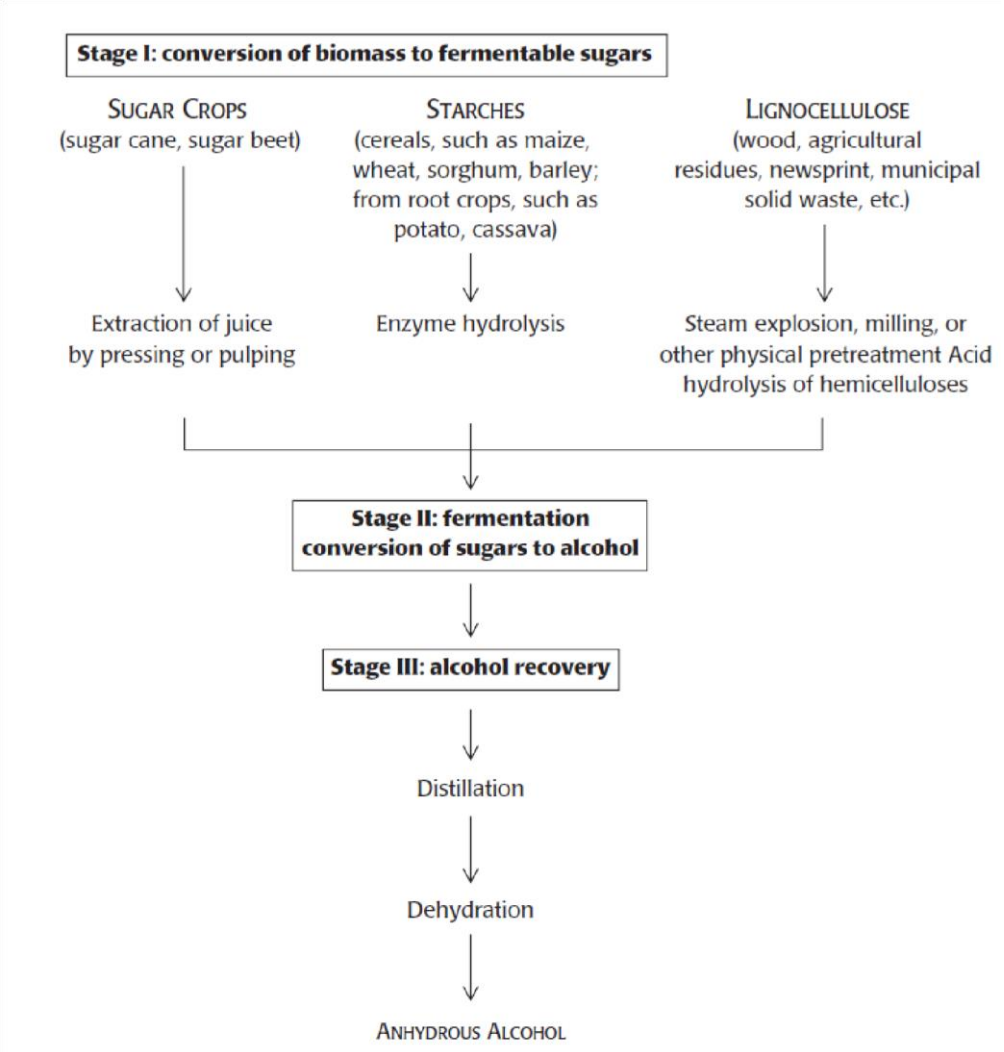


Figure: Stages in the conversion of various feedstocks to ethanol.

- Although yeasts have many of the attributes of an ideal ethanol producer, they have significant limitations such as:

1- **A narrow substrate range they are able to use:** The greatest constraint in employing yeasts as agents of fermentation is the limited range of substrates they are able to use. For instance, yeasts do not ferment most of the oligosaccharides formed during the hydrolysis of starch. Yeasts thus require the addition of glucoamylases to utilize starch completely. Nor can yeast cells utilize cellulose, hemicellulose, cellobiose, or most pentoses.

2- **Limited tolerance to alcohol**: ethanol is toxic to yeast cells at concentrations ranging between 8% and 18% by weight, depending on the strain of the yeast and the metabolic state of the culture. Yeast fermentation is totally inhibited by ethanol concentrations of about 11% by volume.

The conversion of glucose to ethanol and CO_2 is an exothermic reaction: the complete fermentation of an 18%-by-weight glucose solution would raise the temperature of the medium by more than 20°C. Every 5°C increase in temperature increases the evaporative loss of ethanol by 1.5. Yeast metabolism rates also increase with temperature up to an optimum at 35°C; they then decrease gradually between 35°C and 43°C, and drop abruptly above 43°C. These considerations impose a cooling requirement so that the fermenter operating temperature is maintained below 35°C.

- *Saccharomyces* strains are responsible for almost all the current industrial production of alcohol by fermentation. However, another ethanol producer; *Zymomonas mobilis;* has in recent years come under increasing study; because it has a number of potential advantages:

1- Osmotic tolerance to higher sugar concentrations (up to 400 g/l)
2- Relatively higher ethanol tolerance (up to 130 g/l) 3- Higher specific growth rate than yeast.

3- Anaerobic carbohydrate metabolism is carried out through the enter-Doudoroff pathway, where only one mole of ATP is produced per mole of glucose used, thus reducing the amounts of glucose that is converted to biomass rather than ethanol.

- **Flocculence and Cell Recycling**

The objectives of a fermentation process are to convert substrates to alcohol as rapidly as possible, to minimize the cost of alcohol recovery, and to decrease the amount of yeast cells produced as a by-product of the process. Exploitation of cell recycling and of the tendency of yeast cells to *flocculate* (clump) contributes to the attainment of these objectives

- ***Cells recycle***: Yeast Cells can be collected at the end of one batch fermentation to be used as the inoculum for the next. This procedure is termed *cell recycle*.

Because collecting cells by centrifugation or filtration costs more in equipment and attention than any savings that result from such recycling, the key has been finding inexpensive ways of separating yeasts from the fermentation broth. Flocculation provides a partial solution.

- **Flocculation:** is a property in yeasts by which cells stick together because they possess the gene *flo1*-encoded wall protein that binds in a calcium ion–dependent manner to the wall mannans of other cells. As a result, the cells form clumps that sediment rapidly to the bottom of the fermentor and are easily removed from the fermentation mixture for recycling.

Lec-8

Enzyme Technology

Enzymes: are biological molecules that catalyze chemical reactions. They are mainly proteins, generated by an organism to speed up chemical reactions.

Enzyme technology: is the technology associated with the use of enzymes as tools in industry, agriculture or medicine.

- Almost all chemical reactions in a biological cell need enzymes in order to occur at rates sufficient for life. They have an active site on which the substrate is attached, and then broken up or joined. In enzymatic reactions, the molecules at the beginning of the process, called substrates, are converted into different molecules, called products.
- Enzyme activity can be affected by other molecules. Inhibitors are molecules that decrease enzyme activity; activators are molecules that increase activity. Also, activity is affected by temperature, pressure, chemical environment (e.g., pH), and the concentration of substrate
- An enzyme's name is often derived from its substrate or the chemical reaction it catalyzes, with the word ending in ***-ase***. Some of the most common include proteases which break down proteins; cellulases which break down cellulose; lipases which split fats into glycerol and fatty acids; and amylases which break down starch into simple sugars.

For thousands of years natural enzymes made by microorganisms have been used to make products such as cheese, bread, wine, and beer. Enzymes are now used in a wide range of industrial processes. **Commercially produced enzymes are used in**:

1. **Industry**, such as amylases, proteases, catalases, isomerases.
2. **Analytical** purposes, such as glucose oxidase, alcohol dehydrogenase, hexokinase, cholesterol oxidase
3. **Medicine**, such as asparaginase, proteases, lipases

The industrial enzyme producers sell enzymes for a wide variety of applications. Detergents (34%), Textiles (11%), Diary process (14 %), Starch processing (12%), bakery application (5%), beverage and brewing (7%) and animal feed (6%) are the main industries, which use about 75% of industrially produced enzymes.

- The advantages and disadvantages of using enzymes in industry are directly related to their properties

Advantages	Disadvantages
They are specific in their action and are therefore less likely to produce unwanted by- products	They are highly sensitive to changes in physical and chemical conditions surrounding them.
They are biodegradable and therefore cause less environmental pollution	They are easily denatured by even a small increase in temperature and changes in pH. Therfore, the conditions in which they work must be tightly controlled.
They work in mild conditions, i.e. low temperatures, neutral pH and normal atmospheric pressure, and therefore are energy saving	The enzyme substrate mixture must be uncontaminated with other substances that might affect the reaction.

Commercial production of enzyme

Several thousand tonnes of commercial enzymes are currently produced each year. A few animal and plant enzymes are used, for example: papain from plants; pepsin and rennin are derived from animal. Most commercial enzymes are now obtained from microbial sources. **The use of microorganisms as a source material for enzyme production has developed for several important reasons**.

(1) The difficulty of extracting plant or animal enzymes on the scale necessary to meet demand in many industrial sectors.

(2) Seasonal fluctuations of raw materials and possible shortages due to climatic change.

(3) In microbes a wide spectrum of enzyme characteristics, such as pH range and hightemperature resistance, is available for selection.

(4) Industrial genetics has greatly increased the possibilities for optimizing enzyme yield and type through strain selection, mutation, induction and selection of growth conditions.

Microbes are still the most common source of industrial enzymes. Microorganisms produce enzymes inside their cells (**intracellular** enzymes) and may also secrete enzymes for action outside the cell (**extracellular** enzymes).

Intracellular enzymes	Extracellular enzymes
More difficult to isolate	Easier to isolate
Cells have to be broken apart to release them	No need to break cells – secreted in large amounts into
Have to be separated out from cell debris and a mixture of many enzymes and other chemicals	Often secreted on their own or with a few other enzymes
Often stable only in environment inside intact cell	More stable
Purification/down streaming processing is difficult/expensive	Purification/down streaming processing is easier/cheaper

A real breakthrough for large scale industrial production of enzymes from microorganisms occurred after 1950s. Microbial enzymes are predominantly produced by submerged fermentations, although some solid-substrate fermentations are used, particularly for the production of extracellular fungal enzymes. Most industrial enzymes are products of batch processes and few are currently produced via continuous fermentation. Most fermentors are stirred tank reactors that are operated under aseptic conditions and use low-cost undefined complex media.

- In general, the techniques employed for microbial production of enzymes are comparable to the methods used for manufacture of other industrial products. The enzyme production process can be divided into following phases

1. Selection of the production organism.
2. Improvement of the production organism (for example construction of an overproducing strain by genetic engineering.
3. Optimization of culture medium and production condition.
4. Production process
5. Recovery and purification of enzymes.
6. Formulation of a stable enzyme product.

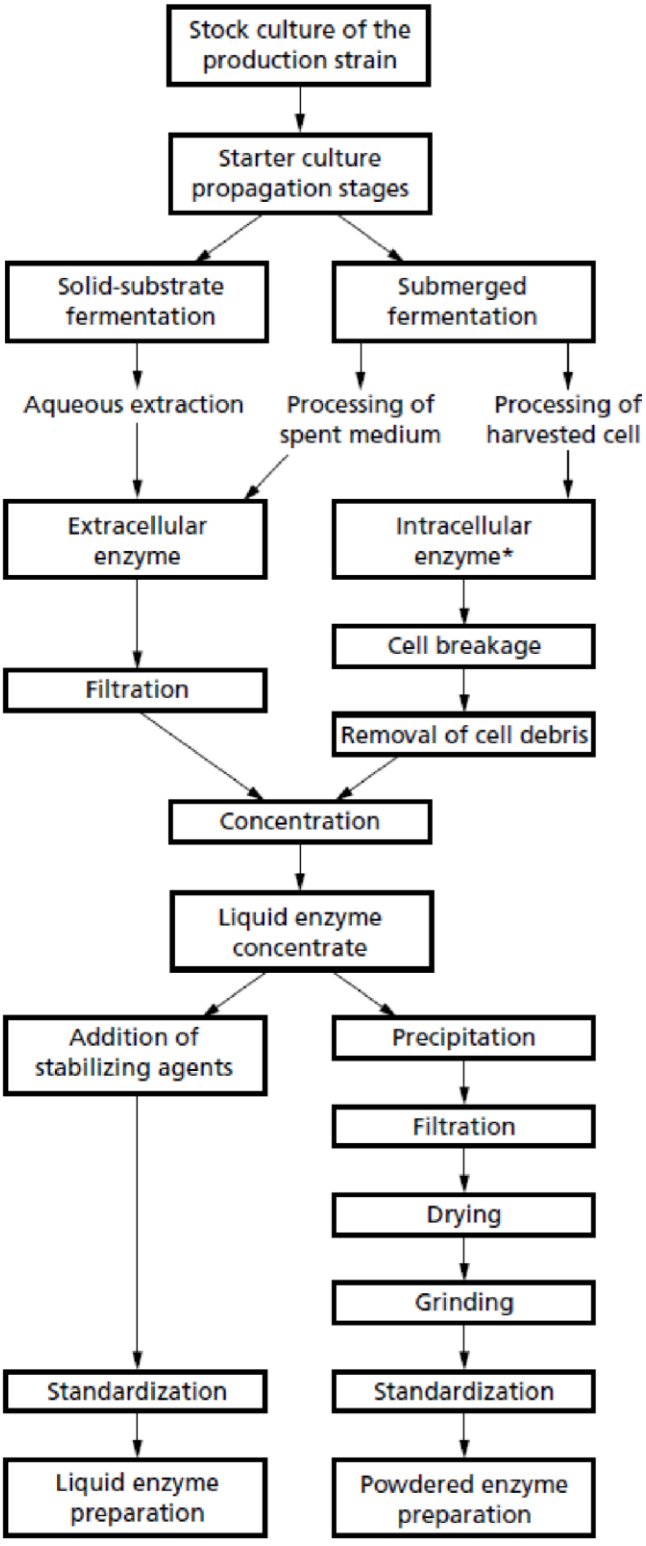


Figure: Production of industrial enzyme

• **Selection of the production strain:** enzyme production processes traditionally begin with the search for a suitable producer organism. A programme of microorganism screening and selection is necessary, to determine enzyme properties, such as optimum pH, heat resistance, and examination of the ability to secrete the target enzyme.

❖ **In choosing the producing strain several aspects have to be considered:**

1- Ideally the enzyme is secreted from the cell.
2- The production host should have a GRAS-status (Generally Recognized As Safe).
3- The organism should be able to produce high amount of the desired enzyme.
4- Can be genetically modified to overproduce the desired activity and not to produce undesired side activities.

The different organisms and their relative contribution for the production of commercial enzymes are given below:

(Fungi – 60%; Bacteria – 24%; Yeast – 4%; Streptomyces – 2%; Higher animals – 6%; Higher plants – 4%)

Once a suitable microorganism is obtained, the fermentation system and conditions for maximum production of the enzyme per unit of biomass must be determined using inexpensive carbon and nitrogen feedstocks.

✶ **Thermostable enzymes**

A thermostable enzyme is one that will not denature at high temperatures. Usually these enzymes have to be extracted from microorganisms that live in extreme heat conditions and have evolved these proteins over time.

➢ Enzymes from thermophilic microorganisms generally provide several advantages:

1. They are thermostable.
2. Able to operate at higher temperatures than enzymes of mesophiles

The level of purification applied varies considerably depending on whether the enzyme is intracellular or extracellular, and on its end use. Downstream processing involves separation, purification, stabilization and preservation.

Improvement of enzyme production

Enzyme production is closely controlled in microorganisms and in order to improve productivity these controls may have to be exploited or modified. Such control systems as:

1. Induction may be exploited by including inducers in the medium.
2. Repression control may be removed by mutation and recombination techniques.
3. The number of gene copies coding for the enzyme may be increased by recombinant DNA techniques.

- The majority of enzymes which are of industrial interest are inducible. Induced enzymes are synthesized only in response to the presence of an inducer in the environment. Inducers are often substrates such as starch or dextrins for amylases, maltose for pullulanase and pectin for pectinases.
- Substrate analogues that are not attacked by the enzyme may also serve as enzyme inducers.
- Most inducers which are included in microbial enzyme media are substrates or substrate analogues, but intermediates and products may sometimes be used as inducers. For example, maltodextrins will induce amylase and fatty acids induce lipase. However, the cost may prohibit their use as inducers in a commercial process.

Improving the Enzyme: Immobilization

As enzymes are catalytic molecules, they are not directly used up by the process in which they are used. However due to denaturation, they do loose activity with time. Therefore, it would be more economic to retain the enzyme and so be able to re-use it for several batches of product.

When the enzymes are used in a soluble form, they can contaminate the product, and its removal may involve extra purification costs.

In order to eliminate wastage and improve productivity, the enzyme and product can be separated during the reaction.

The enzyme can be imprisoned allowing it to be reused but also preventing contamination of the product – this is known as **immobilization**.

Unstable enzymes may be **immobilized** by being attached to or located within an insoluble support, therefore the enzyme is not free in solution. Once attached, an enzyme's stability is increased, possibly because its ability to change shape is reduced.

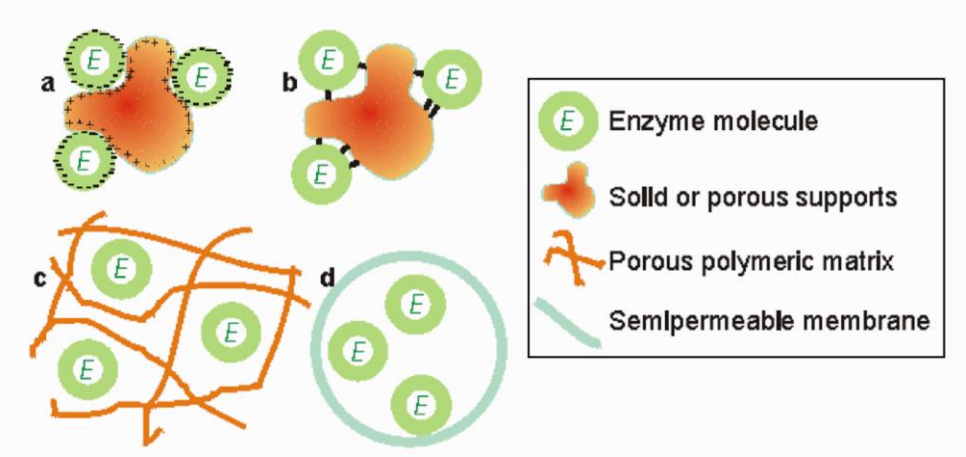
- **There are four main methods available for immobilizing enzymes:**

a. **Adsorption** in glass or alginate beads – enzyme is attached to the outside of an inert material

b. **Cross-linkage** to another chemical e.g. cellulose or glyceraldehydes.

c. Entrapment of enzyme inside a polymer matrix for example in a silica gel, mesh or capsule of an inert material.

d. Membrane confinement



- **Advantages of immobilization**
  1. Easier to separate enzyme and products
  2. Allows catalysis in unfavourable media
  3. Increases stability and can be manipulated easily
  4. Allows continuous production/enzyme used for longer
  5. Enzyme can be recovered and reused
  6. Enzyme does not contaminate product/no purification required
- **Disadvantages of immobilization**
  1. Immobilization may alter shape of enzyme
  2. Expensive.

Lec-9

Production of antibiotics

Antibiotics: are defined as natural antimicrobial agents produced by microorganism and used to kill or inhibit the growth of other organisms.

However, this concept now is broadened to include any chemical compound of natural origin that has an effect against the growth of other organisms. Scientists distinguish this sort of antimicrobial agents from another group called **chemotherapeutic agents** which are chemically synthesized. In addition, a mixture of these two concepts now yields some antimicrobial compounds that are produced by organisms and chemically modified to obtain some desired properties **(semi-synthetic antibiotics)**. Furthermore, some antimicrobial compounds of natural origin have been synthesized by chemical means and therefore they known as **synthetic antibiotics**.

Over the period that followed the first observation of antibiotics by Fleming in 1928, huge efforts were made by scientists to discover and explore these compounds. Thousands of antibiotics were isolated from bacteria and fungi and some of them have been successfully used to fight microbial infections in humans and animals. Moreover, some of them were used to create new generations of clinically valuable antibiotics by chemical modifications.

Antibiotics are secondary metabolites mainly produced by microorganisms living in the soil and are mostly spore forming. Normally antibiotics are produced during the late growth phase of microbial culture. So far, no significant essential role for antibiotics in the growth of producing organisms is known; however, they are important for both human and veterinary medicine.

Most antibiotics are produced by filamentous fungi and bacteria, particularly the actinomycetes. Over 4000 antibiotics have been isolated from various organisms, but only about 50 are used regularly in antimicrobial chemotherapy. The best known and probably the most medically important antibiotics are the β-**lactams**, penicillins and cephalosporins; along with **aminoglycosides**, such as streptomycin, and the broadspectrum **tetracyclines.**

Some antibiotics have applications other than in antimicrobial chemotherapy. For example, actinomycin and mitomycin, produced by *Streptomyces peucetius* and *Streptomyces caepitosus*, respectively, have roles as antitumour agents; and other antibiotics are used for controlling

microbial diseases of crop plants, or as tools in biochemistry and molecular biology research. Several antibiotics are also added to animal feed as growth promoters.

- **Why do microorganisms synthesize antibiotics?**
  - In 1990, Davies suggested that secondary metabolites are ancient molecules and many of them exert their known biological activities (e.g. as antibiotics) through interactions with ancient and conserved sites in macromolecules (or their ancestors.
  - Stone and Williams (1992) argued this opinion and suggested that the producing organisms may have evolved the capability to synthesize antibiotics due to the advantages that may be gained as a result of the functions of these compounds. They made their suggestion based on some evidence:
    I. The role of the antibiotics in their antagonistic abilities in nature.
    II. The fact that all genes necessary for antibiotic biosynthesis, regulation, and resistance are normally located in the same cluster which means that they might be evolved as one group.
    III. The genes necessary for sporulation and antibiotic synthesis are usually expressed simultaneously and that may provide evidence in terms of the defence functions of the antibiotics.
  - So far, the predominant hypothesis regarding the origin of antibiotics is the competition hypothesis which suggests that antibiotics are synthesized to give the antibiotic producing organism an advantage over any possible competing organisms

Production of antibiotics

In general antibiotics are produced by three main groups of microorganisms:

1. Actinomycetes, mainly *Streptomyces* species, produce several types of antibiotics for instance tetracyclines, aminoglycosides, macrolides, chloramphenicol, ivermectin, rifamycins,
2. *Bacillus* species, such as *Bacillus polymyxa* and *Bacillus subtilis*, produce polypeptide antibiotics and *Bacillus cereus,* produces zwittermicin.
3. Fungi (Particularly *Penicillium* and *Cephalosporium*) produce many clinically useful antibiotics such as beta-lactam antibiotics like penicillin and cephalosporin

Biosynthesis of antibiotics is affected by various physiological and environmental factors which can be divided into two types:

1- Positive factors: factors act positively and stimulate production of antibiotics, for instance nutritional stress and quorum sensing signal molecules.

2- Negative factors: factors act negatively causing suppression of antibiotic formation, for example availability of carbon, nitrogen and phosphate sources in the fermentation medium.

➢ **Strategies for the improvement of antibiotics production**

In nature, microorganisms normally produce antibiotics in low quantities; therefore, improving the productivity of antibiotic-producing strains to meet the commercial requirements represents an important challenge in the industrial microbiology.

❖ There are two factors affecting the manufacture of antibiotics in the laboratory:

1- The environmental factors such as nutrient abundance, pH, temperature and the aeration

The biosynthesis of antibiotics can be influenced greatly by manipulating the type and concentration of the nutrients and formulating the culture media. Although, optimizing the environmental factors to increase antibiotics yield is not costly compared with the genetic engineering methods, the results are normally limited because of the regulatory mechanisms in the cell that prevent excessive production of the metabolites which may affect its own survivability.

2- The genetic construction of the microorganism

Genetic manipulations are successively used to get strains that produce many hundreds of times more than that produced by the wild type strain. In this context, random mutagenesis and screening methods have also been widely used. Although a great progress has been achieved, there are certain disadvantages as the method of random mutagenesis is time-consuming and there is the possibility of unwanted mutations.

However, with great advances in genetic, recombinant DNA technology and molecular biology technologies, genetic improvement methods became more directed and led to increases in the fermentation productivity with decreasing the costs tremendously.

Production of Penicillin

Penicillin was discovered by Fleming in 1928 following his famous observation of an inhibitory zone surrounding a fungal contaminant, *Penicillium notatum*, on a plate of *Staphylococcus aureus*. In the late 1930s Florey, Chain and Heatley characterized the inhibitory compound responsible, penicillin, and developed a procedure that allowed it to be produced in a pure form.

Penicillin is a narrow spectrum antibiotic which is only active against Gram positive bacteria. This is because it cannot penetrate the outer membrane of Gram negative bacteria. The mode of action of penicillin is to inhibit the biosynthesis of cell wall peptidoglycan resulting in a bactericidal

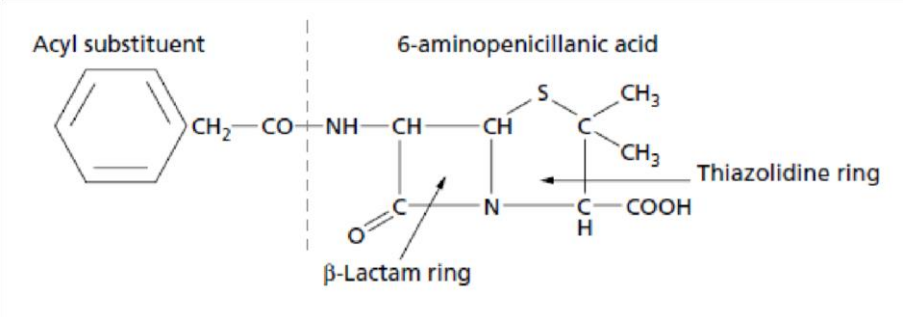
effect. Semi-synthetic derivatives of penicillin (e.g. Ampicillin) have a broader spectrum of activity because they can penetrate the outer membrane of Gram negative bacteria (more hydrophilic, pass through outer membrane pores).

❖ There are two different types of penicillin:

1- **Biosynthetic penicillin** is natural penicillin that is harvested from the mould itself through fermentation.

2- **Semi-synthetic penicillin** includes semi synthetic derivatives of penicillin - like Ampicillin, Penicillin V, Carbenicillin, Oxacillin, Methicillin, etc. These compounds consist of the basic Penicillin structure, but have been modified chemically by removing the acyl group to leave 6-aminopenicillanic acid and then adding acyl groups that produce new properties.

The basic structure of the penicillins is **6-aminopenicillanic acid (6-APA)**, composed of a **thiazolidine ring** fused with a β-**lactam ring** whose 6-amino position carries a variety of **acyl** substituents. This β-lactam– thiazolidine structure (synthesized from **L-αaminoadipate**, **L-cystine** and **L-valine)** is common to penicillins, cephalosporins and cephamycins.



The structure of Penicillin G

Penicillins are most active against Gram positive bacteria. However, an expanded role for the penicillin came from the discovery that different biosynthetic penicillin can be formed by the addition of side-chain precursors to the fermentation medium and that natural penicillins can be modified chemically to produce compounds with improved characteristics. Most penicillins are now semi-synthetic, produced by the chemical modification of natural penicillin, obtained by fermentation using strains of *Penicillium chrysogenum.* Modification is achieved by removing their natural acyl group, leaving 6APA, to which other acyl groups can be added to confer new properties.

These semi-synthetic penicillins, such as methicillin, carbenicillin and ampicillin, exhibit various improvements, including resistance to stomach acids to allow oral administration, a degree of resistance to penicillinase (a penicillin-destroying enzyme produced by some bacteria) and an extended range of activity against some Gram-negative bacteria.

➢ Commercial production of penicillin

Penicillin exhibits the properties of a typical secondary metabolite, being formed at or near the end of exponential growth. Its formation depends on medium composition and dramatic overproduction is possible.

Penicillium notatum, the organism originally found to produce the antibiotic, generated little more than 1 mg/L from the surface cultures initially used for penicillin production. A 20–25-fold increase in yield was achieved when corn steep liquor was incorporated into the fermentation medium. This byproduct of maize processing contains various nitrogen sources, along with growth factors and side-chain precursors, and remains as a major ingredient of most penicillin production media. Even greater penicillin yields were obtained from a closely related species, *Penicillium chrysogenum*, which was originally isolated from a mouldy cantaloup melon. Further increases in yield were achieved when production went over to submerged fermentation. The wartime requirements for penicillin stimulated the rapid development of a large-scale submerged culture system using stirred tank reactors (STRs).

Since the 1940s, penicillin yield and fermentation productivity has been vastly improved by extensive mutation and selection of producer strains. The traditional approach to improving penicillin yields involved random mutation and selection of higher producing strains. Resulting mutants were grown in liquid medium and culture filtrates were assayed for penicillin. This was slow and painstaking as large numbers of strains had to be tested. Nevertheless, such methods were the key to the dramatically increased yields achieved since the discovery of penicillin. Penicillin fermentations now produce yields in excess of 50 g/L, a 50 000-fold increase from the levels first produced by Fleming's original isolate.

- Penicillin production is usually via a fed-batch process carried out aseptically in stirred tank fermenters. The fermentation involves an initial vegetative growth phase followed by the antibiotic production phase. Throughout the process, the oxygen level is very important

and must be maintained at 25–60 mmol/L/h. The processes are maintained at 25–27°C and pH 6.5–7.7, the specific conditions depending upon the *Penicillium chrysogenum* strain used.

- Various carbon sources have been adopted for penicillin production, including glucose, lactose, sucrose, ethanol and vegetable oils. Corn steep liquor is still used as a source of nitrogen, additional nutrients and side-chain precursors. Ammonia, mineral salts and specific side-chain precursors, e.g. phenyl acetic acid or phenoxyacetic acid, may also be added. However, as some precursors are toxic, they must be fed continuously at non-inhibitory concentrations.
- Inoculum development is usually initiated by adding lyophilized spores to a small fermenter. Fungal mycelium may then be grown up through one or two further stages until there is sufficient to inoculate the production fermenter.
- To ensure an optimum yield of penicillin in the following production phase, the mycelium must develop as loose pellets, rather than compact forms. During the following production phase, the carbon source is fed at a low rate and penicillin production increases.
- Penicillin is excreted into the medium and is recovered at the end of fermentation. Antibiotic recovery is often by solvent extraction of the cell-free medium. This involves reducing the pH of the filtered medium to 2.0–2.5 by addition of sulphuric or phosphoric acid, followed by a rapid two-stage continuous countercurrent extraction at 0–3°C using amyl acetate, butyl acetate or methyl isobutyl ketone. The low temperature is necessary to reduce damage to penicillin due to the low pH.

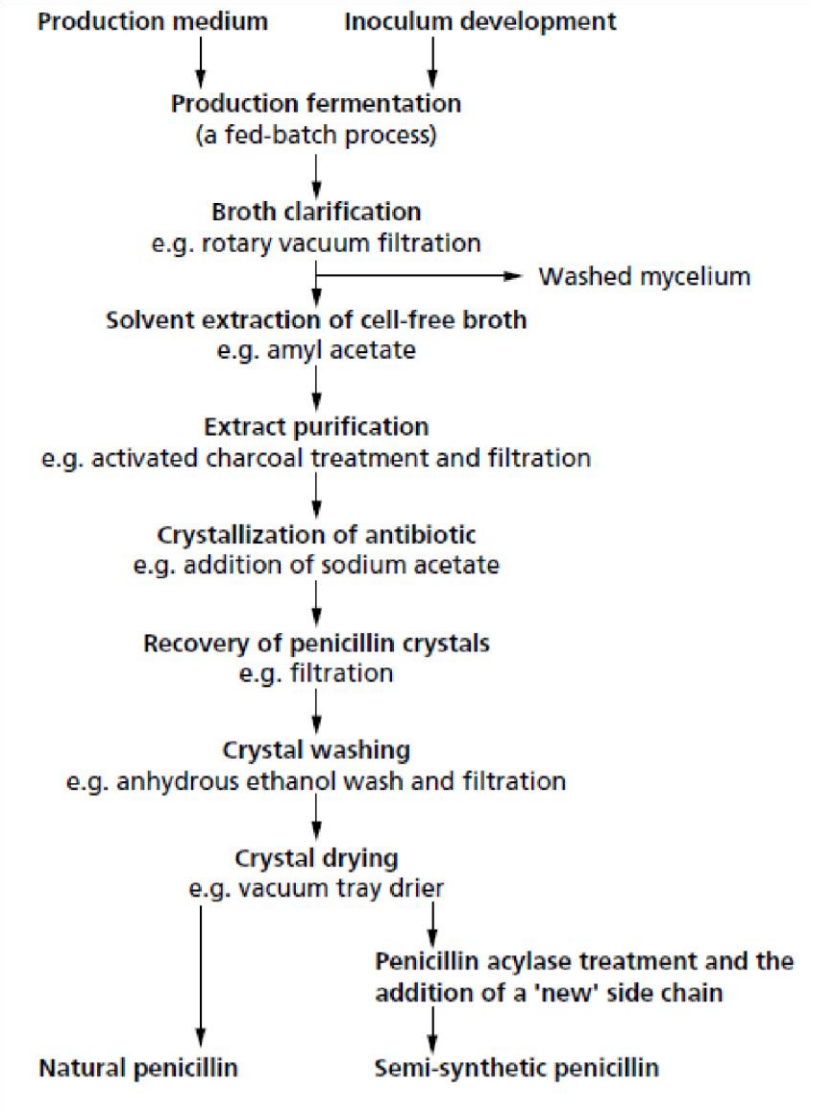


Figure: Production of Penicillin

Lec-10

Production of amino acids

Amino acids are molecules that, when joined together in a long chain and folded into a bundle, create a protein. In nature, 20 different amino acids mix and match to form a wide variety of proteins, whose function depends on the characteristics and order of amino acids comprising the protein structure.

Every amino acid (except glycine) can occur in two isomeric forms, because of the possibility of forming two different enantiomers (stereoisomers) around the central carbon atom. By convention, these are called L- and D- forms, analogous to left-handed and right-handed configurations. Only L-amino acids are manufactured in cells and incorporated into proteins. Some D-amino acids are found in the cell walls of bacteria, but not in bacterial proteins.

Three general approaches are used today for making amino acids: direct chemical synthesis, fermentation and bioconversion using enzymes. Choosing between processes depends on available technology, costs of raw material, market prices, cost of running fermentation versus synthesis reactions, and the environmental impact of the process itself.

Several amino acids are produced in commercial quantities via direct fermentation processes using overproducing microbial strains, or by microbial biotransformation. Amino acids are mostly used as food or animal feed supplements (lysine, methionine, threonine), flavour compounds (monosodium glutamic, serine, aspartic acid). Several amino acids also have uses in pharmaceuticals and cosmetics, and in the chemical industry for the manufacture of polymers.

All twenty amino acids are produced and sold, albeit each in greatly different quantities. Amino acid production is typically carried out by means of **regulatory mutants,** which have a reduced ability to limit synthesis of an end product. The normal microorganism avoids overproduction of biochemical intermediates by the careful regulation of cellular metabolism. The overall strategy for achieving overproduction of the amino acid involves:

1- Increasing the activity of anabolic enzymes.

2- Manipulation of regulation to remove feedback control mechanisms.

3- Blocking pathways that lead to unwanted byproducts.

4- Blocking pathways that result in degradation of the target product.

5-

L- Glutamic acid

Of all the amino acid production processes, that of L- glutamic acid is probably the most important in terms of quantity. Its main use is as the flavour enhancer, monosodium L-glutamate (MSG), which can heighten and intensify the flavour of foods. MSG is naturally present in certain foods and was discovered to be the 'active' component of a traditional flavour-enhancing seaweed stock used in Far Eastern foods.

The industrial production of glutamic acid dates back to 1908, when the Japanese scientists discovered that glutamate was responsible for the characteristic taste of foods cooked with dried kelp (konbu).

For the first 50 years, monosodium glutamate (MSG) was manufactured by expensive chemical processes, based largely on the acid hydrolysis of proteins. This hydrolysis process was costly because glutamate had to be separated from all other amino acids in the hydrolyzate. Significant amounts of MSG were also made by chemical synthesis. This process was expensive too, because it produced a mixture of D- and L-glutamate that had to be resolved to eliminate the d-isomer, which is tasteless.

A revolutionary change was introduced in 1957, when scientists discovered a soil bacterium that excreted large amounts of glutamate into the medium. Similar bacteria were soon isolated by several other companies, ushering in a new industrial technology (amino acid fermentation, the production of amino acids by microorganisms). Except for ethanol, some other organic solvents, and a few vitamins, glutamate was the first organic compound produced on an industrial scale by a microbial fermentation technique.

- **Glutamic acid-producing microorganisms**

Glutamic acid-producing microorganisms include species of the closely related genera *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Microbacterium* and *Micrococcus*. These are Gram-positive, biotin requiring, non-motile bacteria that have intense glutamate dehydrogenase activity.

- **Development of glutamic acid-producing microorganisms**

In order to be useful, glutamate producers must have two things:

1- They must overproduce glutamate in excess of their normal metabolic needs.
2- They must excrete it into culture broth.

Species of ***Brevibacterium*** and ***Corynebacterium*** are used for most industrial fermentations. The wild-type ***Corynebacterium glutamicum***, for example, exhibits feedback inhibition when cellular glutamic acid concentrations rise to 5% on a dry weight basis.

Production of glutamic acid in large quantities is now carried out using mutants of *Corynebacterium glutamicum* that lack, or has only a limited ability to process, the TCA cycle intermediate. This can be achieved by limiting the ability to process the immediate precursor of L-glutamic acid, namely oxoglutaric acid, to the next intermediate of the tricarboxylic acid (TCA) cycle, succinyl coenzyme A (CoA), *i.e.* use of mutants lacking oxoglutaric acid dehydrogenase. During the growth phase these mutants produce essential intermediates from isocitrate via the glyoxylate cycle.

In addition, as these bacteria do not normally secrete glutamate, a range of treatments are employed to make the cells more permeable and aid release of the amino acid into the medium. These treatments include: biotin limitation, restriction of phospholipid biosynthesis by adding C16–C18 saturated fatty acids during the growth phase, and inclusion of surfactants (e.g. Tween 40) and penicillin in the production media.

✶ **Why is biotin starvation necessary for the excretion of glutamic acid?**

The major function of biotin is to serve as a prosthetic group in acetyl-CoA carboxylase, the first enzyme of fatty acid synthesis, so it was hypothesized that the excretion might be related to an increased general permeability of the cell membrane, caused by an insufficiency of fatty acids.

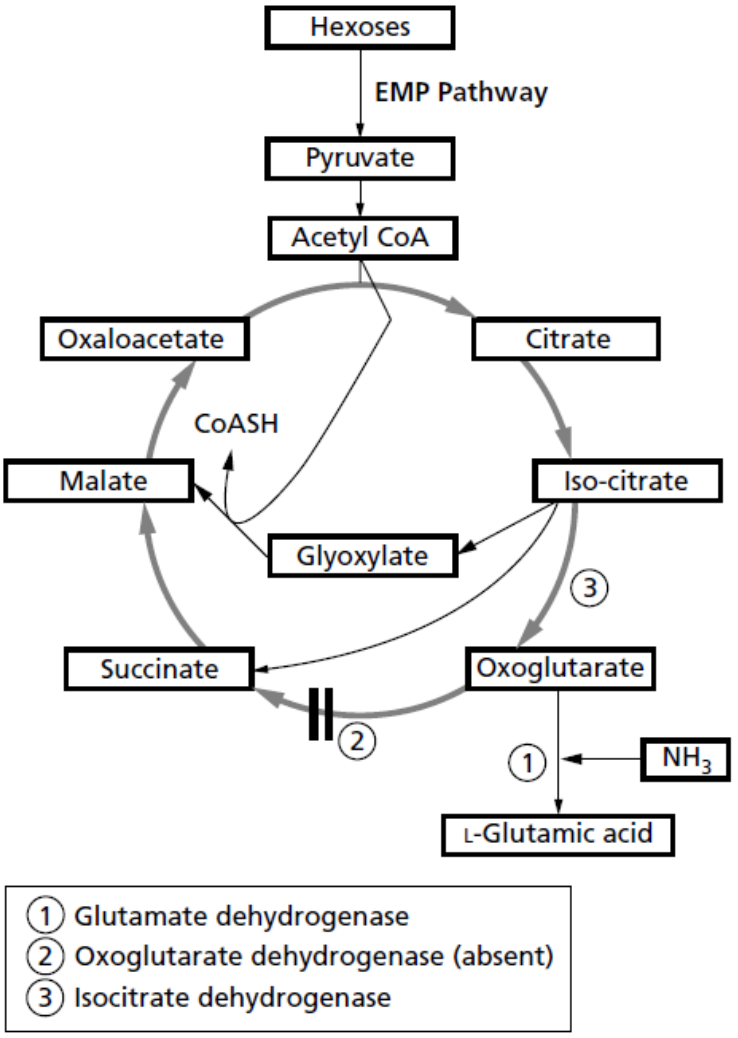


Figure: L-Glutamic acid biosynthesis in mutant strains.

➢ **Industrial production of L-glutamic acid**

Industrial-scale fermentors are normally stainless steel stirred tank reactors. These are batch processes, operated aerobically at 30–37°C, the specific temperature depending on the microorganisms used.

- **Media for glutamic acid production**

Apart from carbon and nitrogen sources, the fermentation medium normally contains inorganic salts, providing magnesium, manganese, phosphate and potassium, and limiting levels of biotin. Corynebacteria are nutritionally fastidious and may also require other vitamins, amino acids, purines and pyrimidines. The preferred carbon sources are carbohydrates, preferably glucose or

sucrose. Cane or beet molasses can be used, but the medium requires further modification as their biotin levels tend to be too high. This can be overcome by the addition of saturated fatty acids, penicillin or surfactants which promote excretion. The nitrogen source (ammonium salts, urea or ammonia) is fed slowly to prevent inhibition of glutamate production. Medium pH is maintained at 7–8 by the addition of alkali, otherwise the pH progressively falls as the glutamate is excreted into the medium. Accumulation of glutamic acid does not become apparent until midway through the fermentation, which normally lasts for 35–40 h and achieves L-glutamic acid levels in the broth of 80 g/L.

- **Product recovery**

Involves separation of the cells from the culture medium and the glutamic acid is then crystallized from the spent medium by lowering the pH to its isoelectric point of pH 3.2 using hydrochloric acid. Crystals of glutamic acid are then filtered off and washed.

L-Lysine

L-Lysine is not synthesized by humans and other mammals therefore; this 'essential' amino acid must be acquired as part of their diet. However, many cereals and vegetables are relatively low in lysine. Consequently, food products and animal feeds derived from these sources are often supplemented with this amino acid.

Over 90000 tonnes of this lysine are currently produced by direct microbial fermentation and biotransformation methods. The remaining portion is produced by chemical synthesis. However, this route has the major disadvantage that a mixture of the D- and L-isomers is synthesized, but it is only L-lysine that the body utilizes. Thus, optical resolution is required following chemical synthesis, whereas microbial production has the advantage that only the L-isomer is formed.

- **Development of lysine-producing microorganisms**

Metabolic control of lysine production in wild-type *Corynebacterium glutamicum* is shown in the figure below. The first key step of this metabolic pathway, aspartate to aspartyl phosphate, catalysed by aspartokinase, is controlled via feedback inhibition by two end-products of this branched pathway, lysine and threonine. Homoserine dehydrogenase activity is also subject to feedback inhibition by threonine and repression by methionine. However, dihydropicolinate synthetase is not inhibited by lysine accumulation, which is unusual for the first enzymes following the branch point of a pathway.

The over-producing strains of *Corynebacterium glutamicum* selected for lysine production have defects in these feedback control mechanisms. They lack homoserine dehydrogenase activity and are thus homoserine auxotrophs. These auxotrophs convert all aspartate semialdehyde to lysine, and because of the lack of threonine synthesis, there is no longer feedback control. However, carefully measured amounts of threonine, methionine and isoleucine must be added to the culture medium to enable this auxotrophic bacterium to grow.

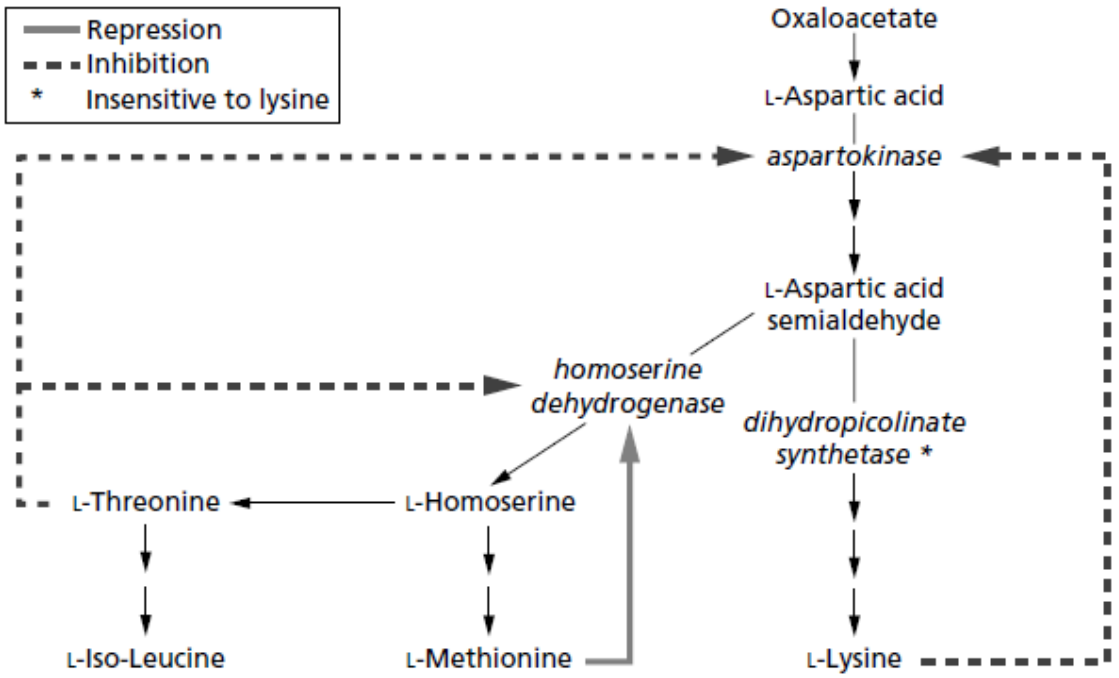


Figure Control of l-lysine production in *Corynebacterium glutamicum*

- **Industrial production of L-lysine**

Most commercial L-lysine fermentations are operated as batch processes in aerated stirred tank reactors. Cane molasses is the preferred carbon source, although other carbohydrates, acetic acid or ethanol can be used, often supplemented with soya bean hydrolysates. The temperature is held at 28°C and the pH is maintained at, or near, neutrality by feeding ammonia or urea, which also act as a nitrogen source.

Control of the biotin level is very important, as concentrations below 30mg/L result in the accumulation of L-glutamate instead of L-lysine. However, cane molasses usually contains sufficient biotin to fulfill this requirement.

Production of lysine starts in the early exponential phase and continues through to the stationary phase. These fermentations last about 60h and yield 40–45 g/L L-lysine from a molasses concentration of 200 g/L, containing 100 g/L sucrose.

Lysine recovery is relatively simple. Once the cells have been removed, the fermentation medium is acidified to pH 2.0 with hydrochloric acid and the lysine is adsorbed onto a cation-exchange column in the ammonium form. A dilute solution of ammonia is then used to elute l-lysine from the column. This eluate is re-acidified and the product is finally crystallized asL-lysine hydrochloride