PG Diploma in Biotechnology

Paper IV- Applications of Biotechnology

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Model Question Paper- IV

1. A. Explain the different preservation methods used for industrially importamt microorganisms?

Or

B. Write in detail about the starin improvement methods employed to industrially important microorganisms.

2. A. Explain how different organic acids being produced through fermentation?

Or

B. Write a detail account on fermentative production of Alcohol?

3. A. What is Immobilisation? Explain different techniques used for enzyme immobilization?

Or

B. What are Biosensors? Write a note on applications of Biosensors in various fields of Biotechnology?

4. A. Discuss the production of β- Lactum antibiotics and their applications?

Or

B. Give a detailed account on production of Tetracyclines? And its applications?

5. A. What is Vaccine? Discuss in detail the role of genetic engineering in production of different Vaccines?

Or

B. What are transgenic animals? Write a note on their applications in Medicine?

Lesson 4.1.1

ISOLATION & SCREENING

Contents

Objective

The objective of this lesson is to learn the different methods used for the isolation and screening of industrial microorganisms

4.1.1.1 Introduction

There are no universal screening methods. The success of a screening program depends upon the selection of appropriate tests as well as appropriate microorganisms to be tested. The capacity of an industrial screening group for isolation of microorganisms and through testing is around 1000-2000 strains per year.

Most screening programs focus on chemotherapeutically useful products for the following areas: activity against antibiotic-resistant strains, tumors, and viruses, as well as a search for enzyme inhibitors and pharmacologically active substances (hormones, etc.). Better starter cultures for the food industry as well as microorganisms that are capable of degrading hazardous and persistent chemicals are also sought.

Of the 10,000 antibiotically active compounds known in the late 1980's, 67% are produced by microorganisms (67% by actinomycets 9% by other bacteria, and 15% by fungi). Additionally, about 2000 other biologically active secondary metabolites are known, as well as a large number of enzymes.

4.1.1.2 Strains used in screening

This success of a screening program depends on both the kinds of organisms used and the methods for detection of activity. Currently, the choice of strain has a 30-40% influence on the outcome, the test procedure a 60-70% influence.

A gram of soil contain between 10^6-10^8 bacteria, 10^4 – 10^6 actinomycete spores, and 10^2-10^4 fungal spores. Less than 1% of the World's microorganisms have been intensively studied. Above all, the approximately 100,00 known fungi have been poorly studied.

In the isolation of new metabolic products, researchers try to isolate strains from extreme or unusual environments in the hope that such strains may be capable of producing new metabolites. For instance, microorganisms from high

altitudes, cold habitats, sea water, deep sea, deserts, geysers, and petroleum fields are being examined. Depending on the inoculum source and enrichment procedure, specific groups of organisms may be isolated.

Enrichment of microorganisms by selection of
Appropriate culture conditions

Enrichment methods	Type of isolate
Extreme pH values (pH 2-4)	Acidophiles
Low temperatures (4-15^{o}C)	Psychrotrophs
High temperatures (42-100^{o}C)	Thermopiles
High NaCl concentrations	Nocardia, halophiles
N_2 atmosphere	Anaerobes
Chitin as growth substrate	Lysobacter
Wood bark, roots	Myxobacteria
Pollen grains	Actinoplanes

The isolation of strains can be carried out with the following procedure.

The soil or water sample is suspended in a definite amount of
Sterile water to which Tween has been added as an emulsifying agent

↓

The sample is vigorously agitated

↓

The supernatant is diluted 10^{-1} – 10^{-10}

↓

Samples from this dilution series are plated on
Various culture media and then incubated

↓

Single colonies from the plates are picked and
Purified by re streaking

Te pure strains are maintained as agar cultures in test tubes

↓

The screening procedure can often be speeded up by testing the initial isolates directly for biological activity.

Test systems for screening of metabolites

Product sought	Test system
Antibiotics	Agar plates with strains of test microorganism For example, Staphylococcus aureus, Proteus Vulgaris, Candida albicans, Penicillium avellaneum, bacteria resistant to or hypersensitive to amino glycoside, macrolides, or β-lactam antibiotics. Inhibition zones as

	indicator of activity
β-Lactamase-resistant antibiotics	Agar plates with test microorganisms to which β-lactamase has been added
Proteases	Agar plates with casein, selection of colonies which produce clear zones on the turbid plates
Amylases	Agar plates with starch, selection of colonies after staining with iodine.
Lipases	Agar plates with oil emulsion, selection of colonies after precipitation of free fatty acids with Ca^{2+}
Phosphates	Agar plates with phenolphthalein-di phosphate and pH indicator, selection based on color change.
NAD	Agar with microorganism auxotrophic for NAD.

The soil or water samples are diluted directly onto the test plates and only those colonies showing activity are isolated.

The variability of metabolites produced by individual genera is some what limited except in the streptomycetes. For instance, except for industrial enzymes, Bacillus strains almost exclusively produce peptide antibiotics.

In one extensive study, 20,000 Actinoplanes strains were isolated, of which 13,000 were screened for the formation of antibiotics. Within a ten year span, 41 new antibiotics were isolated and characterized from these strains. These antibiotics turned out to be almost all either acetylmalomyl or aminoacid derivatives.

Test systems

The success of a screening procedure is quite dependent on the development of "intelligent" tests with which known or undesirable antibiotics can be eliminated and compounds with the required properties can be recognized.

For instance, in one screening programme (Oura et al., 1979), a procedure was set up to discover inhibitors of cell wall biosynthesis, because all known antibiotics with this mode of action have low toxicity. In the 1st phase, screening was done for metabolites which inhibited *Bacillus subtilis*, but did not inhibit acholeplasma laidlawii (which lacks a cell wall). In the 2nd phase, substances were sought which inhibited the synthesis of meso-diaminopimelic acid, a component of the cell wall of bacteria, but did not inhibit the incorporation of leucine, an indicator of protein synthesis. In the 3rd phase, substances whose molecular weights were greater than 1000 were eliminated by use of membranes, because larger molecules often elicit undesirable side effects when used therapeutically.

With these three procedures, culture filtrates of 10,000 strains (fungi, bacteria and actinomycetes) were screened. A new antibiotic, azureomycin, was discovered, and six known antibiotics were also reisolated.

The antibiotic penicillin is a β-lactam, and one mode of resistance is through the production of β-lactamase, an enzyme which splits the β-lactam ring. Inhibitors of β-lactamase might thus prove useful in permitting penicillin therapy against resistant organisms. For the screening of microbial β-lactamase inhibitors, supernatants of the cultures were placed on agar plates containing penicillin or cephalosporin and one of

the β-lactamase – producing microorganisms. Thus it could be determined during the 1st screening whether different β-lactamases could be inhibited.

Nozaki et al. (1987) used a screening procedure with β-lactam-hypersensitive mutants to isolate the antibiotic lactivicin, an antibiotic which combines with the penicillin-binding site of gram-negative and gram-positive bacteria even though it lacks a β-lactam ring.

Fleck and Strauss (1975) used molecular biology tests to discover an antitumor metabolite.

Continuous fermentation can provide a method to isolate from mixed cultures strains with improved properties. By raising the temperature or the alcohol concentration, microorganisms can be selected that are either thermophilic or alcohol tolerant. Similar tests can be used to isolate strains that produce temperature-stable extracellular enzymes.

The most important factor for the success of any fermentation industry is of a production strain. It is highly desirable to use a production strain possessing the following characteristics.

- → It should be a high-yielding strain.
- → It should have stable biochemical characteristics
- → It should not produce undesirable substances.
- → It should be easily cultivated on a large-scale.

4.1.1.3 Screening techniques

Detection and isolation of high-yielding species from the natural source material, such as soil, containing a heterogeneous microbial population is called screening.

There are many screening techniques but only a few are discussed here.
Usually, screening programs include

Primary Screening &
Secondary Screening

4.1.1.4 Primary screening

This includes some elementary tests required to detect and to isolate new microbial species exhibiting the desired property. With antibiotic producers, primary screening programmes serve to remove worthless microorganisms on the basis of relatively simple, fundamental criteria. The important selection criteria are the activity of antibiotics invitro, and possibly in vivo, against a small number of the most important microorganisms.

Primary screening is also needed in the case of other useful microbial species (i.e., microorganisms capable of producing organic acids, amines, vitamins, etc.). The evaluation of the primary screening in industrial research programmes may be made by citing some specific examples of screening procedures as under:

The crowded plate technique

It is the simplest screening & technique employed in detecting and isolating antibiotic producers. In consists of

Preparing a series of dilutions of the soil or other

source material for the antibiotic producing microorganism

↓

Spreading of dilutions on the nutrient agar plates

↓

Agar plates having 300 to 400 or more colonies per plate
are considered since they are helpful in locating
the colonies producing antibiotic activity

The ability of a colony to exhibit antibiotic activity
is indicated by the presence of a zone
Of growth inhibition surrounding the colony

↓

Such a colony is sub cultured to a similar medium

↓

Purified by streaking before making stock cultures

It is necessary to carry on further testing to confirm the antibiotic activity associated with a micro-organism, since the zone of inhibition surrounding the colony may, sometimes, be due to other causes such as a marked change in the pH value of the medium resulting from the metabolism of the colony, or a rapid utilization of critical nutrients in the immediate vicinity of the colony.

The crowded plate technique has a limited application, since it merely provides information regarding the inhibitory activity of a colony against the unwanted microbes that may be present by chance on the plate. Therefore, the technique has been improved upon by introducing the use of a `test-organism'. In this modified technique, agar plates which give well-isolated colonies (roughly 100 to 300 colonies per plate) after incubation are flooded with a suspension of the test organism. The formation of inhibitory zones around certain colonies indicates their antibiotic activity. The diameter of the zones of inhibition is measured in millimeters, to obtain a rough approximation of the relative amounts of antibiotic(s) produced by various colonies. The colonies of the antibiotic producers must be isolated and purified before further testing.

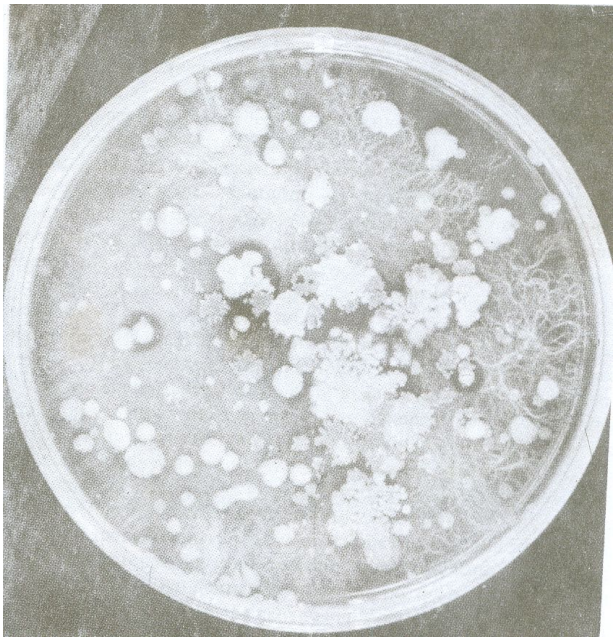

Fig. Crowded Plate Technique

Auxanography

This technique is largely employed for detecting micro-organisms able to produce growth factors (e.g. amino acids & vitamins) extra cellularly. The two major steps of the technique are

A. Preparation of first plate

A filter paper strip (1.5 x 12 cm) is put
Across the bottom of a Petri dish in such a way that
the two ends pass over the edge of the dish

↓

A filter paper disc of Petri dish size is placed over
Paper strip on the bottom of the dish

↓

The nutrient agar (45°C) is poured on the paper disc
In the dish and allowed to solidify

↓

Microbial source material such as soil, is subjected to
Dilution such that aliquots on plating will produce
Well isolated colonies

↓

Plating of aliquots of properly diluted soil sample is done

B. Preparation of second plate

A minimal medium lacking the growth factor under
Consideration is seeded with the test organism

The seeded medium, is poured on the surface
Of a fresh Petri dish

The plate is allowed to set

↓

The agar in the 1st plate, as prepared in step (A), is carefully and aseptically lifted out with the help of tweezers and a spatula and, placed without inverting, on the surface of the 2nd plate as prepared in major steps (B).

The growth factor (s) produced by colonies present on the surface of the 1st layer of agar can diffuse into the lower layer of agar containing the test organism. The zones of stimulated growth, of the test organism around the colonies are an indication that they produce growth factor (s) extracellularly. Productive colonies are sub-cultured and are further tested.

Enrichment culture technique

This technique was designed by a soil microbiologist, Beijerinck, to isolate the desired micro-organisms from a heterogeneous microbial population present in soil. Either medium or incubation conditions are adjusted so as to favour the growth of the desired micro-organisms. On the other hand, unwanted microorganisms are eliminated, or develop poorly since they do not find suitable growth conditions in the newly created environment. Today, this technique has become a valuable tool in many screening programmes meant for isolating industrially important stains.

Generally, it consists of the following steps :

Nutrient broth containing an unusual
substrate (e.g. Cellulose Power) is inoculated
with the microbial source material (e.g. soil) and incubated.

↓

A small position of inoculum from step (1) is plated
onto a solid medium having the same composition.
Well isolated colonies appear after incubation.

↓

Suspected colonies from plate of step (2) are sub-cultured
on fresh media and they are also subjected to further testing.

An example of screening of enzyme producing micro-organisms may be cited. Microorganisms excreting alkaline proteases may be detected from the soil as.

Soil is subjected to serial dilution

All serial dilutions are heated at 80°C for 10 minutes

This treatment kills vegetative cells but spores are main unaffected

The plating of heat treated samples is done by spreading the samples (usually 0.1 ml) from dilutions onto the surface of nutrient agar containing casein at pH 10-12.

The colonies surrounded by a clear zone are sub-cultured

Use of an Indicator Dye

The pH indicating dyes may be employed in some screening methods for detecting micro-organisms capable of producing organic acids or amines, since a pH indicating dye undergoes colour changes according to its pH. Dyes such as neutral red, bromothymol blue, etc., are added to the poorly buffered nutrient agar media. The change in the colour of a dye in the vicinity of the colony suggests the capability of colonical cells to produce either organic acid(s) or amine(s), depending upon the nature of reaction. Such colonies are sub cultured to make stock cultures. But, further testing is required, since inorganic acids or bases are also potential metabolic products of microbial growth.

This differential technique may also be employed in finding out whether micro-organisms are capable of certain microbial transformations or not. E.g. Δ-7 oestrogen formation by bioconversion.

An alternative procedure for detecting organic-acid production involves the incorporation of calcium carbonate in the medium so that organic-acid production is indicated by a cleared zone of dissolved calcium carbonate around the colony.

Another method of isolation is to screen a microbial source in order to find microorganisms capable of utilizing a specific carbon or nitrogen nutrient for growth and biosynthesis. To achieve this, the plating medium is made up so as to contain the particular nutrient as its only source, respectively, of carbon or nitrogen. Dilutions of soil are applied to the plates, and the colonies appearing after incubation are assumed to possess the desired attribute. However, each organism must be attributed to nutrient sources other than those incorporated in the medium. Thus, if only low dilutions of soil are employed, there may be a carry-over of carbon and nitrogen nutrients from the soil which would allow at least limited growth of many microorganisms on the plating medium. Also, a microorganism on the plate which can utilize the specific carbon or nitrogen substrate may only partially degrade it or, in the process, may synthesize other carbon or nitrogen compounds that will allow growth of a few adjacent microorganisms that normally could not grow because of lack of the metabolite.

A modification of the latter technique is employed when volatile substrates such as hydrocarbons, low molecular weight alcohols, and similar carbon sources are being considered. With these substrates, often it is not necessary to incorporate the specific substrate into the agar medium. The dilutions from a microbial source are applied to plates of agar media containing all nutrients but the specific substrate, and the specific substrate is placed in the lid of the Petri plate after inversion of the plate. Enough vapors from the volatile substrate rise to the surface of the agar within this closed

atmosphere to provide the specific nutrient for the microorganisms. Obviously, this procedure is of particular value when the volatile substrate is either immiscible or insoluble in water and, hence, difficult to disperse in an agar medium.

These are only a few examples of primary screening techniques.

4.1.1.5 Secondary screening

Primary screening allows the detection and isolation of microorganisms that possess potentially interesting industrial applications. It does not provide much information needed in setting up a new fermentation process.

Secondary screening in strictly essential in any systematic screening programme intended to isolate industrially useful micro-organisms. Secondary screening helps in detecting really useful micro-organisms in fermentation processes. This can be understood from the following points associated with secondary screening:

- → It is very useful inn sorting out micro-organisms that have real commercial value from many isolates obtained during primary screening. At the same time, microorganisms with poor applicability are discarded. Poor cultures should be discarded as soon as possible, as such studies involve much labour and high expense.
- → Provides information whether the product produced by a microorganism is a new one or not. This information is obtained by paper, thin layer, or other chromatographic techniques. The compound under consideration is compared with previously known compounds.
- → It gives an idea about the economic position of the fermentation process involving the use of a newly discovered culture.
- → Helps in providing information regarding the product yield potentials of different isolates. Thus, this is useful in selecting efficient cultures for the fermentation processes.
- → Determines the optimum conditions for growth or accumulation of a product associated with a particular culture.
- → Provides information pertaining to the effect of different components of a medium. This is valuable in designing the medium that may be attractive so far as economic consideration is concerned.
- → It detects gross genetic instability in microbial cultures. This type of information is very important, since micro-organisms tending to undergo mutation or alteration in some way may lose their capability for maximum accumulation of the fermentation products.
- → It gives information about the number of products produced in a single fermentation.
- → Information about the solubility of the product in various organic solvents is made available. This information is useful in the recovery and purification of the product.
- → Chemical, physical and biological properties of a product are also determined during secondary screening. It reveals whether a product produced in the culture broth occurs in more than one chemical form.
- → It reveals whether the culture is homofermentative or hetero fermentative.
- → Determination of the structure of the product is done. The product may have a simple, complex or even a macromolecular structure.

- → With certain types of products (e.g. antibiotics) determinations of the toxicity for animal, plant or man are made if they are to be used for therapeutic purposes.
- → It reveals whether micro-organisms are capable of a chemical change or of even destroying their own fermentations products. For instance, micro-organisms that produce the adaptive enzyme, decarboxylase, can remove carbon dioxide from the aminoacid, leaving behind an organic amine.

→ It tells us something about the chemical stability of the fermentation product.

Thus, secondary screening gives answers to many questions that arise during the final sorting out of industrially useful micro-organisms. This is accomplished by performing experiments. On agar plates, in flasks or small bioreactor containing liquid media, or by a combination of these approaches.

For ex:

The antibiotic producing Streptomyces species may be taken for an understanding of the screening procedure.

In Primary Screening procedures, the streptomycetes that are able to produce antibiotics are detected and isolated. These organisms, which exhibit antimicrobial activity are subjected to an initial secondary screening where their inhibition spectra are determined. A simple `Giant-Colony Technique' is used for this determination.

Each of the Streptomycin isolates is streaked in a narrow band across the centers of the nutritions agar plates. Then, these plates are incubated until growth of a streptomycete occurs. Now, the test organisms are streaked from the edges of the plates up to, but not touching the streptomycete growth. Again, the plates are incubated. At the end of incubation, growth inhibitory zones for each test organism are measured in millimeters. Thus, the `microbial inhibition spectrum' study extensively helps in discarding poor cultures. Ultimately, streptomycete isolates that have exhibited interesting microbial inhibition spectra need further testing. With Streptomycetes suspected to produce antibiotics with poor solubility in water, the initial secondary screening is done in some different way.

Further screening is carried out employing liquid media in flasks, since such studies give more information than that which can be obtained on agar media. It is advisable to use accurate assay techniques (e.g., paper disc-agar media) to exactly determine the amounts of antibiotic present in samples of culture fluids. Thus, each of the streptomycete isolates is studied by using several different liquid media in Erlen meyer flasks provided with baffles. These streptomycete cultures are inoculated into sterilized liquid media. Then, such needed flasks are incubated at a constant temperature. Usually, such cultures are incubated at near room temperature. Moreover, such flasks are aerated by keeping them on a mechanical shakes, since the growth of streptomycetes and production of antibiotics occur better in aerated flasks than in stationary ones. Samples are withdrawn at regular intervals under aseptic conditions and are tested in a quality control laboratory.

Important tests to be carried out are:

i. Checking for contamination,
ii. Checking of pH,
iii. Estimation of critical nutrients,
iv. Assaying of the antibiotic &
v. Other determinations, if necessary.

The results of the above tests determines which medium is the best for antibiotic formation, and at which stage the antibiotic yields are greatest during the growth of the culture on the various media.

After performing all necessary routine tests in the screening of an actually useful streptomycete for a fermentation process, other additional determinations are made. They are:

1. Screening of fermentation media through the exploitation of which the highest antibiotic yields may be obtained.
2. Determination of whether the antibiotic is new.
3. Determination of the number of antibiotics accumulated in the culture broth is made.
4. Effect of different bioparameters on the growth of streptomycete culture, fermentation process and accumulation of antibiotic.
5. Solubility picture of antibiotic in various organic solvents. Also it is to be determined whether antibiotic is adsorbed by adsorbent materials (e.g. ion-exchange resins or activated carbon). This information is necessary in the recovery & purification of an antibiotic from the fermented broth.
6. Toxicity tests are conducted on mice or other laboratory animals. An antibiotic is also tested for the adverse effects if any, on man, animal or plant.
7. The streptomycete culture is characterized & is classified up to species.
8. Further studies are made on a selected individual streptomycete culture. For example, mutation & other genetic studies for strain improvement are carried out.

In conclusion, tests are designed and conducted in such a way that production streptomycete strains may be obtained with least expenses.

Similar screening analytical techniques could be employed for the isolation of microbial isolates important in the production of other industrial chemical substances.

4.1.1.6 Summary

The screening of microorganisms producing chemotherapeutically useful products includes two different steps .One is primary screening and the second one is secondary screening .The different methods used in primary screening are as follows. The crowded plate technique (detects and isolates antibiotic producers). Auxanography (for detecting micro-organisms able to produce growth factor) Another method is enrichment culture technique in which either medium or incubation conditions are adjusted so as to favour the growth of the desired micro-organisms. On the other hand, unwanted microorganisms are eliminated, or develop poorly since they do not find suitable growth conditions in the newly created environment .The pH indicating dyes may be employed in some screening methods for detecting micro-organisms capable of producing organic acids or amines, since a pH indicating dye undergoes colour changes according to its pH Another method of isolation is to screen a microbial source in order to find microorganisms capable of utilizing a

specific carbon or nitrogen nutrient for growth and biosynthesis. Primary screening allows the detection and isolation of microorganisms but not provide much information needed in setting up a new fermentation process Secondary screening in strictly essential in any systematic screening programme intended to isolate industrially useful micro-organisms. Secondary screening helps in detecting really useful micro-organisms in fermentation processes.

4.1.1.7Model questions

1)What is screening and explain different methods of screening of organisms

4.1.1.8 References

Industrial microbiology by Cassida
Principles of fermentation technology by Stanberry

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Lesson 4.1.2

PRESERVATION OF INDUSTRIALLY IMPORTANT MICROORGANISMS
(General methods)

Contents

Objective

The objective of this lesson is to learn the importance of preservation and different methods used in preservation their advantages and disadvantages

4.1.2.1 Introduction

The isolation of a suitable organism for a commercial process may be a long and very expensive procedure and it is therefore essential that it retains the desirable characteristics that led to its selection. The nature of work being done will determine whether the preservation requirement is only very short-term (e.g., a few days) or for an unlimited time period (e.g., many years). Long-term preservation of a culture is required if a culture is to be deposited in one of the service culture collection with a view to preserving something of scientific value "for perpetuity". Also, the culture used to initiate an industrial fermentation must be viable and free from contamination. Thus, industrial cultures must be stored in such a way as to eliminate genetic change, protect against contamination and retain viability. An organism may be kept viable by repeated subculture into fresh medium, but at each cell division, there is a small probability of mutations occurring and because repeated sub-culture involves very many such divisions there is a high probability that strain degeneration would occur. Also, repeated sub-culture carries with it the risk of contamination. Many methods of preservation for microorganisms have been developed. We know that there exist different types of microorganisms (bacteria, viruses, algae, protozoa, yeasts and moulds). Therefore there are 2 criteria for selecting a method of preservation for a given culture. They are :

i. The period of preservation desired, and
ii. The nature of culture to be preserved.

With the increasing importance of micro-organisms to industry (e.g., in biochemical and antibiotic production, bio-assay, as spoilage microbes, and the like), human, animal and plant pathologists, geneticists, taxonomists and teachers have felt the need for culture collections. There are several large public service collections. These serve as repositories for cultures and as sources of their distribution.

The best known of these are

The Central Bureau Voor Schimmelcultures (C.B.S), founded in 1906

The American Type Culture Collection (A.T.C.C.), founded in 1925, &

The collection of the Common-Wealth Mycological Institute (C.M.I), Founded in 1947.

Any biologist dealing with living material must at least temporarily maintain his own cultures during the course of his studies and preserve them until they are ready for depositing them in one of these major collections.

4.1.2.2 Aims of preservation

There are three basic aims in maintaining and preserving the micro-organisms. They are :

i. to keep cultures alive
ii. uncontaminated, and
iii. as healthy as possible, both physically and physiologically, preserving their original properties until they are deposited in any major collections (i.e., unchanged in their properties).

For very long-term preservation, involving stocks of the strains (as opposed to single specimens of each strain) and where with drawls from stocks are regularly made, a fourth aim is

- to have adequate stocks and appropriate systems for replenishing stocks when necessary.

This fourth aim is concerned with the service culture collections where as the above three are the concern of any maintenance and preservation programme.

The running of the collection and the methods of maintenance used are designed to minimize the following hazards to which cultures are exposed:

→ By repeated transfer selection can occur, either of a mutant strain or of a purely vegetative non-sporulating form. The transfer should, therefore, be done as far as possible by an expert with an eye for the wild strain. However, the fewer transfers made, the less is the risk.

→ Some strains, sometimes, tend to become attenuated under the artificial conditions of culture. Others deteriorate to wet slimy disintegrated mycelium or spores. Simmons (1963) suggests that this may be due to virus infections and there is considerable evidence to support his theory.

→ The maintenance processes to which the microorganism (e.g. the fungus) is subjected are selective, and only adaptive strains survive. These may have some what a typical characteristic.

→ Culture are subject to contamination, infection with mites and adverse conditions of temperature, light, humidity, etc., are responsible for their contamination. The latter may arise through breakdown of apparatus, or by incomplete understanding of the organism.

→ Adequate documentation of the strains must be made. In a culture collection of long standing the strains may well survive several generations of microbiologists,

so to assist in maintaining them in their original condition a clear description of the cultural characteristics supported by dried cultures should be provided at the time of depositing them.

Methods of preservation

4.1.2.3 Serial subculture

→ This is the simplest and most common method of maintaining microbial cultures.

→ The technique involves

Agar slants are prepared

↓

Inoculated with the desired Microorganism

↓

Incubated

After several days (15 days – 1 month) of incubation, the microbes are transferred to fresh media before they exhaust all the nutrients or dry out.

An exception to this is aerobic Streptomyces spp. Where drying up of the medium has been found successful, provided the initial growth showed the production of aerial hyphae. The drying-up of the medium appeared to encourage good sporulation and the preserved specimen became simply a dried out strand of agar coated with spores which remained viable for a few years at room temperature. For some microbial cultures, no other methods have been found satisfactory, but for the majority of species other methods are available.

There are several factors to be borne in mind while choosing a suitable medium. Solid media should be chosen in preference to liquid media, as growth of a contaminant can be more readily observed. However, bacteriophage are often successfully maintained in a liquid medium (e.g. Robertson's Cooked Meat). Some technicians prefer stab cultures for maintenance. But there do not appear to be any published data to show these to be any better than slope cultures. Obviously, if the microorganism is oxygen sensitive, a stab culture would be suggested as an extra safeguard while handling on the bench. While a rich medium may give the best initial growth for heterotrophs, it may also run the risk of accumulating toxic end-products of metabolism. Therefore, the best medium for growth may not necessarily be the best for maintenance and preservation of necessarily be the best for maintenance and preservation of microorganisms. Besides a suitable substratum, other factors affect the growth of cultures for storage. They are :

Light intensity, temperature, humidity, standard growth conditions, method of transfer, culture vessel and storage.

The time period appropriate for subculture may range from a week to even a few years.

Procedure for the preservation of some-bacteria by serial subculture

Bacteria	Medium	Transfer time	Incubation temp-erature ^{o}C	Storage Tempe-rature ^{o}C
Bacillus spp	Nutrientagar	12months or longer	28	10

Pseudomonas Spp	Nutrientagar	3 months	28	10
Clostridium spp	Robertson's cooked meat medium	6 months or longer	28	Room temp-erature

Under normal conditions, cultures have to be re-grown at fairly frequent intervals (e.g., every 4, 6, or 8 months). With a large collection, this requires much lab our. Moreover, there is a risk of occurring hazards as discussed previously, every time a culture is handled. To cut down the frequency of handling of the cultures, it is, therefore desirable to prolong the intervals between sub culturing. There are various means to accomplish this (e.g. cold storage and mineral oil storage).

4.1.2.4 Preservation by overlaying cultures with mineral oil

- → This is a modification of serial sub-culture technique.
- → It was 1st extensively used by Buell and Weston (1947).
- → Of 2000 fungus strains maintained under oil for 10 years at the C.M.I, only 45 were lost.
- → The method is cheap and easy, since it does not require special skills or apparatus such as a centrifuge, dessieator, or vacuum pump.
- → The technique involves.

Preparation of Agar slants

Inoculation of the agar slant contained in a screw-cap tube with a given culture is practiced

|

Subjected to incubation until good growth appears

Using sterile technique, a healthy agar slant culture (from above step)

|

Is covered with sterile mineral oil to a depth of about 1um. Above The top of the agar slant

If a short slant of agar is used, less oil is required

Finally, oiled culture from step 4 can be stored at room temperature

Better viability is obtained when stored at lower temperatures (e.g.15^{o}C)

- → The oil used should be of good quality
- → British pharmacopoeia medicinal paraffin oil of specific gravity 0.865 to 0.890 is quite satisfactory.
- → Sterilization of oil at the C.M.I. is done in Mc Cartney bottles for 15 minutes at 1516/in^{2}.
- → The covering of the culture with oil prevents drying out.
- → This may induce change due to adaptation to growth in oil.
- → Some fungus isolates appear stable and survivals of over 20 years have been obtained at the C.M.I. Others change rapidly, producing a typical culture in a few months (e.g., Fusarium species).

The depth of oil of 1 cm. Is fairly critical (Fennel, 1960), as the oxygen transmission by layers of mineral oil in excess of 1 cm. Becomes less favorable. If less oil is used,

strands of mycelium may be exposed which allows the cultures to dry out (Dade, 1960; Fennell, 1960). If the Mc Cartney bottles are used the rubber liners should be removed from the metal caps as the oil tends to dissolve the rubber and this can be toxic to the cultures.

Advantages

- → Practically all bacterial species or strains tested live longer under oil than in the control tubes without oil. Somme bacterial species have been preserved satisfactorily for 15 to 20 years.
- → Transplants may be prepared when desired without affecting the preservation of the stock cultures.
- → The method is especially advantageous when working with unstable variants where occasional transfers to fresh media or growth in mass cultures results in changes in the developmental stages of the strains.

This method appears to be an ideal method of storage for a busy laboratory with limited funds and a relatively small collection.

4.1.2.5 Other methods of storage for fungi

Every laboratory cannot afford lyophilization or liquid nitrogen storage. Many technicians lack the time for the periodic transfer to fresh media and they would find oil storage messy. Indeed, in these days, when the safety of the personnel is the first consideration, the spatter made by oily needles when they are sterilized is undesirable. Therefore, various other methods of preservations have been developed.

4.1.2.6 Soil cultures

This method is particularly applied for the preservation of sporing microbes (e.g., Bacillus, Penicillium, Aspergillus and Streptomyuces).

Steps involved in this method are:

i. A spore suspension is first prepared which may involve the use of a special medium.
ii. A mixture of soil (20%), sand (78%) and calcium carbonate (2%) is prepared and distributed into tubes (a few grams per tube). They are sterilized for 8 to 15 hours at 130^{o}c and then cooled.
iii. A spore suspension is added to the sterilized loam as prepared in step (ii) and allowed to grow for about 10 days.
iv. The inoculated tubes as in step (iii) are kept in a despiccator under vacuum. The reason behind this is to evaporate the excess water. Then the tubes are sealed.
v. The culture tubes are stored in a refrigerator at about 5 to 8^{o}c temperature. Thus the spores may be preserved for many years without germination.

4.1.2.7 Silica gel cultures

A spore suspension in 5% skimmed milk is added to anhydrous silica gel in screw capped bottles. This is allowed to dry for about 14 days. Then the caps are screwed down. Usually the bottles are stores in a refrigerator, though storage at room temperature can be considered quite satisfactory. The precaution to be taken is to cool everything well before starting the process. The reason behind this is heat liberation when water is added to silica gel. Very good revivals are obtained over several years.

This is an adaptation of the method of Roberts following Ogata (1962) who used it for the preservation of genetic stocks of Neurospora.

4.1.2.8 Water storage

Surprising success has been reported (Boeswinkel, 1976; Mrx and Daniel, 1976) for storage of fungus cultures in water.

Steps involved in this method are:

i. The fungus is grown on a weak medium in a Petri-dish and then cut into small pieces.
ii. By using sterile technique, the pieces are transferred to sterile distilled water in small screw capped bottles.
iii. The cap is screwed down.
iv. The cultures are stored, preferably at a low temperature.

Other methods of preservation are :
Storage of spores in sand,
Drying cultures on filter paper, and
Sealing tubes with paraffin wax.

4.1.2.9 Summary

There are three basic aims in maintaining and preserving the micro-organisms.Theyare to keep cultures alive ,uncontaminated, and as healthy as possible, both physically and physiologically, preserving their original properties. TheMethods of preservation are Serial subculture nothing but continuous subculturing periodically,by overlayering with mineral oil that prevents the penetration of oxygen, the freeze drying,Storage under Liquid Nitrogen Soil cultures for sporing microbes Soil cultures for storin Neurospora,and water cultures for storage of fungus cultures in water.

4.1.2.10 Model questions

1)Discuss in detail different methods of preservation?

4.1.2.11 References

Microbiology by Presscott
Microbiology by Pelzar

Fermentation technology by Stanburry

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Lesson 4.1.3

Preservation of micro organisms (at low temperatures)

Structure

Objective

The Main objective of this lesson is to bring about the different methods for preserving microorganisms at low temperatures.The methods include lyophilization and cryopreaservation

4.1.3.1 Introduction

All practicing microbiologists have felt the need to preserve the viability of micro-organisms with which they work. In addition, all the cultural characteristics of a culture, as they were at the time of preservation, must be conserved. The nature of work being done will determine whether the preservation requirement is only very short-term (e.g. a few days) or for an unlimited time period (e.g. many years). Long-term preservation of a culture is required if a culture is to be deposited in one of the service culture collections with a view to preserving something of scientific value"for perpetutity". Many methods of preservation for micro-organisms have been developed. Here, it is to be noted that there exist different types of micro-organisms (bacteria, viruses, algae, protozoa, yeasts and moulds). Therefore, there are two criteria for selecting a method of preservation for a given culture. They are:

(i) The period of preservation desired, and
(ii) The nature of a culture to be preserved.

With the increasing importance of micro-organisms to industry (e.g. in biochemical and antibiotic production, bio-assay, as spoilage microbes, and the like), human, animal and plant pathologists, geneticists, taxonomists and teachers have felt the need for culture collections. There are several large public service collections. These serve as repositories for cultures and as sources of their distribution.

The long term preservation methods involves the drying and storing the cultures under low temperatures .The important methods are

1)Lyophilization
2)Cryopreservation

4.1.3.2 Lyophilization (or) Freeze-drying

- → It is the most satisfactory method of long-term preservation of microorganisms.
- → It is widely used for the preservation of bacteria, viruses, fungi, sera, toxins, enzymes and other biological materials.
- → Lyophilization, or freeze-drying, involves the freezing of a culture followed by its drying under vacuum, which results in the sublimation of the cell water.

The technique involves.

↓

Growing the culture to the maximum stationary phase

Re suspending the cells in a protective medium
Such as milk, serum or sodium glutamate
(at the Common Wealth Mycological Institute, 10% skimmed milk and 5% inositol in distilled water is found suitable).

↓

Using a sterile technique, the suspension from step 2 is
Distributed in small quantities into glass ampoules.

↓

The ampoules are connected with a high vacuum system
Usually incorporating a desiccant (e.g., phosphorus
Pent oxide, silica gel or a freezing trap), and immersed
Into a freezing mixture of dry ice and alcohol (-78^{o}c)

↓

The vacuum pump is turned on and the ampoules are
Evacuated till drying is complete, after which they may
Be sealed off.

↓

The ampoules may be stored in a refrigerator and the
Cells may remain viable for 10 years or more.

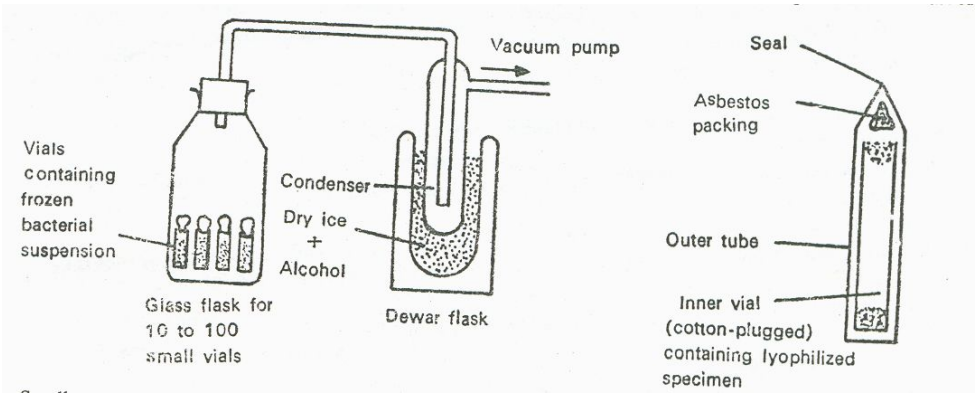


Fig- Lyophilisation of Microorganisms

→ The process of lyophilization was 1st applied to micro fungi on a large scale by Raper and Alexander in 1942.
→ They were successful in processing the cultures at the N.R.R.L. (Northern Regional Research Laboratory) at Peoria and presented their work in 1945.

- → The method of lyophilization may vary from one laboratory to another.
- → At the C.M.I. a centrifugal two-stage freeze dryer is used. In this the cooling is by evaporation and no freezing mixture is required. After drying, the small vials can be filled with sterile dry nitrogen instead of sealing under vacuum.
- → The methods of revival vary from one laboratory to the other. In case of fungi, dry pellets may be transferred to a suitable liquid and allowed to dissolve before it is streaked out on Agar.
- → At the C.M.I., a volume of sterile water equal to the original volume of the spore suspension is placed in the ampoule at room temperature. Then, the ampoule is left for about 20 to 30 minutes for the water to be absorbed slowly before streaking out. This delay seems to produce more satisfactory cultures. The degree of viability is assessed visually or by spore counts.

Factors affecting the viability of freeze-dried cultures include.

i. Chemical composition of the protective (suspending) medium.
ii. Addition of certain compounds to the culture suspension before freeze-drying to give protection to the culture against the toxicity exerted by moisture and oxygen when stored in unsealed ampoules.
iii. Insufficient elimination of oxygen and moisture on the survival rate of freeze-dried culture, and
iv. Storage temperature of freeze-dried and sealed cultures.

Advantages

i. As the ampoules are sealed there is no risk of contamination or infection with mites.
ii. The prepared ampoules are easily stored, they are not readily broken and most species remain viable for many years (more than 20 years in case of many bacterial species.
iii. There is less opportunity for cultures to undergo changes in characteristics (i.e., they remain unchanged during storage period).
iv. During to the small size of glass ampoules, hundreds of lyophilized cultures can be stored in a small storage space. In addition to this, the ampoules' small size makes them ideal for postage.
v. Lyophilization cuts down the number of transfers.

Checking of viability may be done at long intervals. This may be done when cultures are required or by routine sampling. Usually, many replicates are made so that the material from a constant source can be supplied over a considerable period. It makes the `Seed stock' system possible, which is used at the American Type Culture Collection (Clark and Loegering, 1967) for conserving living reference microbes over long periods.

Clark and Loegering describe this method as follows :

"In this system the ampoules of freeze-dried or frozen culture material from original stocks are set aside as seed stock, and stored under optimum temperature conditions. This material is never distributed. Periodically an ampoule of seed stock is opened, and the culture grown from it re-frozen or re-lyophilized in sufficient quantity for distribution. It is also used to prepare new seed stock if the viability of original material declines or the original seed stock becomes nearly depleted. In this way original material can be conserved over a long period".

4.1.3.3Storage at very low temperatures (or) Nitrogen storage (or) Storage under Liquid Nitrogen (or) Cryogenic storage

The metabolic activities of micro-organisms may be reduced considerably by storage at the very low temperatures (-150^{o} to –196^{o}) which may be achieved using a liquid nitrogen refrigerator.

Snell (1991) claimed that this approach is the most universally applicable of all preservation methods. Fungi, bacteriophage, viruses, algae, yeasts, animal and plant cells and tissue cultures have all been successfully preserved. This technique is also a satisfactory method for the long-term preservation of micro-organisms.

According to Meryman (1956), life is regarded as "at a standstill at –130^{o}c, and below, so at the temperature of liquid nitrogen (-196^{o}c), provided the cultures survive the treatment, the period of preservation should be indefinite. Thus, long-term preservation (storage) without any change in the cultural characteristics is now attainable.

Hwang protects her material with 10% glycerol and cools it slowly at the rate of about 1^{o}c. Per minute to a temperature of –35^{o}c. Thereafter, further cooling of the temperature from –165^{o}c to –196^{o}c was uncontrolled and faster. The procedure and apparatus are fully described (Hwang, 1966). She is able to preserve mycelial forms, Saprolegniaceae, Pythiaceae, Entomophthoraceae and Other fungi which would not survive the vigorous process of lyophilization as well as the more resistant moulds.

The technique involves:

Growing a culture to the maximum stationary phase

Resuspending the culture as a cell or a spore suppension, as finely broken-up particles of mycelium, or as a piece of fungus mycelium in a suitable cryoprotective agent (such as 10% glycerol used at the Common Wealth Mycological Institute)

The suspension as prepared in step II, is distributed into ampoules (These must be resistant to cold-shock) At the A.T.C.C. 1.2ml. Wheaton `Goldband' prescored borosilieate Glass ampoules have been found satisfactory. The ampoules must not be overfilled (less than 0.5ml being recommended)

Ampoules filled with a culture suspension are frozen and are hermetically sealed. Hwang (1966) recommends precooling to 7^{o}c, before freezing begins. Freezing can be attained by plunging the ampoules straight into the liquid nitrogen by suspending them over the liquid nitrogen for a short period and then lowering them into the liquid nitrogen or by controlled cooling. There are many interacting factors concerning the choice of the method of freezing and it will depend largely on he micro-organisms to be frozen, the degree of revival required and the apparatus available.

The frozen ampoules prepared as in (iv) are usually clipped on metal (aluminimum) canes, one above the other and six to each cane. The canes, in turn, are packed in metal boxes or canisters (aluminium), which hold about 20 canes. These are perforated to allow the free running of the liquid nitrogen. The cultures are revived by removing from the container, rapidly thawing and culturing them in the usual way.

Precautions

Cracked or faulty ampoules are dangerous. The reason is that the liquid nitrogen may penetrate and fill the ampoules. At the time of thawing, such ampoules (filled with the liquid nitrogen) may explode due to the sudden expansion of the nitrogen into gas. The storage nitrogen may also become infected.

If the thawing is done in water and if an explosion occurs, the water will tend to hold the fragments of glass. But it is recommended that gloves and eye or face shield be worn when thawing out ampoules. The nitrogen itself should be handled carefully because of the risk of cold burns. Great care should be taken while handling any metal parts in direct contact with the liquid nitrogen. There is a small risk of asphyxiation while handling it in a confined space, since the gas is odourless and colourless. Hence, the presence of nitrogen in the atmosphere may not be noticed. Therefore, the nitrogen containers should be housed in a well-ventilated room.

Advantages

- → It is an effective method of preservation.
- → No subculturing is required.
- → The cultural characteristics remain unchanged. This could be of particular importance while storing highly specialized strains employed in industrial processes.
- → The ampoules are not open to contamination or infection by mites, since they are sealed.
- → The living material of a type which would not normally grow in a culture and would not be preserved in a culture collection can be retained in a viable state.
- →

Dis advantages

- → The method is expensive, since a costly apparatus is required.
- → A reliable supply of nitrogen is needed.
- → There is a possibility of a minor accident, since the method involves the use of explosive gas (the liquid nitrogen).

4.1.3.**4 Summary**

Both lyophilization and the cryopreservation are effective methods for preserving the microorganisms for a long time . They reduces the need for repeated sub culturing during the maintenance of cultures.Both are advantageous techniques for all most all types of organisms**.**

4.1.3.5 Model questions

1) Explain the process of cryopreservarion technique?
2) Explain the advantages and disadvantages of lyophilization?

4.1.3.6 Reference books

Microbiology by Presscott
Microbiology by Pelczar
Fermentation technology by Stanburry

Author-**P.Jaganmohan Rao,**
Ch.Chakravarthi.

Lesson 4.1.4

IMPROVEMENT OF INDUSTRIALLY IMPORTANT MICROORGANISMS

Structure

Objective:

The main objective of this lesson is to describe different methods for improvement of industrially important microorganisms

4.1.4.1Introduction

It is highly desirable that the industrial fermentation process should be made more and more economical. This largely depends upon the efficiency of the production strain involved in the fermentation process. Therefore, a person interested in starting a fermentation industry or in competing with other industries must procure an efficient strain. Thus it is clear that, the use of a high-yielding strain in any fermentation process is the most critical factor. Usually, newly isolated strains obtained by screening techniques are not so efficient as could be used in industrial fermentation processes. Therefore, such strains require improvement, so far as the yield of a particularly desired compound is concerned. This is accomplished by producing the mutant fermentation strains with the help of physical or chemical methods. These mutants may be grouped into two major categories:

(i) auxotrophic mutants, and
(ii) mutants resistant to analogues.

4.1.4.2 Auxotrophicmutants:

Micro-organisms, usually, have regulatory mechanisms that control the amount of metabolites synthesized. Therefore, micro-organisms cannot synthesize excess of the metabolites over limiting the cells' requirements Obviously, suppression of these regulatory mechanisms is necessary to develop the strains for higher yields of the desired metabolites. Microbial cultures which have multivalent mechanisms, concerted repression or feed-back inhibition may be used for strain improvement. Subsequently, a search is made for mutants which have lost the ability to synthesize one of the end products capable of feed-back inhibition or repression. This may be explained by considering a situation where three end products ($E.P._1$, $E.P._2$ and $E.P._3$) are synthesized via a branched biosynthetic pathway from an intermediary metabolite (A) as shown in

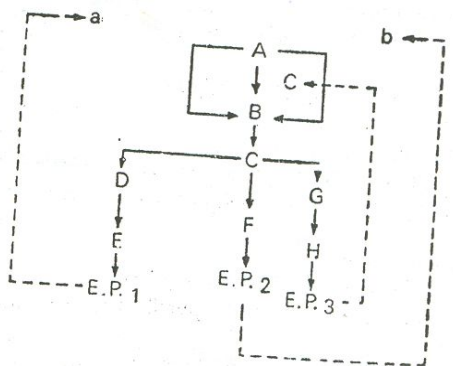


Fig- Rgulatory System of Isoenzymes involved in a branched Biosynthetic Pathway.

There are two main regulatory mechanisms that differ from each other. There may be three distinct iso-enzymes (a, b and c) capable of effecting the first reaction in the pathway (A to B). And each may be inhibited or repressed by one of the three end products.

With the multivalent or concerted regulatory mechanism repression is only apparent if all the three end products are present together as illustrated in

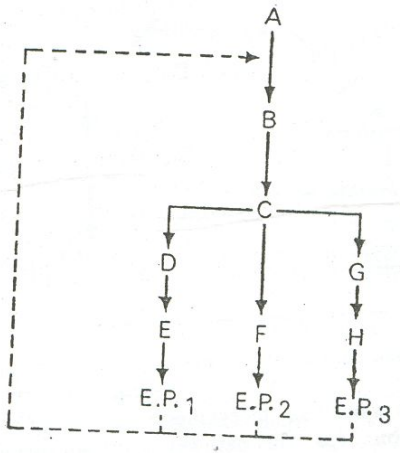


Figure- Multivalent regulatory mechanism of the branched Biosynthetic pathway.

Here it is to be noted that, there is only a single enzyme for the reaction A to B. It should also be noted that there is sometimes a cumulative action of the third end products as shown schematically in Table

End Product (in slight excess)	Percent inhibition (multivalent)	Or repression (cumulative)
$E.P._1$	0	50
$E.P._2$	0	50
$E.P._3$	0	50
$E.P._1 + E.P._2$	0	75

$E.P._2 + E.P._3$	0	75
$E.P._1 + E.P._3$	0	75
$E.P._1 + E.P._2 + E.P._3$	100	87.5

4.1.4.3 Industrially important mutants

Different types of industrially important mutants have been summarized as under:

(a) A mutant strain of Corynebacterium glutamicum can excrete about 60 g. of lysine per litre in a medium based on glucose and minerals. This mutant strain needs homoserine. On the other hand, wild strain of this bacterium does not need homoserine and fails to excrete lysine. This discrepancy can be well explained by the schematic illustration as shown in Figure.3

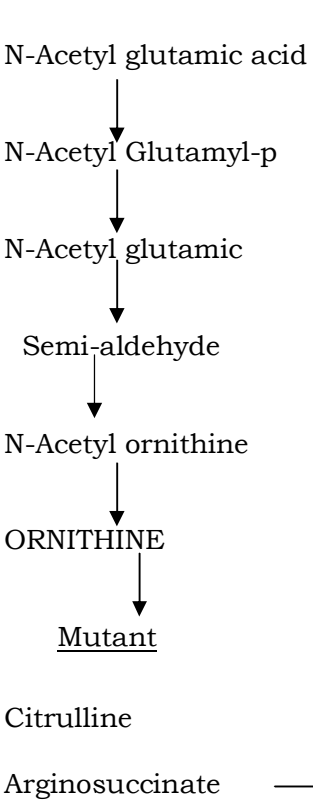
b)In the case of a wild strain, there is a common biosynthetic pathway to the biosynthesis of lysine and threonine, for the first few reaction steps. This pathway is subject to fee-back inhibition by a mixture of lysine and threonine controlling the activity of aspartate kinase. But a mutant strain requiring homo serine can no longer synthesize threonine. Moreover, feed-back inhibition no longer occurs, and lysine gets accumulated in the medium. Optimum production of lysine takes place in a medium containing 400 μg of homo serine per ml., and a high concentration of biotin (20 μg/I). It may be shown that inhibition due to threonine is increased by methionine. Methionine reacts competitively with a regulatory site on aspartate kinase. The ration of threonine to methionine also plays an important role as shown in Table.

Amino acid added	Concentration (in M)	Lysine produced (μ M)	Percent of control
Lysine	10^{-2}	12.3	64
Methionine	10^{-2}	15.9	83
Threonine	10^{-2}	0	0
Homo serine	10^{-2}	0	0
Lysine	10^{-3}	28	145
Methionine	10^{-3}	21.8	113
Threonine	10^{-3}	0	0
Homoserine	10^{-3}	0	0
Threonine	10^{-4}	14.2	74
Methionine	10^{-4}	10.6	55
Homoserine	10^{-4}	2.4	12

(b) It should be noted that in the wild strains of Escherichia coli, the biosynthetic pathway to lysine is the same, but its regulatory mechanism is different. In this case, there are three aspartate kinases, each separately controlled by either lysine, methionine of threonine. This regulatory system may be represented by Figure $E.P._3$ being lysine. There is also feed-back inhibition of dihydropicolinate synthetase by lysine.

(c) Those mutant strains in which one of the enzymes of a biosynthetic pathway is missing are also valuable strains, since they may be employed in the production of an intermediary metabolite of that particular pathway. This

may be exemplified by a mutant strain of Corynebacterium glutamicum. The biosyntheses of amino acid, arginine, occurs by a biosynthetic pathway as illustrated in Figure.



Now, a mutant strain which has lost the enzyme acting on ornithine will excrete that amino acid to long as just sufficient arginine is provided for growth, without enough being present to cause feed back inhibition.. Optimum production of ornithine occurs in a medium containing 200 μg of arginine ml. And 5 μg of biotin per litre. Moreover, this medium should be rich in glucose and ammonium salts.

(d)There are some mutant strains with enzymes that offer resistance to feed-back control. Lookingto the regulatory mechanism of feed-back inhibition, interaction between the end product and the regulatory site of an enzyme changes the enzyme configuration. Subsequently, the enzyme becomes non-functional. A mutant strain may be produced having the enzyme with an altered regulatory site. Such an altered regulatory site fails to interact with the inhibitor. Therefore, feed-back inhibition does not take place.

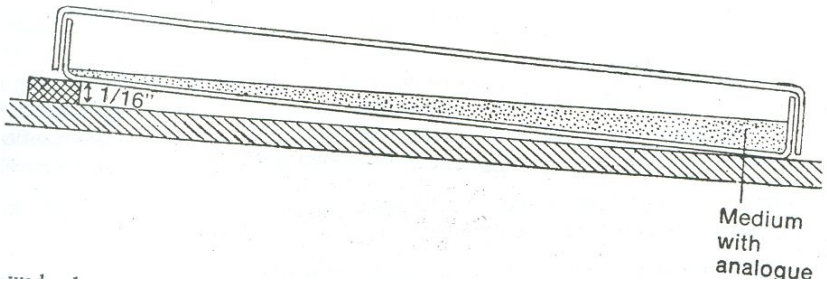
4.1.4.3Mutants resistant to analogues

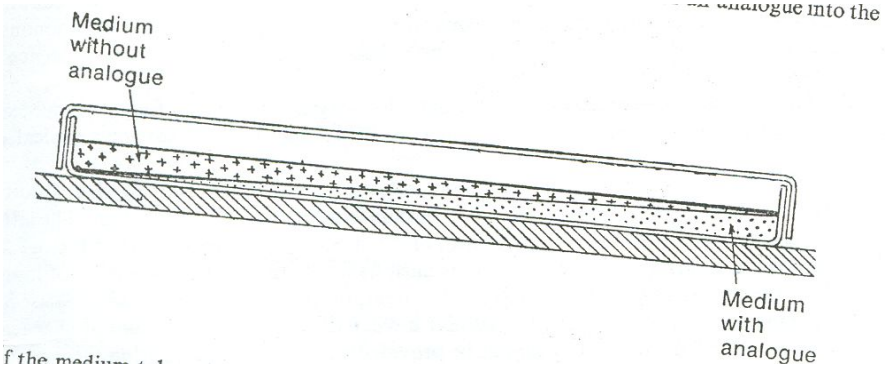
It is also possible to use an analogue in the selection of industrially important strains. An analogue can interact with the regulatory site associated with feed-back inhibition. Such an analogue often exerts toxic effect. And, this toxicity eliminates all sensitive mutant cells in a population. For example, α-amino β-hydroxyvaleric acid is

the analogue of threonine. By the use of this antimetabolite, the selection of a mutant strain may be done at the following two stages:

(1)The analogue of an amino acid, threonine, is added during the preparation of a nutrient agar medium that is poured into a sterile petri-dish. Then, the medium is allowed to solidify at an angle as shown under:

When the wedge has set, a second layer of the same medium, without analogue is poured onto it and allowed to set with the plate level as under: After some time, diffusion of an analogue into the upper layer of the medium takes place. As a result of this, there is development of a concentration gradient at the surface. Now, a culture, previously treated with a mutagen, is spread on the surface of this medium. Then, selection of any mutants offering resistance to high concentrations is done.





(2) Lastly, a search is to be made to find out resistant mutants capable of producing threonine. This may be accomplished by inoculating the mutants, as point cultures, onto an agar medium seeded with a threonine dependent culture. The growth of seeded culture (i.e. threonine requiring culture) around each colony of threonine excreting mutant strain may occur. The diameter of the zone of seeded culture growth depends upon the quantity of threonine produced by the mutants. Thus, analogueresistant mutant strains excreting higher yields of threonine may be obtained.

Using the above technique, a mutant strain of *Brevibacterium flavum* capable of excreting threonine up to 12.6 g. per litre is obtained.

(f) Mutant strains may, sometimes, undergo reversion since mutations are not always stable. As a result of this, revertants may develop in the microbial population of a mutant strain. The revertant strain possesses an enzyme different from that which has been lost due to the previous mutation. Also, the enzyme is not sensitive to feed-

back inhibition. This may be exemplified by the threonine deaminase of a revertant strain of the bacterial genus, Hydrogenomonas. The situation is represented as under:

(i) The wild strain did not require the amino acid, isoleucine. Moreover, it did not Produce this amino acid.

(ii) The auxotrophic mutant required isoleucine for growth and multiplication.

(iii) The revertant strain did not require isoleucine.

(g) Constitutive mutants are also important in a fermentation industry, since they may be used to produce increased yields of particular enzymes. These mutants produce particular enzyme in the absence of inducing substrates or other substrates that offer resistance to catabolite repression. There are numerous techniques for selecting these mutants. Since simple methods have been briefly discussed here:

(1) The microbial cells are cultured on a medium containing a carbon source with the following two characteristics:

(2) The microbial culture is cultivated in a cyclic manner, alternatively with and without an inducing substrate. For example, the microbial culture may be grown in the presence of glucose and then of lactose in a cyclic manner. After a certain number of cycles, the bacterial population will contain an increased proportion of constitutive mutants.

(3) Us of inhibitors of the inducer in a medium may also be made in the selection of constitutive mutants. For example, 2-initrophenyl β-fucoside is an inhibitor of lactose and it may be used in selecting mutants constitutive for the production of β-galactosidase. Thus, mutants of Escherichia coli produce β-galactosidase without induction, provided the medium contains lactose as the sole source of carbon.

(4) It is possible to isolate constitutive mutants which offer resistance against toxic substances. The enzyme, for which the mutants are constitutive, destroys the toxic substances present in the environment. For example, when a culture of the photosynthetic bacterium, *Rhodopseudomonas spheroids*, is repeatedly exposed to 0.1 M H_2 O_2, 25 per cent of the surviving mutants are constitutive mutants for the production of catalase.

(5) Use of toxic anti metabolites for selecting constitutive mutants having ability to produce an increased yield of enzymes involved in the bio-synthesis of the metabolite concerned, may be made. For example, mutants of Lactobacillus casei resistant to dichloroamethopterin excrete eighty times more thymidylate synthetase than those of the parent culture.

Apart from different methods for inducing high-yielding mutant strains, there is also another method for obtaining high-yielding strains. In this, case, the genetic constitution of the microbial cells is changed. This is accomplished by transferring all, or part of the DNA to the recipient culture from the donor culture. Again, there are many techniques for the transfer of genetic material. They have been listed as under:

(i) Transformation
(ii) Transduction
(iii) Lysogeny
(iv) Conjugation, and
(v) Parasexuality.

4.1.4.4 Summary

The main reason for the failure of most fermentative processes is nothing but not having a high yielding strain .Even the strain is available it may give low yield. To improve the yield the strain should be improved to get the high yield. The improvement is by means of either by autotrophic mutant selection or by selecting the mutants resistant to analogues.]

4.1.4.5 Model questions

1)Explain different methods for microbial strain improvement

4.1.4.6 References

1. Microbiology by Presscott
2. Microbiology by Pelczar
3. Fermentation technology by Stanburry

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Lesson 4.2.1

BREWING, FERMENTATIVE PRODUCTION OF ALCOHOL

Structure

Objective

Brewing is the production of malt beverages. Brewing also differs from most other industrial fermentations because flavor, aroma, clarity, color, foam production, foam stability, percentage of alcohol and sterilities all are factors associated with the finished product. Various malt beverages like beer are produced by microbial fermentation.

4.2.1.1 Introduction

Industrial solvents like ethanol production is a very important fermentation industry. Alcohol production was the first fermentation known to mankind. Distilleries began to appear in Europe in the middle of the seventeenth century. At first alcohol was used only for human consumption. Later the demand for alcohol as a universal solvent and chemical raw material developed. The distillery industry grew very rapidly.

4.2.1.2 Brewing

Brewing is the production of malt beverages. This is one of the oldest of fermentations, dating back thousands of years and probably originating in the Nile valley. In early times, the type of local agriculture most likely influenced the particular fermented beverage produced. The cereal grains are used for beer production, fruits for wine production and honey for mead.

The various characteristics of a finished malt or other alcoholic beverage are difficult if not impossible to duplicate by chemical synthesis or by mixing chemically synthesized products with natural materials, even though the ethanol itself can be easily synthesized chemically. There is a high demand by the public for this type of fermentation product. Thus these considerations place alcoholic – beverage fermentations in a unique situation in regard to fermentation industries, in that they are among the most stable of the various fermentation processes.

A. Medium

The medium must contain the carbon, nitrogen, vitamin, growth factors sources. Apart from these common needs it must contribute to the aroma, flavor, foam, color, clarity, stability of the finished product. This will be further processed to produce wort.

B. Microorganisms

Brewing utilizes strains of sacchoromyles carlsbergensis, and S. cerevisiae. Yeast strains are specially selected for their fermenting ability and for their ability to flocculate at the proper time near the end of the fermentation. Brewing is different from many other industrial fermentations in that the cells for inoculating are often those recovered from a previous fermentation. Before being employed as inoculum, the yeast cells from a previous fermentation are washed with phosphoric acid, tartaric acid or ammonium persulphate by setting. This is a procedure that reduces the pH value to approximately 2.5, and removes bacterial contamination, if present.

C. Production

The aerated medium is collected to approximately 10 to 11^{o}C and then placed in a closed fermentation tank containing cooling coils. The closed tank is preferred so that evolved carbon dioxide can be collected for later carbonation of the product. Approximately three Quarters to one pound of yeast are added for each 31 gallons of wort, and within 24 hours after inoculation, foam begins to appear on the surface of the medium, first along the wall of the tank and then gradually across the surface. The carbon dioxide evolution then increases so that the yeast cells become suspended in the medium. At this point, the wort is often transferred to a second fermentation tank so that dead and weak yeast cells, precipitation proteins and insoluble hop regins can be left behind as a deposit on the bottom of the original settling or starting tank or trapped in the foam. This procedure also provides some additional aeration to the medium.

By approximately 40 to 60 hrs after inoculation, the surface foam layer becomes very thick and can measure up to almost 12 inches in depth. It is during this time that the most rapid yeast – cell multiplication occurs, and considerable heat is evolved. This heat evolution causes a temperature rise to approximately 12 to 13^{o}C, the peak temperature for this fermentation.

By approximately the fifth day of fermentation, there is no longer enough carbon dioxide evolution to support the heavy foam and the foam begins to collapse. From seven to nine days, the last phase of the fermentation, the yeast become inactive and floclulate, the yeast break. The fermentation is usually completed by approximately five to seven days. The yeasts settle to the bottom, and the medium is further cooled to hasten settling. At this time, some of the surface scum may be removed to improve flavor.

The completed fermentation is transferred to storage flasks and held at approximately 0 to 3^{o}C for a period of time. During this “Cold storage maturation”, coagulated nitrogenous substances, regins, insoluble phosphates and yeast cells sediment from the beer. In addition esters are formed and the beer matures so that it losses its harshness. Antioxidants are added during cold storage maturation to prevent later oxidative changes in beer which affect Havor. Sulfur dioxide and ascorbic acid are commonly used to accomplish this.

D. Carbonation

Carbonation of the beer is accomplished either by injection of cleaned CO_2 recovered from the evolved fermentation gas, or by the knowsen process in which actively fermenting yeast is added to provide the so called natural carbonation. Addition of carbon dioxide, which is themost common practice, provides a final dissolved CO_2 content of approximately 0.5% in the beer. The CO_2 displaces dissolved

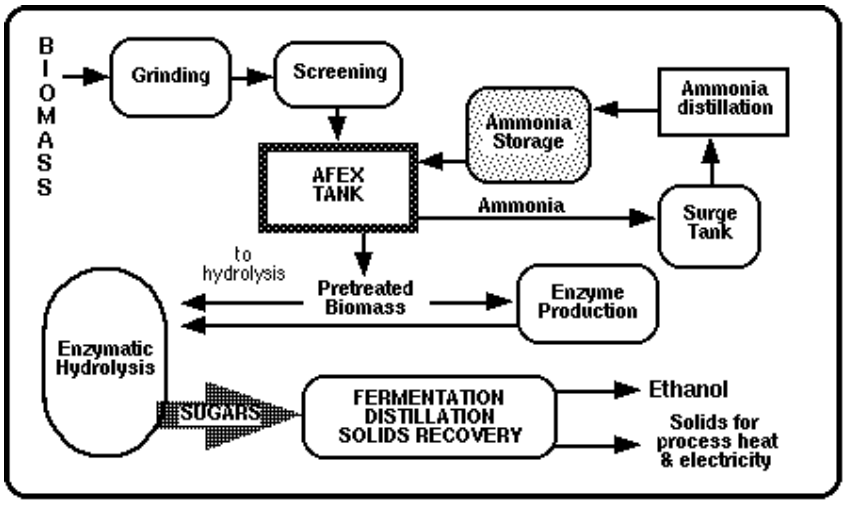
oxygen, which is detrimental to the stability of the bear, and helps in the production and retention of foam and in the preservation of the beer.

The krausening process is accomplished by the addition to beer undergoing cold storage maturation of approximately 15% of active fermentation broth and cells from an early stage in the fermentation. These yeast cells are allowed to slowly ferment and remove residual sugar, a process that requires approximately 3 to 4 weeks of cold storage maturation time. The excess CO_2 evolved during this period is maintained so that the final level will be similar to that for CO_2 injection. After the sugar is gone, the beer undergoes several weeks further cold storage maturation so as to complete the clarification of beer.

E. By products

The spent grains separated without drying from the wort find commercial use as animal feed. However, they also are dried to about 10% moisture to provide an animal feed product known as dried brewer's grain which contains protein, fat, carbohydrate, crude fiber, and B complex vitamins.

4.2.1.3 Alcohol Production



1. Figure 1. Ethanol production (Fig.10.3, 148, Waites)

Ethanol is used as a solvent and a precusor of many synthetic chemicals. This ethanol have been produced by fermentation processes. Today, fermentation processes have virtually disappeared as a result of the development of chemical production. This chemical production development is because of

(i) high prics of the fermentable substrates
(ii) increasing economic competition from the petrochemical industry.

Ethanol is an attractive fuel because it may be used alone or mixed with other liquid fuels like gasohol. The basohol is a blend of 10 – 22% ethanol with gasoline. In the 1970s Basil and a few other countries under took the full scale production of

ethanol from indigenous renewable biomass resources to offset the growing costs of oil imports. Brasil is now responsible for over 46% of annual world production, some 14.5 billion litres of ethanol.

A. Microorganisms

The micro organisms responsible for alcoholic fermentation of molasses are yeast species of Saccharomyces, Torulopsis, Kloekera, Candida and certain species of Mucor and bacteria. The most important among them are the yeast species belonging genus Saccharomyces. The two important species are S. cerevisiae and S. carls bergensis. The S. cerevisiae is called top fermenter yeasts, as it is active at the top surface of the fermenting liquid. They remain either in an evenly distributed layer, or as a ring along the walls of the fermentation tank. S. carls bergensis is called bottom fermenters. Bottom fermenters develop at the bottom of the fermentation tanks. These are whirled up in the liquid owing to CO_2 evolution.

The yeast strains used for alcoholic fermentation should possess the following features :

(i) It should produce a large quantity of alcohol.
(ii) It should be a fast growing strain.
(iii) It should have a high tolerance to alcohol.

Usually the strains of yeast, S. cervisiae, are used for commercial production of a ethyl alcohol. The choice of microorganism also depends on the type of raw material to be used in fermentation. When starch or molasses are used as raw materials S. cere visiae is selected. When whey and waste sulphite liquor are employed as raw material candida strains are preferred.

B. Raw materials for ethanol fermentations

45 Kg of fermentable sugar yield from 18 to 23 Kg of ethanol. For starchy materials the yield is 40 to 50% on the dry weight of carbohydrate. For cellulosic raw materials, the yields of ethanol are substantially less because α-cellulose is quite resistant to enzymatic attack.

Types of raw materials can be usefully divided into (i) sugar containing materials, which can be fermented directly, (ii) a starchy materials, which can be easily hydrolyzed by enzymes or acids to fermentable sugars and (iii) cellulosic materials, which are difficult to hydrolyze.

(i) Sugar

Ethanol may be produced from any sugar containing fruits, fruit juices or extracts, such as grape juice, apple juice, honey, date syrup or sugar containing effluents of canneries. Such sources are usually too costly in comparison with sugar beets, sugar cane and sweet sorghum. The production of crystalline sucrose yields a by-product, molasses, which until recently has been the cheapest source of fermentable sugar. Molases containing about 50 – 55% fermentable sugar can be fermented without difficulty.

(ii) Starch

Starch which has been gelatinized by heating can be readily hydrolyzed to fermentable sugars by enzymes. Such starches occur in cereal grains, root crops or tubers. All of these materials have been used for the production of distilled alcoholic

beverages; the use of wheat or corn for production of whiskey, and the use of potatoes for production of voolka are well known. In the united states corn is the most abundant source of starch.

(iii) Cellulose

The utilization of cellulosic materials for the production of ethanol is ultimately the most promising, but it also presents the most difficult technological and economic problems. One of the most promising developments in using cellulose is the 2-step hydrolysis of cellulosic materials with (a) dilute acid to hydrolyze the hemicellulose to pentoses, followed by (b) acid or enzymatic hydrolysis of the separated cellulose.

C. Medium

The optimum sugar concentration ranges between 10 to 18% in the fermentation medium. Also, a suitable source of nitrogen such as ammonium sulfate is added in optimum concentration. When beet – molasses is used, usually a small quantity of black – strap molasses is also added to meet the biotin deficiency in the beet molasses. The pH of the medium is adjusted to 4.8 to 5. The pH can be adjusted by the use of H_2SO_4 or lactic acid. Alternatively, it can be adjusted by growing lactic acid bacteria prior to fermentation. The high acidic pH in the medium hinders the growth of micro organisms.

D. Fermentation Conditions

(i) Carbon sources

Sugar concentration in the range of 10 to 18% is found satisfactory. Sugar concentration of 12% is often used. For example, cane molasses contain sucrose as a carbon source. The sucrose content of the raw material is about 48 to 55% wort is prepared to contain about 10% sugars. High concentration affects yeast adversely while low concentration makes the process uneconomical.

(ii) Nitrogen source

Many organic as well as inorganic nitrogenous compounds may be used in this fermentation. Ammonium sulfate is generally used with success to supply nitrogen for growing yeast cells. The level of nitrogen in the medium is limited. Excess nitrogen is not acceptable, since it promotes the growth of yeast, inhibiting the fermentation.

(iii) pH

pH of the fermentation medium is adjusted to 4.8 to 5. The higher pH increases chances of contamination, where as very low pH values inhibit the yield of ethyl alcohol.

(iv) Temperature

Usually, the temperature range of 70^{o} to $80^{o}F$ is preferred. As there is heat evolution during fermentation, the temperature in the fermentation tank rises gradually and is controlled by means of cooling coils. Temperature can also be controlled by cold water spray around the fermentation tank. High temperatures favours the growth of bacteria but causes loss of ethyl alcohol due to evaporation.

(v) Time

Fermentation starts within a few hours after the addition of yeast. The process becomes very rapid after 24 hours to complete the fermentation. Duration for ethyl alcohol fermentation is usually 30 to 72 hours when the specific gravity of the fermented liquid becomes constant.

E. Production types

There are two types of fermentations for alcohol production using microorganisms.

(i) Batch fermentation
(ii) Continuous fermentation

(i) Batch fermentation

If the fermentation is in a tank which filled with media and inoculated with microorganisms. This tank has no continuous supply for substituting the depleted requirements during fermentation in the fermentor. The total fermentation time varies between 48 and 72 hr, and final alcohol concentrations of 6-8% can be obtained. The proteinaceons residue which is a valuable feed product is economically important to the operation of the process.

Batch fermentations are carried out without the need for establishing pure culture conditions, that is without the need for complete sterilization. This fermentation inhibits the growth of the other microbes by depleting the available nutrients, by a lowering of the pH and most importantly by the formation of ethanol.

(ii) Continuous fermentation

Most of the experimental work has been carried out with homogenous fermentations with a single stirred fermentor. Fresh medium is continuously pumped into the fermentor and an equal volume of the fermentor liquid is continuously pumped out for recovery of ethanol and yeast. The rate at which medium is added is usually expressed as dilution rate. The dilution rate is the ratio of withdrawn liquid to the volume of the total liquid in the fermentor.

(F) Recovery

The fermented liquid is allowed to settle for a few hours and then distilled in analyser and rectifier columns to obtain ethyl alcohol. The highest concentration can be got by fractional distillation is the constant boiling mixture, which contains 95.6% ethyl alcohol and 4.4% water.

4.2.1.4 Summary

- Wort is the diluted molasses form, which is the mostly used medial source in Brewing industry.
- The carbonation of the brewing product is needed as this increases the stability and help in the production and retention of foam, and in preservation of beer.
- During ethanol production all the medium components must be maintained at the exact levels otherwise if more concentration are used it effects the fermentation process and if low levels are used it effects the yield.

- The batch and continuous fermentation systems operation need to have experience in operating the system as they need the sterilization conditions and continuous monitoring of fermentation.

4.2.1.5 Model Questions

1. What is brewing
2. How can you produce the important industrial solvent ethanol using microbial fermentation.

4.2. 1.6 Reference Books

1. Industrial microbiology – A.H. Patel
2. Industrial microbiology – L.E. Casida
3. Industrial microbiology – Prescott and Dunn's.

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Lesson 4.2.2

Production of Citric Acid

Structure

Objective

Organic acids productions is an important fermentation industry. Various types of organic acids can be produced by using microorganisms like Citric acid, Lactic acid, Acetic acid, Gluconic acid, Pyruvic acid, Fumaric acid, etc. The generally used microbial sources in this process are aspergillus, pseudomonas, penicillium, candida. They very important organic acid fermentation industry is production of citric acid, acetic acid, gluconic acid.

4.2.2.1.Introduction

Citric acid is widely used in the food industry, as an acidulant and flavoring agent in beverages, confectionary and other foods, and in leavening systems for backed goods. The non-food applications of organic acid are in maintaining metals in solution for electroplating, as a cleaning and pickling agent for metals, and as a replacement for polyphosphates in detergent industry lactic acid is largely used in pharmaceutical industries and in some plastic industries gluconic acid is used as a mild acidulant in metal processing, leather tanning and foods. So these organic acids enhancing the need of further improvements in the organic acid fermentation technology.

4.2.2.1. Citric Acid Production

Citric acid is a tricarboxylic acid. It was first isolated from lemon juice and crystallized in 1784 by Scheele. It is found as a natural constituent of number of fruits. Initially, citric acid was produced mostly from lemon juice. Today, most of the citric acid used in food and other industries is from fungal fermentation.

About 70% of the citric acid produced is used in the food and beverage industry, about 12% in pharmaceutical industry, about 8% in Other industrial applications. The food and beverage industry uses citric acid mostly as an acidulant, because of its high solubility and extremely low toxicity and a pleasant sour taste.

A.Synthetic pathway of citric acid

The first stages of citric acid formation involve the breakdown of hexoses to pyruvate in glycolysis, followed by its decarboxylation to produce acetyl COA. Oxaloacetate would largely supplied through the completion of the TCA cycle, oxaloacetate condenses with acetyl COA to form citrate, catalysed by citrate synthase. However, in order to accumulate citrate, its onward metabolism must be blocked. This is achieved by inhibiting aconitase, the enzyme catalyzing the second step i.e.,

formation of cis-aconitate, in the TCA cycle. Inhibition is accomplished by removal of iron, an activator of aconitase. Consequently, during citrate accumulation, the TCA cycle is largely inoperative beyond citrate formation, hence the importance of the anaplerotic routes of oxaloacetate formation.

B. Microorganism used

The citric acid can be produced by yeast, fungi and bacteria. The fungal strains useful for citric acid production are aspergillus niger, a. awamori, Penicillium, Janthinellum, P. restrictum, etc. The yeasts producing citric acid are species of Candida, Hansenula, Saccharomyces, etc. The bacterial species used in producing citric acid are of bacillus and brevibacterium.

C. Citric acid production by fungi

Currie first reported that strains of A. niger, when cultured in a sugar containing medium of low pH, produce citric acid. Strains of aspergillus niger has major advantages in using this organism for producing citric acid are the case with which it can be handled, the cheap raw materials that it can utilize for citric acid production and high consistent yields, thereby making the process commercial.

1. Medium

Sucrose is the best source of carbon among various tested organic substances, particularly sugars, in producing high yields of citric acid. It was also reported that sucrose concentration exceeding 15% should not be used, since the excess of sugar remained unconverted to citric acid. When a part of sucrose was substituted by fructose or glucose, it results in lower yields. Beet molasses is extensively used as a carbon source in the fungal production of citric acid on commercial basis. Apart from the carbon, hydrogen and oxygen supplied by the added carbohydrate, the trace metals, namely nitrogen, potassium, phosphorus, sulphur and magnesium are in the fermentation medium of citric acid production.

The composition of medium proposed by Currie is

a. Sucrose – 125 to 150 g/lit
b. NH_4NO_3 - 2.0 to 2.5 g/lit
c. KH_2PO_4 – 0.75 to 1.0 g/lit
d. $MgSO_4.\ 7H_2O$ – 0.2 to 0.25

Using HCl pH must be maintained to 3.4 to 3.5.

2. Production process

The fermentative production of citric acid is two types

a. Surface and solid substrate fermentations
b. Submerged processes

a. Surface and solid substrate fermentations

These methods use simple technology and have low energy cost, but are more labour intensive liquid surface methods involving placing the sterilized medium, usually containing molasses plus various salts, into shallow aluminium or stainless steel trays stacked in an aseptic room. The medium is formulated with relatively low levels of iron, otherwise the citric acid yield is reduced. The trays are inoculated by spraying with A. miger spores, either a spore suspension or dry spores. The fungus then develops on the

surface of the medium. Sterile air blown over these cultures, which is important for maintaining aerobic conditions, temperature control in lowering the CO_2 level. Medium pH gradually falls to below 2, at which point citric acid production begin. At 30° C, the fermentation takes about 8-12 days to complete and achieve a productivity of around 1.0 kg/m^3 per day.

Solid state fermentation processes for citric acid production are small –scale operations. Each plant generates only a few hundred tones per annum, and uses a solid medium of steam-sterilized wheat bran or sweet potato waste that has a 70-80% moisture content. This mash is inoculated with spores of A.niger and then spread on trays or a clean floor to a depth of 3-5 cm. Air circulation helps to maintain the temperature at about 28°c. This process runs for 5-8 days after which the mash is collected and the citrate is extracted using hot water.

Various solid food processing residues are being evaluated to determine whether they too could serve as low cost substrates for citric acid production. In addition, technological developments are being sought, such as the use of packed bed reactors, preliminary trials have produced high levels of citric acid with low levels of fungal biomass.

b. Submerged processes

More than 80% of the world wide supply of citric acid is produced using submerged batch fermentation in stirred tanks of 40-200m^3 capacity or larger air lift fermenters of 200-900m^3 capacity. The fermenters are corrosion – resistant, made of stainless steel, or steel lined with special glass or plastic.

These fermentations mostly use beet or cane molasses as the carbon source. Unlike surface methods, vegetative inocula, rather than spores, are normally used. Consequently, the culture organism is taken through several propagation stages in order to generate a sufficient quantity of inoculum. Initially spores of the production strain of A.niger are produced on solid medium and then used to inoculate a small-scale submerged fermentation where fungal pellet formation takes place. Quantities of stable fungal pellets are then developed for the inoculation of the production fermenter.

The structure of these pellets has a major influence on productivity. Small pellets of less than 1 mm diameter, with fluffy centers and smooth surfaces are preferred. The structural properties and their physiology are strongly dependent on medium composition and operating conditions. Pellets that produce high levels of citric acid are characterized by short forked, bulbous hyphae. The presence of even low levels of some heavy metals, particularly manganese, can be detrimental to pellet formation, resulting in hyphae that are long and unbranched. Thus, it is necessary to pretreat all raw materials to reduce manganese concentrations to below 0.02 m mol/lit. Low manganese levels also limit the operation of the pentose phosphate pathway, which would otherwise divert flux away from glycolysis and reduce citrate production. Citric acid yields are also improved by formulating the medium with minimum levels of iron. This reduces onward metabolism of citrate because aconitase has a requirement of iron. The addition of copper further diminishes aconitase activity, as it acts as an antagonist to iron.

In order to maintain good citric acids yields, media sugar concentrations must be at least 140 g/lit, which promotes the activity of both glycolytic enzymes and pyruvate

carboxylase. It is also important to restrict growth through nitrogen limitation. This is normally accomplished by providing ammonium salts at levels of 0.1 to 0.4 g/lit. The ammonium ions also stimulate citric acid production by counteracting the inhibitory effect of citrate on phosphofructokinase. These fermentations are highly aerated and maintained at 30°C. For the initial growth phase, the pH starts at 5-7, but must then be kept below 2, otherwise oxalic and gluconic acids accumulate at the expense of citric acid, i.e., low pH inhibits glucose oxidase. Overall yields of 0.7-0.9 g citrate per gram glucose can be attained in these submerged fermentations with productivities of upto 18.0 kg/m^3 per day.

3. Recovery

The crude fermented liquor containing citric acid, obtained either from surface or submerged culture, and the extract from solid state culture, is filtered to remove mycelia or cells and other suspended impurities.

The waste mycelia are pressed to recover most of the fermented liquor. The mother liquor is heated to 80° – 90°C by the addition of small amounts of hydrated lime to allow precipitation of oxalic acid. Citric acid is then precipitated as calcium citrate using 1 part of hydrated lime for every 2 parts of liquor added over a 1 hr period while the temperature is raised to 95°C.

The precipitated calcium citrate is filtered and washed with water several times. It is then transferred to acidulators and treated with H_2SO_4. The solution is again filtered to remove $CaSO_4$. The mother liquor containing citric acid is decolorized by charcoal and passed through ion exchange resin columns. The liquor is concentrated in vacuum and finally run into low temperature crystallizers where citric acid crystallizes as citric acid monohydrate.

The quality of citric acid can be improved by using $Ca(OH)_2$ free of contaminating metals such as Mg^{++}, Fe^{++}. By the addition of 9 to 12% calcium ferrocyanide to the mother liquor at 95°-97°c for 5-8 min the recovery of citric acid has been found to improve.

4.2.2.3 Summary

- The various types of organic acids produced by using microorganisms are citric acid, lactic acid, acetic acid, gluconic acid, pyruvic acid, etc.
- About 70% of citric acid produced is used in the food and beverage industry, about 12% in pharmaceutical industry and about 18% in other applications.
- Aspergillus and penicillium species are generally used in organic acids production.

4.2.2.4 Model Question

1. Describe the various methods for production of Citric Acid. Add a note on the various applications of it.

4.2.2.5 Reference Books

1. Industrial microbiology – A.H. Patel
2. Industrial microbiology – Cruger

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Lesson 4.2.3

FERMENTATIVE PRODUCTION OF ORGANIC ACIDS

Structure

Objective:

This lesson explains the fermentative production of organic acids like vinegar and Gluconic acid. It also states the importance of these acids in various fields.

4.2.3.1 Introduction.

Organic acids productions is an important fermentation industry. Various types of organic acids can be produced by using microorganisms like Citric acid, Lactic acid, Acetic acid, Gluconic acid, Pyrunic acid, Fumaric acid, etc. The generally used microbial sources in this process are aspergillus, pseudomonas, penicillium, candida. They very important organic acid fermentation industry is production of citric acid, acetic acid, gluconic acid.

4.2.3.2. Acetic Acid Production

The normal course of events when a natural solution of fermentable sugars is exposed to indigenous microbial activities is an alcoholic fermentation by yeasts, followed by bacterial oxidation of the ethanol to acetic acid. Acetic acid is also called vinegar. The term vinegar is derived from the French words vin (wine) and aigre (sour). Generally, vinegar is classified as a condiment that contains a minimum of 4% acetic acid.

Vinegars have been produced for several thousand years from local fermentable substrates and alcoholic beverages. They are mainly produced from fruit juices and sugar syrups, including molasses and honey.

A. Vinegar fermentations

There are two stages in the formation of vinegar using sugar-containing media.

a. Alcoholic fermentation
b. Acetic acid fermentation

a. Alcoholic fermentation

During this stage sugar containing materials like fruit juices, honey or hydrolyzed starchy materials are fermented to ethyl alcohol. This biconversion is brought about by the agency of enzymes of yeast, saccharomyces cerevisiae.

$C_6H_{12}O_6$ ----------→ $2C_2H_5OH + 2CO_2$

One gram of glucose should yield 0.5114 g of ethylalcohol. It is necessary to adjust the fermentable sugar concentration in the raw material undergoing fermentation in the range of 8-20% with certain fermentations it is necessary to add potassium and ammonium phosphates to the production medium on the other hand,

these mineral salts are not needed in case of fermentations using crushed grapes and grape juice or with apple juice. A temperature of 75 to 80° F, has been found to be satisfactory.

This stage is carried out in two stages :

i. Primary stage – It lasts from 3 to 7 days. It is usually conducted in open fermentation vats.

ii. Secondary stage – It lasts over a period of several weeks. It is carried out in closed fermentors, Sulfur dioxide is added to the production medium at the start of fermentation and prior to the addition of the starter culture of yeast. This inhibits the growth and activity of unwanted yeasts and bacteria. An initial level of 50-100mg of sulfur dioxide per litre is enough. The yield of alcohol is usually only about 85-90% of the stoichiometrical yield.

b. Acetic acid fermentation

The acetic acid fermentation is a highly aerobic process, essentially a biotransformation by acetic acid bacteria, involving incomplete oxidation of ethanol to acetic acid. Other minor products include acetaldehyde, ethylacetate and acetoin.

Industrial acetic acid bacteria are members of the genera acetobacter and glucanobacter, mostly A. aceti, G.oxydans. In some instances, mixed cultures are more efficient than a single organism. These bacteria may be divided into two main groups by their action on acetic acid.

Conversion of alcohol to acetic acid is largely a problem of aeration technology, which must bring into intimate contact the liquid to be oxidized with acetic acid bacteria and oxygen.

The three methods used are :

i. Traditional surface methods

Surface processes involve natural microflora that first carry out an alcoholic fermentation of sugars derived from plant juices or saccharified starch. The resultant alcoholic solution, contained with in part-filled barrels, them undergoes a spontaneous natural fermentation to convert ethanol to acetic acid. This is reliant upon acetic acid bacteria establishing themselves on the liquid surface where ethanol oxidation occurs. These traditional batch methods are generally slow and unpredictable.

ii. Trickling generators

These methods involve the movement of alcoholic liquid over surfaces on which films of acetic acid bacteria have been attached, and a good air supply is provided to facilitate ethanol oxidation.

This process was first developed by Schutzenbach for the commercial production of vinegar. Traditional generator consists of a cylindrical wooden vessel of upto $60m^3$, divided horizontally into three sections. The upper section receives the alcoholic solution, which then trickles down through the main middle section that is loosely packed with inert materials, such as beech wood or rattan shavings, corn cobs and charcoal. These materials provide a very large surface area on which acetic acid bacteria can become established and over which forced air is passed from below. The bottom section serves to collect the vinegar, from where it may be returned to the top of

the same generator for recirculation or to a second generator in series. Cooling jackets are provided to prevent overheating.

iii. Submerged methods

These are five times faster than the trickling methods and require less capital investment. Since their introduction there have been several modifications that have improved aeration efficiency. The process frequently used, often based on frings acetators, consists of a highly aerated stainless steel stirred tank reactor. This vessel is continuously stirred at 1450-1750 rpm and is normally maintained at 24-30^{o}c, although temperatures of upto 40^{o}c have been reported. Specially selected strains of acetic acid bacteria are used that function well in suspension cultures. They grow in a suspension of very fine air bubbles within the fermenting liquid. Initial cell concentrations of around 1.5 x 10^{8} cells/ml at the start of a cycle rise to 2.25 x 10^{8} cells/ml, a 1.5 fold increase in population. Submerged processes are usually operated automatically on a semi continuous basis. Starting medium for each cycle contains 7-10% acetic acid and 5% ethanol, higher ethanol concentrations being inhibitory.

4.2.3.3. Gluconic Acid

Gluconic acid finds many commercial uses, being marketed usually as the calcium or sodium gluconate salt. Thus, calcium gluconate is employed as a pharmaceutical to supply calcium; and calcium and sodium gluconates in alkaline solution are good metal sequestering agents, particularly for iron, aluminium and copper. In addition ferrous gluconate supplies iron for the treatment of anemia. So gluconic acid is used as a mild acid acidulant in metal processing, leather tanning and foods.

Gluconic acid production is by using the micro organisms of acetobacter species like bacteria and fungus like penicillium and aspergillus. A.niger is the highly used organism for gluconic acid production.

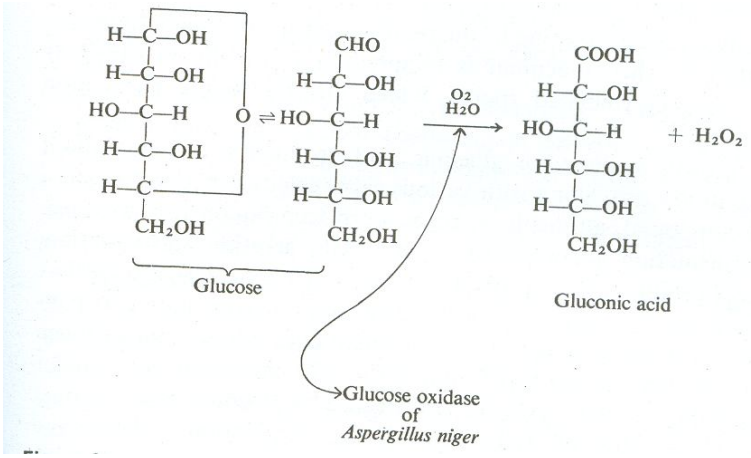

Fig. Gluconic acid from glucose

The fermentative production of gluconic acid by A. niger is a one – step enzymatic transformation of glucose to gluconic acid with the aldehyde of glucose being oxidized to the carboxyl of gluconic acid. The enzyme mediating this reaction is glucose – oxidase.

The A. niger gluconic acid fermentation can be accomplished by both stationary and submerged culture, but the submerged culture is presently utilized in industrial production. Once formed in an initial gluconic acid-growth fermentation, the A.niger mycelium is reused in successive replacement culture fermentations.

The growth medium of gluconic acid production contains about 25% glucose, along with various salts, calcium carbonate and a boron compound. The fermentation is conducted at 30^{o}c with aeration and agitation, and heat evolution is controlled by cooling coils, water – jacketed fermentors and so forth. At the termination of either the growth or replacement culture fermentations, the gluconic acid is recovered by neutralization of the broth with calcium hydroxide so as to allow crystallization of calcium gluconate. Free gluconic acid then is recovered from the calcium gluconate by the addition of sulfuric acid.

4.2.3.4 Summary

- The various types of organic acids produced by using microorganisms are citric acid, lactic acid, acetic acid, gluconic acid, pyruvic acid, etc.
- About 70% of citric acid produced is used in the food and beverage industry, about 12% in pharmaceutical industry and about 18% in other applications.
- Aspergillus and penicillium species are generally used in organic acids production.

4.2.3.5 Model Questions

1. How vinegar can be produced by microbial fermentation.
2. Give the organisms and procedure for the production of Gluconic acid

4.2.3.6 Reference Books

1. Industrial microbiology – A.H. Patel
2. Industrial microbiology – Cruger

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Lesson 4.2.4

ANEROBIC FERMENTATION WITH REFERENCE TO ACETONE AND BUTANOL

Structure

Objective

This lesson explains the process of fermentation involved in production of acetone and butanol and their applications

4.2.4.1. Introduction

Anaerobic fermentation

Anaerobic fermentations are those fermentations that are carried out in the absence of oxygen by strictly anaerobic or facultative anaerobic bacteria or yeasts. The property of anaerobic growth is associated with strict anaerobes, such as members of the genus clostridium and with facultative anaerobes like lactic acid bacteria and yeasts. There is no evidence for the anaerobic fermentation from the members of the order Actinomycetales, which includes the anaerobic *Actinomyces* genus or form the fungi although organisms from there groups carry out aerobic fermentation.

The strict anaerobes usually lack catalase activity, and possess little peroxidase activity which is not sufficient to remove the highly toxic hydrogen peroxide produced during aerobic growth. Certain organisms normally considered as being aerobic are capable of anerobic growth, if they can reduce nitrate of sulfate in the medium to obtain oxygen atom. There are however not considered in anaerobic fermentation because they employ similar mechanisms of terminal respiration for both aerobic and anaerobic growth, but grow better in air. In fermentations utilizing facultative anaerobes like yeasts, employ aeration during inoculums buildup to increase cell number before anaerobic fermentation conditions are imposed. This shows that the rate and amount of cell growth is usually greater for aerobic conditions than for anaerobic.

Anaerobic growth recovers less energy per unit of carbon substrate utilized than aerobic. Carbon substrates in there fermentations undergo only partial decomposition so that various organic acids, amines etc accumulate in the growth medium, presenting problems in pH maintenance of the fermentation. Thus this makes use of more carbon substrates which can be beneficial to fermentation yields that involve conversion of substrate carbon to fermentation product. A specific difference in the aerobic and anaerobic metabolism of microorganisms is that in anaerobic fermentations the product

of interest are the normal decomposition products for a given carbon substrate while in case of aerobic fermentations the products are the partially decomposed carbon substrates subjected to normally minor biosynthetic sequence of the cells so that the structures of the fermentation products bear little resemblance to the normal catabolic intermediates of the carbon substrates. This can also be a reason for the possibilities of developing new types of fermentation products from aerobic organisms.

Anaerobic fermentations are intriguing for commercial use because they do not require the expense of large volumes of sterile air or the expense of energy input into the fermentation in the form of vigorous impeller action. Although difficult to handle and control on an industrial scale, there organisms could be used in future for fermentations utilizing inexpensive carbon sources.

4.2.4.2. Anaerobic fermentation with reference to acetone and butanol.

Chaim Weizman performed fundamental research on the fermentation of *Clostridium acetobutylicum* for the production of acetone, butanol and ethanol. During World War I, the production of interest was acetone used for production of the explosive trinitrotoluene. But after World War, butanol became more important for the production of nitrocellulose – lacquers.

Through *Clostridial* fermentation of starch, molasses, sucrose, wood hydrates and pentoses, many alcoholic products like butyric acid, butanol, acetones and isopropanol are obtained. The relative proportions of each of these products in the fermentation depend on the bacterial strain used and on the fermentation conditions. Three fermentation types can be categorized, according to their fermentation products.

1. Acetone – butanol fermentation with *Clostridium acetobutylicum*
2. Butanol – isopropanol fermentation with *Clostridium butyricum*
3. Butyric acid – acetic acid fermentation with *C.bytyricum*

Our current interest is only the acetone-butanol fermentation. During fermentation processes, concentration of less than 0.5% has no influence on the cells, but in higher concentrations damage to the phospholipids of the cell membrane will occur. Above 1.3% concentration of butanol, production ceases. Depending on the strain and fermentation conditions, autolysins may cause cells to lyse.

Stock cultures of *Clostridium acetobutylicum* are stored as spores in sand for up to 30 years . Although production requires only low inoculum ration 1:3000, build up of inoculum has several advantages, like greater resistance to contamination.

Clostridium acetobutylicum and related acetone – butanol producing bacteria is anaerobic, motile, spore forming rods whose spores are heat resistance. Certain *Clostridium* species producing N-butanol first synthesize butyric and acetic acids, then they convert these products to butanol and acetone. There are two groups of butyric acid producing clostridium species. One is *Clostridium butyricun* which produce acetic acid butyric acids & CO_2 and hydrogen gas without reducing the acids to the corresponding solvents. The other group, *Clostridium acetobutylicum* is able to reduce the solvents to acids.

4.2.4.3. Production mechanism

It is known that acetic and butyric acids are produced first and then converted to acetone and butanol, and some enzymatic steps are reversible. From isotope labeled studies showed that some of the carbon of each acid ended up in the alternate solvent. However, a general picture of biosynthetic sequence has evolved.

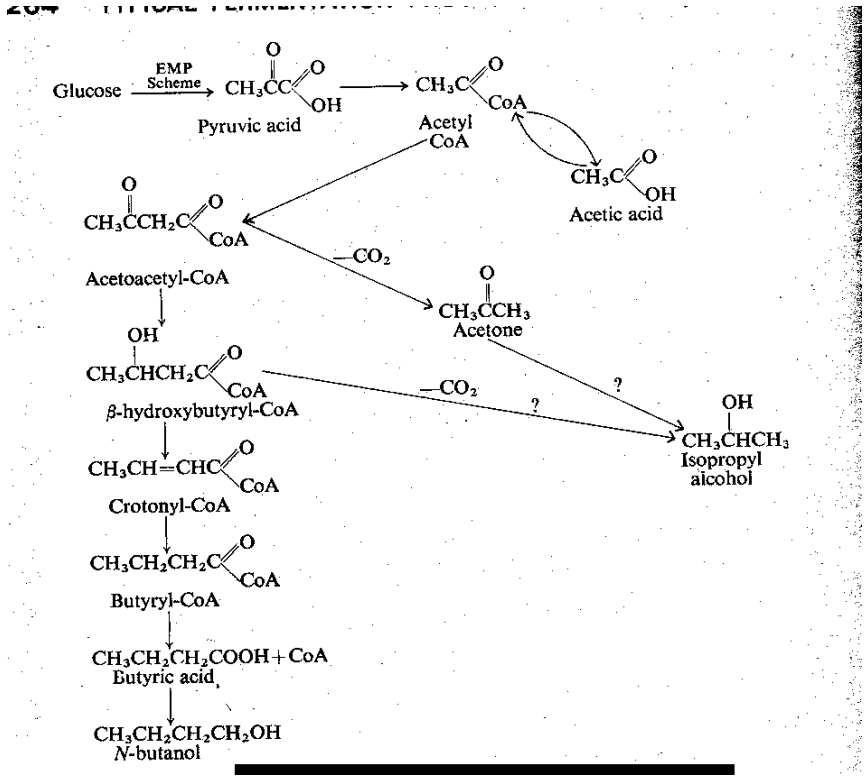

Fig: cycle: Reactions in butanol fermentation.

Glucose is degraded via the EMP scheme to pyruvate and the excess reducing power liberated as gaseous hydrogen. Then pyruvate is converted to acetyl COA plus, CO_2 and hydrogen. A part of acetyl COA is converted to acetic acid directly and additional is condensed to yield acetoacetyl COA which is then converts to β - hydroxybutyric acid, crotonic acid followed by butyric acid. The butyric acid is then reduced to N-butanol. The acetoacetic acid combines with acetyl COA to yield acetone.

4.2.4.4. Fermentation procedure

In the commercial process two slightly different species are used. One is *Clostridium – autobutylicum* and *Clostridium saccharo-aceto-butylicum.*

1.Inocculum preparation : Spores from soil stock are added to deep tubes of semisolid potato-glucose medium for molasses cultures and to deep tubes of potato corn medium for the corn type. The addition of spores to the bottom provides protection for vegetative cells from oxygen after germination. The inoculated cultures are heat shocked & cooled rapidly to incubation temperature. Then tubes are incubated at 31 to 32^{oC} for *C.saccharo-aceteobutylium*; & at 37^{o} ^{C} for *C. acetobutylium* for 20 hours in an inoculum medium same as that of large batch medium i.e. molasses or corn media respectively. Further increased volumes of inoculum are produced by successive transfer of 2 to 4% inoculum by volume to larger & larger volumes. The active evolution of spores during the fermentation provides the anerobic conditions required for growth.

During the last stage of transfer of inoculum from molasses culture to the production fermentor, it is checked for pH values, Brix values (density), rate of gas evolution, presence of facultative anaerobes by aerobic plating on an agar medium, and presence of contaminants by hanging-drop preparation. For corn-culture rate of gas formation, pH, microscopic observation aerobic plating, and titrable acidity are checked.

The final inoculum stage for molasses medium is 26 to 28 hours incubated culture before addition to the production fermentor, where as for corn culture incubation until the titrable acidity demonstrates a distinct acid break, indicating a lack of gross contamination is used.

The inoculum is added first to the production fermentor followed by the medium, since inoculum mixing can be proper.

2. Preparation of medium

For corn fermentation corn meal is prepared by passing corn through a magnetic field to remove dust and metallic debris followed by degerming the corn. The degermed corn is then ground to a relatively fine state in a roller or hammer mill. 8 to 10 percent of corn meal is added to water with or without stillage followed by heating approximately to 20 minutes at 65^{o}c to gelatinize the starch to form corn-mash production medium which is then sterilized.

The molasses production medium contains 6 percent sugar as sucrose, either black strap or high – test molasses and to this is added ammonia and ammonium sulfate, calcium carbonate, super phosphate and some times corn steep liquor. The calcium carbonate is added to prevent development of gross acidity, although excess calcium carbonate hinders solvent formation and ammonia is actually added within first 18 to 24 hours of fermentation. Over cooking of molasses has to be avoided.

Some procedures involve using of the aqueous residue remaining after the natural fermentation. Solvents have been stripped (distilled) from completed fermentations (stillage) to provide 30 to 40 percent of total aqueous volume for the preparation of fresh medium. This is called `slopping back'. During this process case must be taken to prevent toxic fermentation by products and unfermentable residues that accumulate.

Duration of fermentation is 2 to 21/2 days in large volume fermenters . Antifoarming oils are used in small amounts to prevent problems in solvent recovery stills.

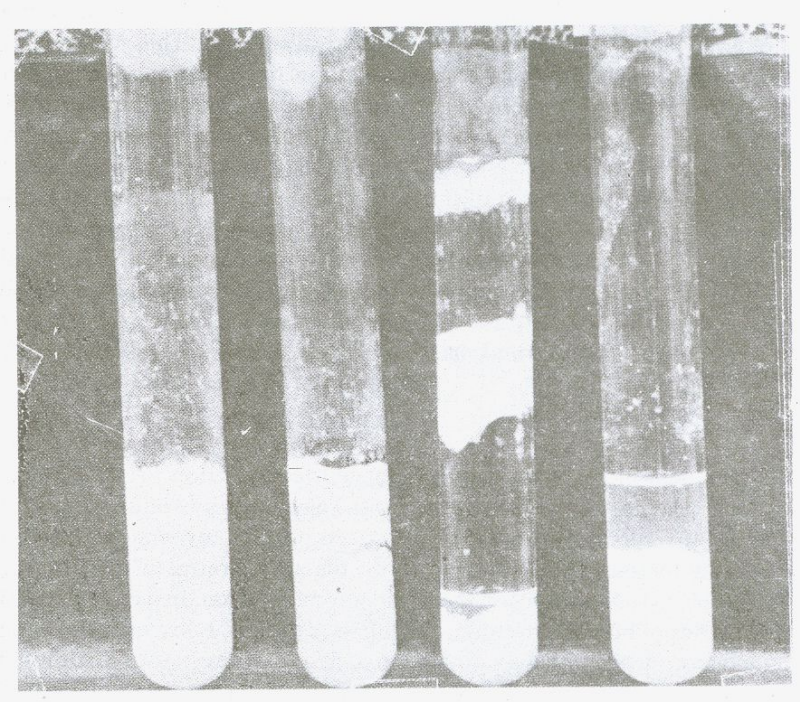

Fig.Progress of Acetone-Butanol fermentation by Clostridium acetobutylicum in Corn mash.

3. Kinetics of fermentation

The fermentation process involves three phases.

First phase

In this phase there is rapid growth of organism. Acetic and butyric acid Production. Carbon dioxide and hydrogen are evolved in large amounts. The pH at initial stage is at 6 to 6.5 for corn medium and 5.5 to 6.5 for molasses medium, it will then decrease & remains fairly constant. After 13 to 17 hours of incubation, `filtrable acidity' increases to maximum. Enzymes are produced for converting acidic products to neutral. This titrable acidity is determined as the milliliters of 0.1 N alkali required to neutralize the acids in a 10 ml fermentation broth and reported as milliliters 0.1 N acid per 10 ml sample.

Second phase

Here there is a sharp decrease in titrable acidity due to rapid conversion of acids to neutral solvents. Rate of gas evolution maximizes. Then gradually slows down.

Third phase

The gas evolution is decreased markedly and by decreased rate of solvent production is seen. Titrable acidity increases and pH of corn fermentation approximates 4.2 to 4.4 and that of molasses is from 5.2 to 6.2. Many cells will autolyse at this stage and riboflavin content of medium will increase.

3. Product recovery

Approximately 2 percent total solvents are present in the final harvested broth. This is passed through a `beer still' which contains approximately 30 perforated plates, in order to strip off solvents continuously. As the broth enters from the top of the still and descends down opposite to the flow, steam is passed which vaporizes the solvent. The steam and solvents are then collected and condensed by cooling so as to provide a solution with 40% /w total mixed solvents. The individual fractions are further separated by fractional distillation, with the acetone and butanol occurring in separate fractions.

The recovered fermentation gases like CO_2 has been converted to dry ice and methanol and ammonia have been prepared from there gases. The residual stillage which contains riboflavin and other B vitamins and bacterial cells after concentration and drying serves as vitamin feed supplement.

4.2.4.5. Yield.

In corn medium, the ratio of butanol, acetone and ethanol are 6, 3, 1 respectively but in molasses medium there ratios are 6, 5, 3 and 5 respectively. 3 parts carbon dioxide and 2 parts hydrogen by volume are the major products of this fermentation.

The total obtainable yields of butanol are influenced strongly by the toxicity of butanol for the fermentation organism. Thus the highest possible concentration of butanol with commercially used strains is 13.5g/l. This toxicity limits the initial concentration of sucrose at approximately 6%.

4.2.4.6. Contamination

Corn medium is more prone to microbial contamination than molasses medium. With either medium, attempts were made to detect contamination during inoculum production so that clean inoculum can be employed for production fermentors. Microscopic observations, aerobic plating and titrable acidity and gas evolution are followed closely.

Lactic acid producing bacteria, particularly the *Lactobacillus leichmannii* type can also produce slime that causes foam. There contaminants utilize carbon source resulting in high concentrations of lactic and other acids which prevents formation of the *Clastridium* adaptive enzymes required for conversion of acetic and butyric fermentation products to neutral solvents.

Phages also affect the processes regardless of type of media employed. The first indication of phage attack for corn medium is a slow or sluggish fermentation with decreased formentation gas evolution and for molasses cessation of gas evolution can be observed. Due to phage attack most of the bacteria may be killed and lysed within few hours. Although fermentation may start up within 24 to 48 hours because of the presence of resistant organisms, neutral solvents will not be produced unless the phage attack has occurred during phases 2 or 3 of the fermentation, that is, after the adaptive enzymes have been produced for converting fermentation acids to neutral solvents.

Cleanliness during operation of fermentation production plant is an asset to prevent phage contamination. Use of resistant or survived strains may prevent the loss.

4.2.4.7 Summary

Anaerobic fermentations are those fermentations that are carried out in the absence of oxygen by strictly anaerobic or facultative anaerobic bacteria or yeasts. During World War I, the production of interest was acetone used for production of the explosive trinitrotoluene. But after World War, butanol became more important for the production of nitrocellulose – lacquers.

Through *Clostridial* fermentation of starch, molasses, sucrose, wood hydrates and pentoses, many alcoholic products like butyric acid, butanol, acetones and isopropanol are obtained. In corn medium, the ratio of butanol, acetone and ethanol are 6, 3, 1 respectively but in molasses medium there ratios are 6, 5, 3 and 5 respectively Cleanliness during operation of fermentation production plant is an asset to prevent phage contamination. Use of resistant or survived strains may prevent the loss.

4.2.4.8 Model questions.

1) Explain the process of acetone fermentations

2) Describe the kinetics of butanol fermentation

4.2.4. 9 Reference books

1. Industrial microbiology – A.H. Patel
2. Industrial microbiology – Cruger

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Lesson 4.3.1

FERMENTATIVE PRODUCTION OF AMINO ACIDS

Structure

Objective

Amino acids are an important class of biomolecules. They are required by living cells for many activities like protein synthesis precursors. The production of amino acids through the fermentation process was initiated by Kinoshita and his coworkers. They produced L-glutamic acid as the first amino acid by the fermentation method.

Several amino acids are produced in commercial quantities via direct fermentation processes using overproducing microbial strains, or by microbial biotransformation. The amino acids are mostly employed as food or animal feed supplements and flavors compounds. However, several amino acids also have uses in pharmaceuticals and cosmetics, and in the chemical industry for the manufacture of polymers. In this chapter we will see the synthesis of different types of amino acids.

4.3.1.1 Introduction

Many processes for the production of various amino acids have been developed. Total world production of L-glutamic acid is considered to be in excess of 1,50,000 MT per year. It is used mainly as a flavoring agent. L-lysine has also been produced on a large scale by fermentation and is used mainly as a feed – supplement. Total world production L-lysine is probably in excess of 3,85,000 T per year. Methionine, alanine & glycine are not produced commercially by fermentation.

4.3.1.2. Production of Acidic Amino Acids

Among all the amino acids productions, the L-glutamic acid is probably the most important in terms of quantity. Its main use is as the flavour enhancers, monosodium L-glutamate (MSG), which can heighten and intensify. The flavour of foods without adding significant flavour of its own. This compound was first isolated from the seaweeds, *Laminaria japonica*.

Since 1960's the classical production methods using plant sources have largely been replaced by fermentation processes, production of glutamic acid from carbohydrate in high yield is carried out by a group of bacteria represented by *Corynebacterium glutamicum* and others like Brevibacterium, Microbacterium, etc.
L-Glutamic acid may be produced in many ways:

a. by the hydrolysis of wheat gluten, soyabean cake or other proteinaceous material.
b. By the cleavage of the pyrrolidone carboxylic acid found in molasses.
c. By a one stage fermentation process involving a single micro-organism.
d. By a two-stage fermentation process were ---- ketoglutaric acid is produced by one microbe and
------ ketoglutaric acid is converted to L-glutamic acid by another microbe.

A one stage process utilizing corynebacterium glutamicum is mainly adopted to produce L-glutamic acid on a large – scale.

Industrial production of L-glutamic acid using glucose as the carbon source was initiated in 1957 by Kyowa Hakko Kogyoco. Industrial scale fermenters are normally stainless steel stirred tank reactors of upto 450m^3. These are batch processes, operated aerobically at 30-37^oC, the specific temperature depending on the microorganisms used. Medium pH is maintained at 7-8 by the addition of alkali, otherwise the pH progressively falls as the L-glutamate is excreted in the medium.

Glucose and starch hydrolysates are the principal raw materials for the fermentative production of amino acids as carbon sources. More efforts have been made to replace these materials with such cheaper and more easily available ones as molasses, ethanol, pentose, acetic acid and other petrochemicals.

Apart from carbon and nitrogen sources, the fermentation medium normally contains inorganic salts, providing magnesium, manganese, phosphate and potassium. It is very necessary to add a limited amount of biotin to the production medium. The quantity of biotin needed is in the range of 1 to 5 ug/lit. The Corynebacteria are nutritionally fastidions and may also require vitamins, amino acids and 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 high. This can be overcome by the addition of saturated fatty acids, penicillin or surfactants, which promote excretion. The nitrogen source is fed slowly to prevent inhibition of L-glutamate production. Urea and ammonia may be used as nitrogen sources. The production media is slightly alkaline.

The duration of fermentation is 40 hours, and achieves amino acid, L-glutamic acid levels in the broth of 80 g/lit. Production recovery involves separation of cells from the culture medium. The L-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 L-glutamic acid are then filtered off and washed. Monosodium L-glutamate is prepared by adding a solution of sodium hydroxide to the crystalline L-glutamic acid followed by recrystallization.

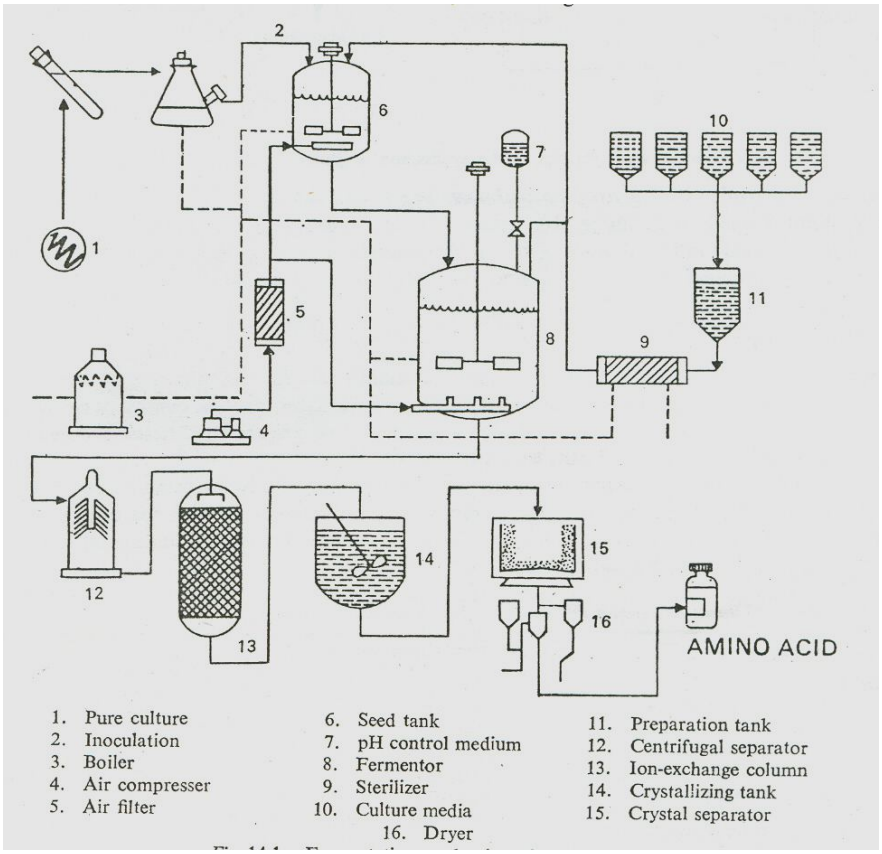

Fig . Fermentative production of amino acids

4.3.1.3. Production of Basic Amino Acids

L-lysine is an essential amino acid for the nutrition of humans. It is used for supplementing cereal proteins lacking this amino acid. Thus, protein quality of cereal foods is improved resulting in an improved growth and tissue synthesis. This amino acid has also been used medically as a nutrient.

The annual world production of L-lysine necessary to fulfil the requirement is now in excess of 3,85,000 tonnes. Over 90,000 tonnes of this lysine are currently produced by direct microbial fermentation and biotransformation methods. The remaining portion is produced by chemical synthesis. However, this method has the major disadvantage that a mixture of D and L-isomers is synthesized, but it is only L-lysine that the body utilizes. Thus optical resolution is required following chemical synthesis, where as microbial production has the advantage that only the L-isomer is formed.

A. Microbial sources

The fermentative production of L-lysine using homoserine auxotrophs of *Cornebacterium glutamicum*. This yields 39 g litre of L-lysine. The other microorganisms used in L-lysine production are Brevibacterium, Nocardia.

B. Anabolic pathway of L-lysine

The first key step of this anabolic pathway is aspartate to aspartyl phosphate, catalyzed by aspartokinase is controlled via feed back inhibition by two end products,

lysine and threonine. Homoserine dehydrogenase activity is also subject to feed back 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.

C. Medium

Production of media containing suitable amounts of L-aspartic acid are used for securing better yields of L-lysine. Cane molasses is the preferred carbon source. Although other carbohydrates, acetic acid or ethanol can be used, often supplemented with soya bean hydrolysates. Ammonia and urea used as nitrogen source at the same time to maintain the pH to neutrality. Control of biotin level is important, as concentrations below 30 ug/litre result in the accumulation of L-glutamate instead of L-lysine. However cane molasses usually contains sufficient biotin to fulfil this requirement. The function of biotin is to stimulate the carbon dioxide fixing enzyme, phospho enolpyruvate carboxylase.

D. Production

Most commercial L-lysine fermentations are operated as batch processes in aerated stirred tank reactors. The lag phase is shortened by using a high concentration of inoculum, normally about 10% of the fermentation volume. Production of lysine starts in the early exponential phase and continues through to the stationary phase. These fermentations last about 60 hours and yield 40-45 g/lit L-lysine from a molasses concentration of 200g / lit, containing 100g/lit sucrose.

E. Recovery

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 L-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 elute is reacidified and the product is crystallized as L-lysine hydrochloride.

4.3.1.4. Production of Hydroxy Amino Acids

A. L-Thercomine

Production from L-homoserine has been studied, but it has been unsuccessful as an industrial process because of the high cost of homoserine using diamino pimelate anxotroph and diaminopimelate plus methionine double auxotroph of E. Coli, with a carbohydrate source, Haung obtained L-threomine production at 2-4 g per lit.

The cultivation was carried out in 5 liter jar fermentors containing 3 liters of a medium having the composition :

7.5% fructose,
1.4% $(NH)_2\ SO_4$,
0.3% KH_2PO_4,
0.3% $MgSO_4.7H_2O$
2.0% $CaCO_3$

B. L-Serine

Corynebacterium glycinophilum ATCC 21341, produced 10g of L-serine per liter with a medium containing 2% glycine under favorable conditions. This strain was

isolated from a putrefied banana and resistant to a high concentration of glycine. A lencine – methionine double anxotroph, AJ3414, derived from C.glycinophilum ATCC 21341 produced 14g of serine per liter in a medium containing 3% glycine. The serine dehydratase activity of these mutants was reduced to 31 and 1.3% respectively, of the parent strain. The serine hydroxymethyl transferase is induced by serine formed during fermentation. Chloramphenicol addition at 16 and 48 hours in the culture of the parent strain increased serine production to 11g per liter compared with 4.6g per liter in the control culture.

Pseudomonas 3 ab, a facultative methylotropic organism, was incubated for 1 day in a medium containing 1% methanol. After supplementation of this medium with methanol and glycine and incubation at pH 8.5 for 3 days, 4.7 g of serine per liter were produced.

4.3.1.5. Production of Branched Chain Amino Acids

a. L-Isoleucine

The wild type strains of bacteria do not excute appreciable amounts of isoleucine in a salt sugar medium. Microbial production of isoleucine was first established by addition of precursors such as ---- aminobutyrate, D-threonine, and ----- hydroxybutyric acid which will escape the regulation. DL- ---- Bromobutyric acid could also be utilized as a precursor for isoleucine production. In addition to its role as a precursor by deamination to ---- Ketobutyrate, it stimulates isoleucine production by releasing feed back inhibition of threonine of threonine dehydratase by iso-leucine.

b. L-leucine

A leucine producing mutant was obtained as a leaky-type isoleucine auxotroph from an ----AB-resistant mutant of serratia marcescens. The isoleucine auxotroph produced L-leucine by long incubation in a medium lacking isoleucine. S-13 and S-11, partial revertants of strain No. 149, produced 13g per liter of L-leucine in 48 hrs in a medium lacking isoleucine.

The medium composition is

2% glucose,
10% dextran,
1% urea,
0.1% K_2HPO_4,
0.05% $MgSO_4$. $7H_2O$,
2% $CaCO_3$

C. L-valine

Vemura etal reviewed L-valine production by a fermentation process. Valine producing bacteria are often found in nature, but processes using auxotrophic or regulatory mutants have also been developed. The addition of certain drugs induce valine production. Most of the wild type of organisms capable of copions valine production belong to the Entero bacteriaceae family, especially to the genera Aerobacter and Escherichia.

Isoleucine and leucine auxotrophs of c-glutamicum produce a large amount of L-valine in culture broths. This is 11g per liter in a medium containing 7.5% glucose. Addition of leucine and valine markedly increased valine production in a synthetic medium.

4.3.1.6. Production of Aromatic Amino Acids

A. L-tryptophan

Tryptophanase, which catalyses synthsis of L-tryptophan was utilized for the production of L-tryptophan and related compounds such as 5-hydroxytryptophan. The culture broth of proteins rettgeri was used as the enzyme source for the reaction. The medium contains 6.0g of indole in 10ml of methanol, 0.8 g sodium pyruvate, 8.0g of ammonium acetate, 0.001g of pyridoxal phosphate, 0.1g of Na_2SO_4 and 100 ml of the cultured broth in a total volume of 120ml. 1+ of medium adjusted to 8.8 using 6N KOH, and it was inculated at 34°C for 48 hours.

B. L-phemylalanine

A tyrosine auxotrophic mutant, resistant to DEP and PAP, produced 9.5 g of L-phenylalamine per liter in the molasses – containg medium. The medium composition is

10% cane sugar
2% $(NH)_2SO_4$
0.05% KH_2PO_4
0.05% K_2HPO_4
0.025% $MgSO_4$. $7H_2O$
0.25% NZ-amine
2% $CaCO_3$

C. L-tyrosine

A phenylalamine auxotroph, which became multiply resistance to analogs of aromatic aminoacids, phenylalamine, tyrosine produced 13.5 g of L-tyrosine per liter in a cane molasses medium containing 10% reducing sugars as invert. Some mutants obtained produced larger amounts of L-tyrosine than the parent strain. The medium composition used for L-tyrosine production is 10% cane molasses.

2% $(NH_4)_2SO_4$,
0.05% K_2HPO_4,
0.05% KH_2PO_4,
0.025% $MgSO_4$. $7H_2O$
2% $CaCO_3$

4.3.1.7. Production of Other Amino Acids

A. Alanine

Many bacteria, fungi, yeasts and actinomycetes isolated from natural sources produce alanine in culture media. Prominent producers are corynebacterium gelatinosum, brevibacterium. In contrast to other amino acids, the alamine produced in these cultures is usually the racemic form. Psudomonas sp. No. 483 and micrococcus sodonensis are rare examples of L-Alamine producers. D-Alanine is produced by corynebacterium foscians under certain conditions using pseudomonas docunhae and xanthoonas orysae the L-Alanine is produced from L-aspatic acid with P. docunhae, high yield can be obtained by shaking a culture at 30°c in a medium containing ammonium fumarate, sodium fumarate, corn-steep liquor, peptone and inorganic salts.

B. L-proline

L-proline, which has a characteristically sweet taste, is not an essential amino acid, but recently medical and food industries have begun to make use of it. An auxotroph of C.glutamicum produced 31g of L-proline per liter in a medium containing 15% reducing sugars as invert. One of the characteristic conditions for L-proline production is a high concentration of ammonium salts. An excessive supply of biotin and unusually high concentrations of magnesium ions were also required for proline production by auxotrophic mutants.

4.3.1.8 Summary

- The amino acids are required in large amounts as they have number of applications in food industry chemical industry, pharmaceutical industry and cosmetic industry.
- The strains of Corynebacterium an brevibacterium are important microorganisms used in aminoacids production.
- As a flavouring L-glutamic acid and as a feed supplement L-lysine are highly used.
-

4.3.1.9 Model Questions

1. Describe in detail about the microbial fermentative production of L-glutamic acid.
2. Explain how the aromatic acids can be produced by microbial fermentation.

4.3.1.10 Reference Books

1. Industrial microbiology – Crueger
2. Industrial microbiology – Prescott and Dunn's
3. Industrial microbiology – L.E.Casida

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Lesson 4.3.2

FERMENTATION PRODUCTION OF VITAMINS AND ENZYMES

Structure

Objective

This lesson gives an idea about the fermentative production of different enzymes and vitamins

4.3.2.1 Introduction: Vitamins are very important compounds in the diet because they are of great value in the growth and metabolism of the living cell. All vitamins may be synthesized by prototopic microorganisms. Even though almost all the vitamins are synthesized by microorganisms, only riboflavin and B_{12} have found extensive commercial production. For the production of some vitamins, direct fermentations are operated, where as for others, biotransformations or combined chemical and microbiological processes are employed.

Enzymes, are the biological catalysts, for the biochemical reactions, leading to the microbial growth and metabolic and other physico chemical reactions. Enzymes depending on localization considered into two types – exocellular and endocellular. The exocellular enzymes include most of the enzymes produced by microbial fermentations for commercial use.

4.3.2.2. Fermentative Production of Vitamins

Most vitamins were previously prepared from animal and plant tissues, although dried backer's and brewer's yeast preparations, from saccharomcyces cerevisae, have long been employed as a rich source of B vitamins. Microorganisms are now used as sources of wide range of vitamins, including thiamin, riboflavin, pyridoxine, cobalamine, L-ascorbic acid, B-carotene, etc. Certain microorganisms excrete vitamins greatly in excess of their metabolic needs if grown under highly specified and artificial conditions. Thus, such microorganisms are of industrial important and interest.

1. Cobalamine (Vitamin B_{12})

Vitamin B_{12} is an important dietary component for normal growth in human beings and domestic animals. The daily requirement for human beings is 0.001 mg/day. Its deficiency causes anaemia. This is the only vitamin which contains metal and porphyrin ring. In the cobalamine cobinamide is linked to a nucleotide, 5, 6-dimethyl benzimidazole. The cobinamide molecule has a central atom of cobalt linked to a cyanide group, surrounded by four reduced pyrrole rings joined to form a macro-ring.

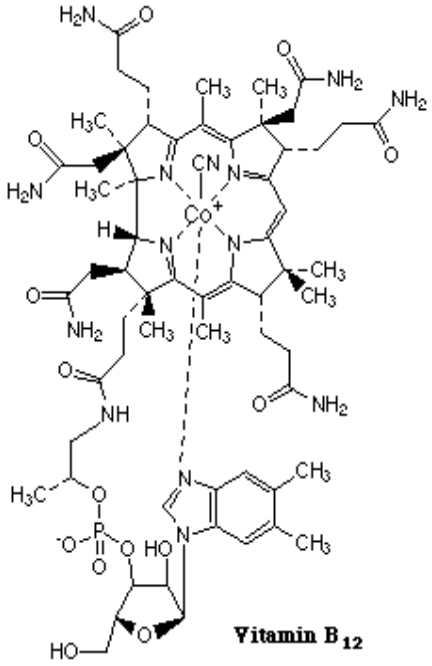

Fig 1. Structure of vitamin B_{12}

Vitamin B_{12} is entirely produced on a commercial basis by the fermentation. Generally, it is manufactured by primary fermentation. The first commercial production of vitamin B_{12} was as a by-product of various streptomyces antibiotic fermentations, particularly that for streptomycin, and as a by product of the acetone – butanol fermentation. The mutation of streptomyces to higher antibiotic yield capacity decreased vitamin B_{12} yields. The sub sequent discovery of relatively high concentrations of this vitamin inn sewage – sludge solids also allowed its recovery and commercialization from this source in some industries. All this is largely a matter of economics, since maximum production of the antibiotic and maximum production of cobalamin generally do not take place under the same conditions of fermentation.

a. Microorganisms

The microorganisms used for production of vitamin B_{12} are streptomyces griseus, S.olivaceus, Bacillus megaterium, B.coagulans, pseudomonas denitrificans, propionibacterium freudenreichii, proteins species and pseudomonas species. The streptomyces olivaceus NRRLB-1125 is the specifically used strain for commercial production of vitamin B_{12}.

b. Medium

The production medium used for the fermentative production consists of carbohydrate, proteinaceous material and a source of cobalt and other salts.
The components of the medium are

1. Solubles - 4%

2. Dextrose - 0.5% to 1%
3. $CaCO_3$ - 0.5%
1. $COCl_2$. $6H_2O$ - 1.5 to 10 ppm

The pH of the medium must adjust to about 7 with NaOH. The organism scavenges low levels of cobalt from the medium, but higher levels are toxic. Most forms of distiller's solubles, soya bean meat, yeast, casein, etc. are found satisfactory under suitable conditions. It is essential to add cyanide to the medium to aid the conversion of other cobalamins to vitamin B_{12}.

C. Production procedure

Sterilization of the medium may be practiced batchwise or continuously. In batchwise sterilization, the medium contained in the production tank is heated at 250°F for 1 hour. In the continuous method, the production tank is charged at 330°F for 13 minutes by mixing it directly with live steam during sterilization, steam is blown into openings and all transfer lines are kept filled with live steam when not in use to ensure sterility.

During the fermentation process temperature needed to be maintained at 80°F. In the first 24 hours the pH of the medium decrease rapidly with consumption of sugar. After 2 to 4 days, lysis of the mycelium begins resulting in the rise of pH. The stabilization is maintained by reducing the pH to about 5 with sulphuric acid and adding small amounts of a reducing agent.

A proper rate of aeration and a correct speed of agitation are essential because the rate of the growth of the streptomycete strain depends on the rate of aeration and agitation. Aeration rates higher than optimum causes foaming. The optimum rate is 0.5 volume air / volume medium / minute. The important antifoaming agents are : soya bean oil, corn oil, lard oil and silicones. An antifoaming agent in its sterile form is added to the medium during foaming according to the requirement.

The duration of the fermentation is about 3 to 4 days, or until mycelium are usually in the range of 1 to 2mg per liter in the fermented broth. During the major part of the fermentation period, most of the cobalamine is associated with the mycelium, But a considerable portion of the cobalamine is in the solution at the end of the fermentation period. Heating the mixture to boiling at pH 5 or below, liberates the cobalamine quantitatively from the mycelium. Broth containing cobalamine is subjected to further work-up depending on the type of the product to be produced.

In order to obtain crystalline vitamin B_{12}, the very first operation is of filtration to remove mycelium. Then the filtered broth is treated with cyanide to bring about the conversion of cobalamin to cyanocobalamin. Alternatively, this conversion may also be done after some concentration has been secured while fermentation is in progress. The adsorption of the cyano cobalamin from the solution is practiced by passing it through an adsorbing agent packed in columns. Several adsorbants are available for this purpose like activated charcoal. Finally elution of cyano cobalamin from adsorbent is accomplished by the use of an aqueous solution of materials ranging from organic bases to hydrochloric acid.

Another purification step applicable to aqueous concentrates consists in dissolving a zinc salt in the slight acid solution and then raising the pH to bring about precipitation of zinc hydroxide, which eliminates many impurities, chromatography on alumina and binal crystallization from methanol-acetone, ethanol-acetone, or acetone – water usually complete the process.

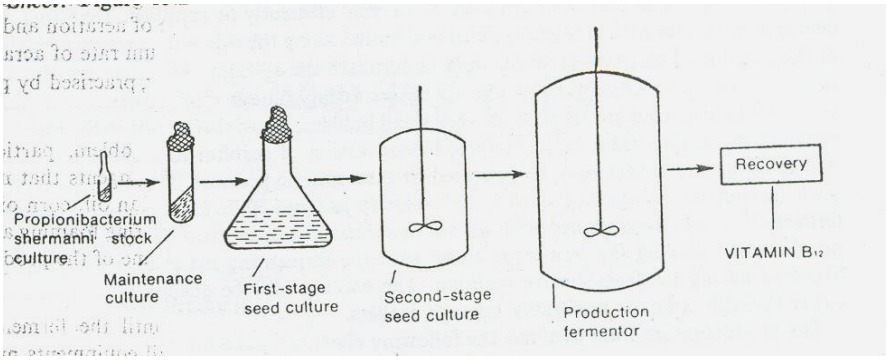

Fig . Vitamin B_{12} fermentation process

Species of the genus propiomibacterium, p. frendenreichii has been reported to produce the highes yields of vitamin B_{12}. The medium for this species is glucose, corn-steep, betain, and cobalt.

2. L-ascorbic acid (Vitamin 2)

L-ascorbic acid was isolated in pure form from lemon juice for the first time by king and wangh. It is a strong reducing agent and plays a part in cellular oxidation – reduction reactions.

Annual production of ascorbic acid is now over 40,000 tonnes. The established process involves chemical stages and a microbial biotransformation. It starts with the chemical catalytic reduction of D-glucose and D-sorbitol, the sorbitol is then oxidized to L-sorbose using Acetobacter auboxydans or A.xylinum. Media for this biotransformation step consists of glucose, yeast extract or corn steep liquor, a slight excess of calcium carbonate and 15-30% D-sorbitol. The biotransformation is performed at 30^{o}C under vigorous aeration and with in 1-2 days a 90-95% conversion is achieved. L-sorbose is recovered from the medium following filtration and concentration of the filtrate to a syrup. This sugar crystallizes on cooling and about 65% is recovered. The sorbose is then chemically converted to ascorbic acid via 2-keto – L-gluconic acid.

A much more direct route from glucose to ascorbic acid has now been made possible by the introduction of a gene encoding 2,5-diketo – D – gluconic acid reductase from corynebacterium into eruinia. This simplifies the process to a single biotrasformation and a single chemical step. The genetically modified Eruinia strain transforms glucose to 2-keto-L-gluconic acid, which can then be simply converted to ascorbic acid in one chemical step.

3. Riboflavin (Vitamin B_2)

Riboflavin is used to fortify processed foods, particularly breakfast cereals and soft drinks. It can be produced chemically or by germentation processes, often using the yeasts Ashbya gossypii, Candida flareri and Eremothecium ashbyii. Annual production via fermentation is around 600 tonnes and the main processes utilize strains of A.gossypii, largely because of their greater stability. These strains have been developed to produce 10-15 g/lit in aerobic fermentation, carried out at 34-37^{o}C and pH

6.5-7.0. Each fermentation is divided into two stages; the first promotes growth and second stage maximizes riboflavin production. This is achieved through the restriction of growth by controlling the periodic or continuous addition of the carbon source or a micronutrient such as iron. The riboflavin is secreted into the medium, but some remains cell bound and is released by heat treatment prior to further purification.

4.3.2.3. Fermentative Production of Enzymes

Enzymes are proteins produced by living cells and utilized by these cells to catalyze specific chemical reactions. Their biological function is to bring about and regulate, by their concerted action, the metabolic processes in the organism. Commercial production and utilization of enzymes are based on two factors.

1. Enzymes are produced by living cells.
2. Enzymes can exert their specific action independent of living cells.

Enzymes are used for a variety of purposes. They are employed in three major fields.

1. Laboratory
2. Industrial
3. Clinical

In the usage of enzymes some applications use the enzymes in crude form and others use in highly purified state.

Industrial enzymes are produced from plants, animals and microorganisms, but manufacture from the first two groups is limited for several reasons. As the concentration of enzymes in plants is generally low, processing of large amounts of plant material is necessary. On the other hand, animal enzymes are by products of meat industry and for this reason limited in supply.

In contrast, microbial enzymes can be produced in amounts meeting all demands of the market. Seasonal fluctuations of raw material do not account and there are possibilities for genetic and environmental manipulation of bacterial and fungi to give increased yields of desired enzymes in a way not possible with higher organisms. So microbial enzymes present a wide spectrum of characteristics that makes them utilizable for quite specific applications.

A. Selection of microorganisms

The first step in the manufacture of an enzyme involves the selection of an organism suitable to produce the desired enzyme in amounts as large as possible.

For selection:

1. Extracellular enzymes are preferred, because difficult and costly methods of cell disruption are not necessary. As compared with intracellular enzymes, they are present in a relatively pure form in the culture liquor. Intracellular enzymes are industrially used to a lesser extent because of difficult procedures of cell disruption and separation of contaminating cell components.
2. High yields of enzymes should be obtained with an economical time required for culture production.
3. The strain must be stable with respect to productivity, requirement for culture conditions, and sporulation.

B. The synthetic activity should be as far as possible in the direction of the desired enzyme. Formation of interfering by-products should be low.

C. The organism should be able to grow on cheap substrates.

D. Clarification of the culture liquor or extract should be possible with our difficulties.
E. The strain must not produce toxic substances and should be free of antibiotic activities. It should not belong to related strains that synthesize toxins. Mostly enzymes with particular properties eg., with respect to stability and activity are desired.

Enzyme(s)	*Micro-organism(s)*
α-Amylase	*Aspergillus oryzae, A. niger, Bacillus subtilis, B. amyloliquefaciens.*
Glucoamylase	*A. niger, Rhizopus niveus, R. delemar, Endomycopsis* spp.
Protease	*A. oryzae, A. niger, A. saitoi, Mucor pusillus, B. subtilis, Streptomyces griseus*
Lipase	*A. niger, Rhizopus* spp. *Candida cylindracea*
Cellulase	*A. niger, Trichoderma viride*
Pectinase	*Coniosthyrium diplodiella*
Glucase oxidase and others	*Penicillium amagasakiense*
Invertase	*Saccharomyces cerevisiae*
Naringinase	*A. niger*
Melibiase	*Mortierella vinacea*
Aspartase	*Escherichia coli*
Aminoacylase	*A. oryzae*
Antocyanase	*A. niger*
Glucose isomerase	*Streptomyces* spp.
Catalase	*A. niger*

Table 1. Microbial enzymes

B. General aspects of enzyme production

1.Cultivation techniques

Enzymes are commercial produced by two methods:

a. Semisolid culture
b. Submerged culture

It is evident that the submerged culture method has been gaining ground during the past three to four decades.

a. Semisolid culture

The enzyme producing culture is grown on the surface of a suitable semisolid substrate. The substrate usually consists of moistened wheat or rice bran, supplemented with nutrient salts. The production medium is prepared by mixing bran with a solution containing any desired nutrient salts. The desired pH for optimum growth of the mould is adjusted with acid. Then the medium is steam – sterilized in an autoclave while stirring. This sterilized medium is spread on metal trays upto a depth of 1-10 cms., the total quality being of the order of thousands of kilogrammes. Such a transfer is performed under aseptic conditions. Alternatively, the cultivation may be carried out in rotating drums. The fungal spores are inoculated, either in the autoclave after cooling or in trays. A series of trays are enclosed in a large vessel. Aeration is ensured by the circulation of suitably humidified air over the surface of the culture. It is necessary to keep the temperature with in narrow limits. Moreover, heat generation occurs during fermentation. Therefore, the trays should be equipped with a cooling system. It should be borne in mind that, direct air cooling is not practical, since drying

of the cultures takes place. Subsequently extraction of enzyme is performed with water. Examples for the enzymes production by fermatation technology using semisolid culture are: α-amylase production using aspergillus oryzae, protease production using A.niger and A.oryzae.

The advantages of the semisolid cultivation are mainly low investment, It allows the use of substrates with a high dry matter content, useful for cultivating some moulds which are very difficult to grow in fermentors.

The disadvantages of semisolid cultivation are mainly requires more space, requires more labour, involves a greater risk of infection.

b. Submerged culture

The submerged culture methods are widely used for enzymes production. The fermentation tank is a cylindrical tank of stainless steel. The tank is equipped with an agitator, an aerating device, a cooling system and various ancillary equipment. The quantity of production medium taken in the fermentation tank is in the range of 1000-30,000 gallons or more.

The formulation of the production medium and to a lesser extent control of fermentation conditions play major roles in the success of the process. The production medium should basically contain an energy source, carbon and nitrogen sources and any special growth requirements. Certain compounds present in the medium may induce or inhibit enzyme formation. For example, the presence of lactose in the medium induces B-galactosidase.

The enzymes are pH dependent, so it is necessary to control the pH within the desired limits of enzyme during fermentation. This can be achieved by adding a buffer system. The alternative is to add certain compounds which upon metabolism, bring about a change in pH in the desired direction.

After the completion of fermentation, the fermented liquior is subjected to rapid cooling to about 5^{o}C to reduce deterioration. Separation of microorganisms is accomplished, either by filtration or centraifugation of refrigerated broth with adjusted pH. The colloidal particles present in the filtrate are eliminated with coagulating or flocculating agents. Removal of the suspended solids is carried out by vacuum drum filtration or by a disc-type centrifuge equipped with a self-cleaving bowl. In order to obtain a higher degree of purity, the enzyme is precipitated with acetone, alcohols or inorganic salts. Fractional precipitation gives purities higher than one-step precipitation. Extraction of endocellular enzymes involves the disintegration of microbial cells. This can be accomplished by a homogenizer or a bead mill. There after, purification methods being employed for exo-enzymes are used for purification.

2. Extraction methods

The first step in the isolation of enzymes is their extraction. Techniques that fall into this group are employed either to separate enzymes from solid substrate culture or to release enzymes from the interior of microbial cells. This is two types

a. Extraction of solid substrate cultures
b. Extraction of cells

a. Extraction of solid substrate cultures

Enzymes produced by this method are of the extracellular type. The cultures can be produced in relatively small equipment all the year round, while the extraction is

conducted in times of enzyme demand. The enzymes will be extracted into water, however, which may contain acids, salts, buffers or other substances to facilitate solubilization of the enzyme or to improve its stability in solution.

b. Extraction of cells

The cell suspension passes a homogenizing value at the selected operating pressure and imringes on an impact ring. The strong shearing forces combined with the sudden decompression lead to a disruption of the cell wall.

3. Separation methods

The different types of enzyme separation methods are

a. Solids separation techniques
b. Membrane separation techniques
c. Precipitation techniques

a. Solids separation techniques

These are involved in the clarification of culture liquors and extracts, in the separation of precipitates and in the sterilization of liquid enzyme preparation by mechanical methods. Solid substance may be greasy, sometimes colloidal and often density differences between solid particles and liquid phase are very small. Therefore pretreatment of solid is necessary which is by acidification, mild heating, etc. the solids separation is by either centrifugation or filtration.

b. Membrane separation techniques

The membrane processes allow separation of solutes from one another or from a solvent, with no phase change ultrafiltration is the only membrane process of importance in large-scale enzyme production. From normal filtration processes it differs just by the size range of the particles to be separated.

c. Precipitation techniques

Separation from solution by salting out is one of the oldest and yet most important procedures of concentration and purification of enzymes.

The commonly used salt for precipition is ammonium sulfate. In ammonium sulfate solutions precipitated enzymes are often storable for years without significant loss when kept at room temperatures. As the solubility of a protein molecule is lowest at its isoelectric point, successive precipitation of different enzymes from a solution can be achieved by changing the pH.

4. Conversion to storage form

Commercial enzyme products are available either in solution or in solid state. Generally, users prefers solutions because of their easier handling, but enzymes are very unstable in aqueous solutions. To improve the stability dissolve the enzyme in a highly concentrated solution of salts and sugars.

Commercially available solid enzyme preparations are dried mold brans, dried precipitates or dried solutions. Spray drying is preferred method for removal of water from enzyme solutions. Freeze drying is most preserving, but its use is limited by cost considerations.

c. Particular enzyme preparations

Fewer than 20 are more commercially used on a scale that has significant impact on either the enzyme industry or the user industries.

1. Proteases

Complex mixtures of true proteinases and peptidases are usually called proteases. The proteases are of endoenzymes and are all extracellular enzymes. The proteases from microorganisms are classified into four main groups based on the mechanism of their action: serine proteinases, thiol proteinases, metallo proteinases and acid proteinases. The proteases are produced by bacteria like bacillus species and by yeast like Asperigillus species. Subtilisin Carlsberg is the most widely used detergent protease. It is obtained from Bacillus licheniformis by submerged – culture method. It is essential to take care during their production, because they are unstable, and tend to lose their activity during dehydration.

a. Serine proteases

These proteases are widespread in bacteria and fungi. They show maximum activity at neutral to alkaline pH. Among the different types of serine proteases, serine alkaline proteases have high economic importance.

Serine alkaline proteases are more active at pH 9.5-10.5. These are produced by numerous species of bacteria and fungi. The best sources are Bacillus subtilis and Aspergillus.

Keay and Moser have proposed that alkaline serine proteases produced by different strains of Bacillus subtilis are two types : subtilisin Carlsberg and subtilisin novo.

Bacillus megaterium produce the protease during the log phase of growth, while B subitilis, B.Cereus, produce in the stationary phase.

The concentration of parely carbonaceous medium components should normally be kept on a low level. High concentrations of carbon source yields excess organic acids leading to a decrease in pH, which is accompanied by a decrease inn alkaline protease production. It has been shown that NH_4^+ ions strongly inhibit production of the enzyme, while nitrates and Na salts of aspartic acid and glutamic acids promotes its formation.

Serine alkaline proteases of bacterial origin are used in large amounts in laundering and to a lesser extent in leather tanning and the food industry.

B. Metallo proteinases

The activity of metallo proteinases is very poor, so has less commercial importance. They exhibit maximum activity at pH 7 to 8. Strains used for production are Aspergillus, Bacillus and streptomyces.

C. Acid proteinases

The acid proteinases are more used in food industry. They are characterized by maximum activity and stability at pH 2.0-5.0. They are usually produced using Aspergillus niger, Penicillium, Rhizopus.

2. Amylases

Amylases play the most important part in food technology like bread-making, beer making, etc. Therefore, concentrates α- and B-amylases are prepared and used in a variety of ways. These enzyme preparations must be carefully standardized for activity, according to the purpose for which they are to be used.

Amylases are characterized by their ability to hydrolyze 1, 4-glucosidic linkage in polysaccharides the two main groups of amylases are --- amylase and B amylase.

α-amylases are endo enzymes. They attack all the linkages be between glucose units in the starch molecule. α-amylases vary in their effectiveness, depending on their source.

α-amylases are produced by the use of fungi A. niger and A. oryzae as well as bacteria B.licheniformis, and B.amyloliquefaciens. The media used for the production of α-amylases by sub merged culture using fungal source is in Table 2 and using bacterial source is given in Table 3.

Table 2. Yeast α-amylase medium

Table 3. Bacterial α-amylase medium

(Patel page. 142)

A temperature in the range of 30^{o} to 40^{o}c is satisfactory. The optimum pH for the fermentation medium is 7.0. It is necessary to maintain the pH near neutrality, since the amylase is denatured below 6. Calcium carbonate is used as the buffer to maintain neutral pH. The production of α-amylase begins when the bacterial count reaches 10^{9}-10^{10} cells per milliliter after about 10 to 20 hours, and continues for another 100-150 hrs. Preservation of liquid preparations of bacterial α-amylases is done by 20% sodium chloride. The most active preparations contain 2% active protein.

4.3.2.4 Summary

- Vitamin derivatives and enzymes are important biological catalysts.
- For production of cyanocobalamin, the only metal containing vitamin, streptomyces and Bacillus species are used.
- Among the surface semisolid and subculture methods used for production of enzymes subculture method is gaining more importance.
- While producing the enzymes the activity must not be lost.

4.3.2.5 Model Questions

1. What are the enzyme extraction methods from the crude fermented product
2. How can you produce L-ascorbic acid using fermentation ?

4.3.2.6 Reference Books

1. Industrial microbiology – A.H. Patel
2. Industrial microbiology – Crueser
3. Industrial microbiology – L.E. Casida

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Lesson 4.3.3.

METHODS OF IMMOBILIZATION

Structure

Objective

The main objective of this lesson is to make you familiar about immobilization technology, various methods used to immobilize and the applications of this technology in various aspects.

4.3.3.1 Introduction

An important factor determining the use of enzymes in a technological process is their expense. Several hundred enzymes are commercially available at prices of about £1 mg^{-1}, although some are much cheaper and many are much more expensive. As enzymes are catalytic molecules, they are not directly used up by the processes in which they are used. Their high initial cost, therefore, should only be incidental to their use. However due to denaturation, they do lose activity with time. If possible, they should be stabilised against denaturation and utilised in an efficient manner. When they are used in a soluble form, they retain some activity after the reaction which cannot be economically recovered for re-use and is generally wasted. This activity residue remains to contaminate the product and its removal may involve extra purification costs. In order to eliminate this wastage, and give an improved productivity, simple and economic methods must be used which enable the separation of the enzyme from the reaction product. The easiest way of achieving this is by separating the enzyme and product during the reaction using a two-phase system; one phase containing the enzyme and the other phase containing the product. The enzyme is imprisoned within its phase allowing its re-use or continuous use but preventing it from contaminating the product; other molecules, including the reactants, are able to move freely between the two phases. This is known as **immobilisation** and may be achieved by fixing the enzyme to, or within, some other material. The term 'immobilisation' does not necessarily mean that the enzyme cannot move freely within its particular phase, although this is often the case. A wide variety of insoluble materials, also known as substrates (not to be confused with the enzymes' reactants), may be used to immobilise the enzymes by making them insoluble. These are usually inert polymeric or inorganic matrices.

4.3.3.2 Advantages of immobilization

Immobilisation of enzymes often incurs an additional expense and is only undertaken if there is a sound economic or process advantage in the use of the immobilised, rather than free (soluble), enzymes. The most important benefit derived from immobilisation is

1. the easy separation of the enzyme from the products of the catalysed reaction. This prevents the enzyme contaminating the product, minimising downstream processing costs and possible effluent handling problems, particularly if the enzyme is noticeably toxic or antigenic.
2. It also allows continuous processes to be practicable, with a considerable saving in enzyme, labour and overhead costs. Immobilisation often affects the stability and activity of the enzyme, but conditions are usually available where these properties are little changed or even enhanced.
3. The productivity of an enzyme, so immobilised, is greatly increased as it may be more fully used at higher substrate concentrations for longer periods than the free enzyme. Insoluble immobilised enzymes are of little use, however, where any of the reactants are also insoluble, due to steric difficulties.

4.3.3.3Charecters of supporting material

Carrier matrices for enzyme immobilisation by adsorption and covalent binding must be chosen with care. Of particular relevance to their use in industrial processes is their cost relative to the overall process costs; ideally they should be cheap enough to discard. The manufacture of high-valued products on a small scale may allow the use of relatively expensive supports and immobilisation techniques whereas these would not be economical in the large-scale production of low added-value materials. A substantial saving in costs occurs where the carrier may be regenerated after the useful lifetime of the immobilised enzyme. The surface density of binding sites together with the volumetric surface area sterically available to the enzyme, determine the maximum binding capacity. The actual capacity will be affected by the number of potential coupling sites in the enzyme molecules and the electrostatic charge distribution and surface polarity (i.e. the hydrophobic-hydrophilic balance) on both the enzyme and support. The nature of the support will also have a considerable affect on an enzyme's expressed activity and apparent kinetics. The form, shape, density, porosity, pore size distribution, operational stability and particle size distribution of the supporting matrix will influence the reactor configuration in which the immobilised biocatalyst may be used. The ideal support is cheap, inert, physically strong and stable. It will increase the enzyme specificity (k_{cat}/K_m) whilst reducing product inhibition, shift the pH optimum to the desired value for the process, and discourage microbial growth and non-specific adsorption. Some matrices possess other properties which are useful for particular purposes such as ferromagnetism (e.g. magnetic iron oxide, enabling transfer of the biocatalyst by means of magnetic fields), a catalytic surface (e.g. manganese dioxide, which catalytically removes the inactivating hydrogen peroxide produced by most oxidases), or a reductive surface environment (e.g. titania, for enzymes inactivated by oxidation). Clearly most supports possess only some of these features, but a thorough understanding of the properties of immobilised enzymes does allow suitable engineering of the system to approach these optimal qualities.

4.3.3.4 Methods of Immobilisation

There are four principal methods available for immobilising enzymes adsorption

a. covalent binding
b. entrapment
c. membrane confinement

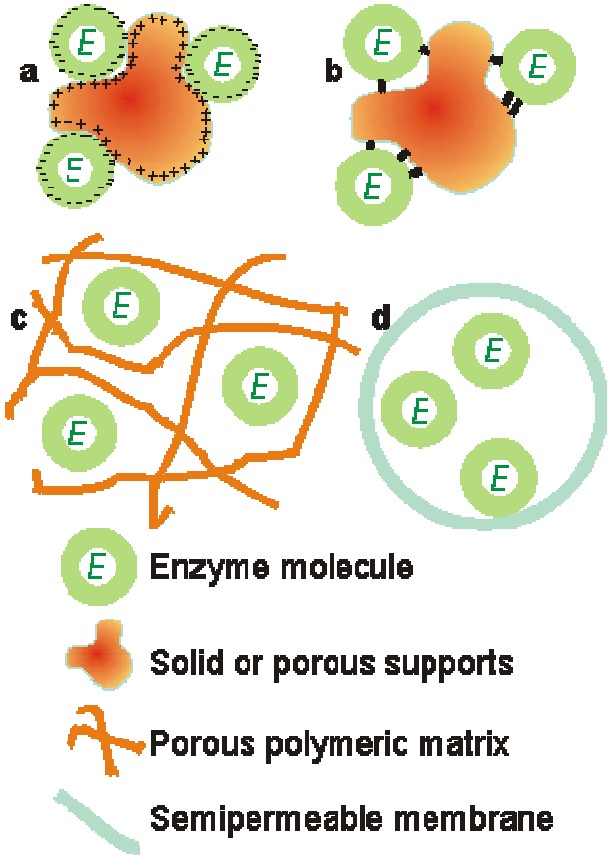


Figure 3.1. Immobilised enzyme systems. (a) enzyme non-covalently adsorbed to an insoluble particle; (b) enzyme covalently attached to an insoluble particle; (c) enzyme entrapped within an insoluble particle by a cross-linked polymer; (d) enzyme confined within a semipermeable membrane

4.3.3.5 a. Adsorption:

Adsorption of enzymes onto insoluble supports is a very simple method of wide applicability and capable of high enzyme loading (about one gram per gram of matrix). Simply mixing the enzyme with a suitable adsorbent, under appropriate conditions of pH and ionic strength, followed, after a sufficient incubation period, by washing off loosely bound and unbound enzyme will produce the immobilised enzyme in a directly usable form(**fig3.1**). The driving force causing this binding is usually due to a combination of hydrophobic effects and the formation of several salt links per enzyme molecule. The particular choice of adsorbent depends principally upon minimising leakage of the enzyme during use. Although the physical links between the enzyme

molecules and the support are often very strong, they may be reduced by many factors including the introduction of the substrate. Care must be taken that the binding forces are not weakened during use by inappropriate changes in pH or ionic strength. Examples of suitable adsorbents are ion-exchange matrices (**table 3.1**), porous carbon, clays, hydrous metal oxides, glasses and polymeric aromatic resins. Ion-exchange matrices, although more expensive than these other supports, may be used economically due to the ease with which they may be regenerated when their bound enzyme has come to the end of its active life; a process which may simply involve washing off the used enzyme with concentrated salt solutions and re-suspending the ion exchanger in a solution of active enzyme

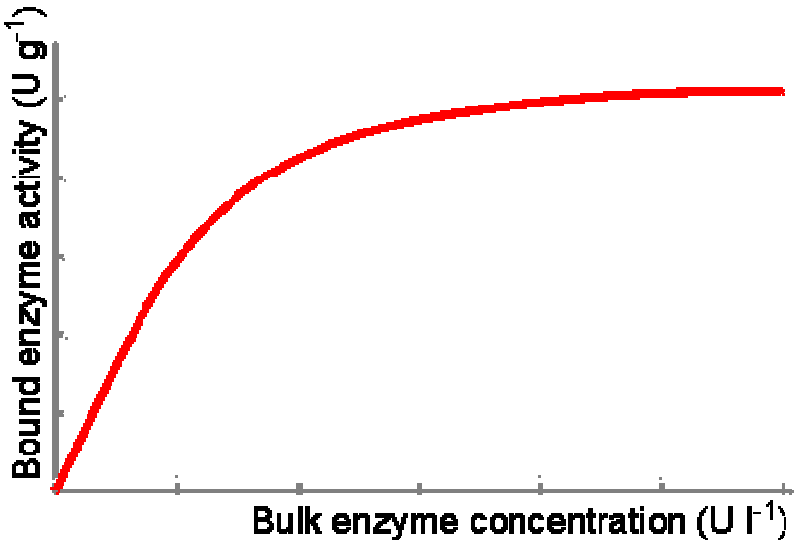


Figure 3.2. Schematic diagram showing the effect of soluble enzyme concentration on the activity of enzyme immobilised by adsorption to a suitable matrix. The amount adsorbed depends on the incubation time, pH, ionic strength, surface area, porosity, and the physical characteristics of both the enzyme and the support

Table 3.1 Preparation of immobilised invertase by adsorption.

	Support type	
% bound at	**DEAE-Sephadex anion exchanger**	**CM-Sephadex cation exchanger**
pH 2.5	0	100
pH 4.7	100	75
pH 7.0	100	34

4.3.3.4.b covalent coupling:

Immobilisation of enzymes by their covalent coupling to insoluble matrices is an extensively researched technique. Only small amounts of enzymes may be immobilised by this method (about 0.02 gram per gram of matrix) although in exceptional cases as much as 0.3 gram per gram of matrix has been reported. The strength of binding is very strong, however, and very little leakage of enzyme from the support occurs. The relative usefulness of various groups, found in enzymes, for covalent link formation depends upon their availability and reactivity (nucleophilicity), in addition to the stability of the covalent link, once formed (**table3.2**). The reactivity of the protein side-chain nucleophiles is determined by their state of protonation (i.e. charged status) and roughly follows the relationship $-S^-$ > -SH > $-O^-$ > $-NH_2$ > $-COO^-$ > -OH >> $-NH_3^+$where the charges may be estimated from a knowledge of the pK_a values of the ionising groups and the pH of the solution. Lysine residues are found to be the most generally useful groups for covalent bonding of enzymes to insoluble supports due to their widespread surface exposure and high reactivity, especially in slightly alkaline solutions. They also appear to be only very rarely involved in the active sites of enzymes

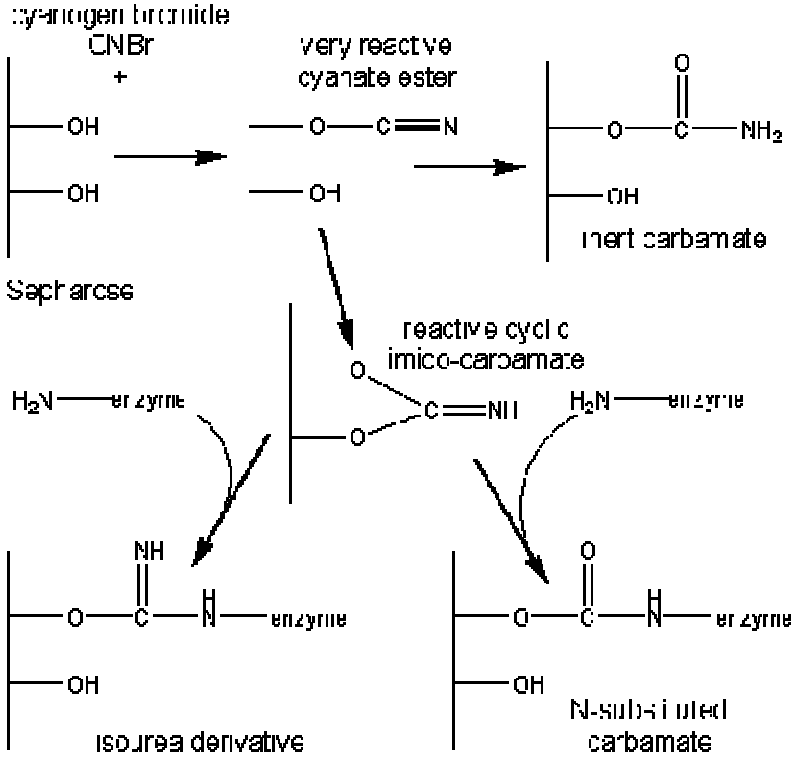
Table 3.2 Relative usefulness of enzyme residues for covalent coupling

Residue	**Content**	**Exposure**	**Reactivity**	**Stability of couple**	**Use**
Aspartate	+	++	+	+	+
Arginine	+	++	-	±	-
Cysteine	-	±	++	-	-
Cystine	+	-	±	±	-
Glutamate	+	++	+	+	+
Histidine	±	++	+	+	+
Lysine	++	++	++	++	++
Methionine	-	-	±	-	-
Serine	++	+	±	+	±
Threonine	++	±	±	+	±
Tryptophan	-	-	-	±	-
Tyrosine	+	-	+	_+	+
C terminus	-	++	+	+	+
N terminus	-	++	++	++	+
Carbohydrate	- ~ ++	++	+	+	±
Others	- ~ ++	-	-	- ~ ++	-

The most commonly used method for immobilising enzymes on the research scale (i.e. using less than a gram of enzyme) involves Sepharose, activated by cyanogen bromide. This is a simple, mild and often successful method of wide applicability. Sepharose is a commercially available beaded polymer which is highly hydrophilic and

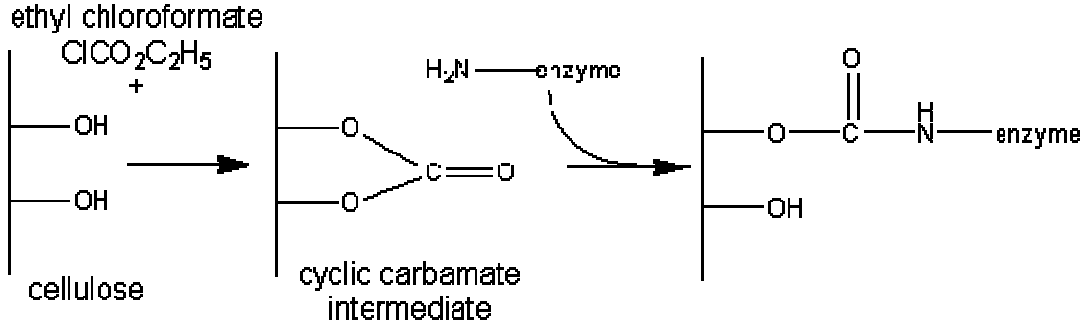
generally inert to microbiological attack. Chemically it is an agarose (poly-{□-1,3-D-galactose-□-1,4-(3,6-anhydro)-L-galactose}) gel. The hydroxyl groups of this polysaccharide combine with cyanogen bromide to give the reactive cyclic imido-carbonate. This reacts with primary amino groups (i.e. mainly lysine residues) on the enzyme under mildly basic conditions (pH 9 - 11.5, Figure 3.3a). The high toxicity of cyanogen bromide has led to the commercial, if rather expensive, production of ready-activated Sepharose and the investigation of alternative methods, often involving chloroformates, to produce similar intermediates (Figure 3.3b). Carbodiimides (Figure 3.3c) are very useful bifunctional reagents as they allow the coupling of amines to carboxylic acids. Careful control of the reaction conditions and choice of carbodiimide allow a great degree of selectivity in this reaction. Glutaraldehyde is another bifunctional reagent which may be used to cross-link enzymes or link them to supports (Figure 3.3d). It is particularly useful for producing immobilised enzyme membranes, for use in biosensors, by cross-linking the enzyme plus a non-catalytic diluent protein within a porous sheet (e.g. lens tissue paper or nylon net fabric). The use of trialkoxysilanes allows even such apparently inert materials as glass to be coupled to enzymes (Figure 3.3e). There are numerous other methods available for the covalent attachment of enzymes (e.g. the attachment of tyrosine groups through diazo-linkages, and lysine groups through amide formation with acyl chlorides or anhydrides

(a) cyanogen bromide



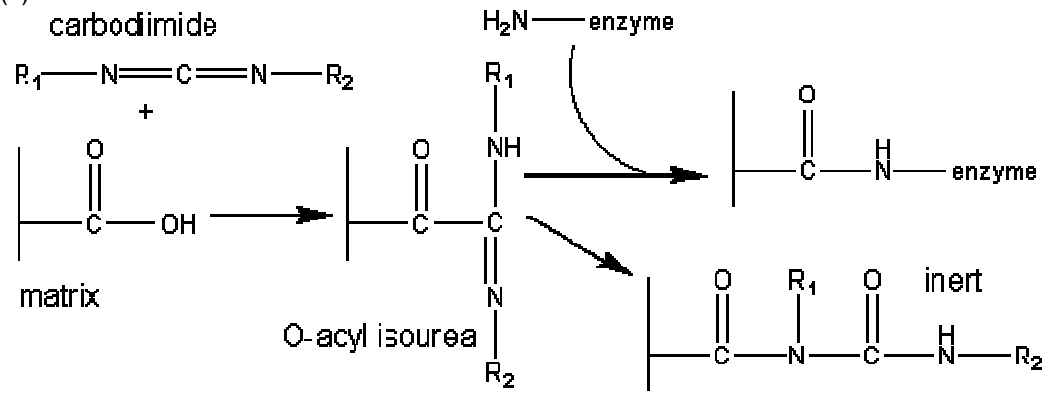
(3.3a)

b) ethyl chloroformate



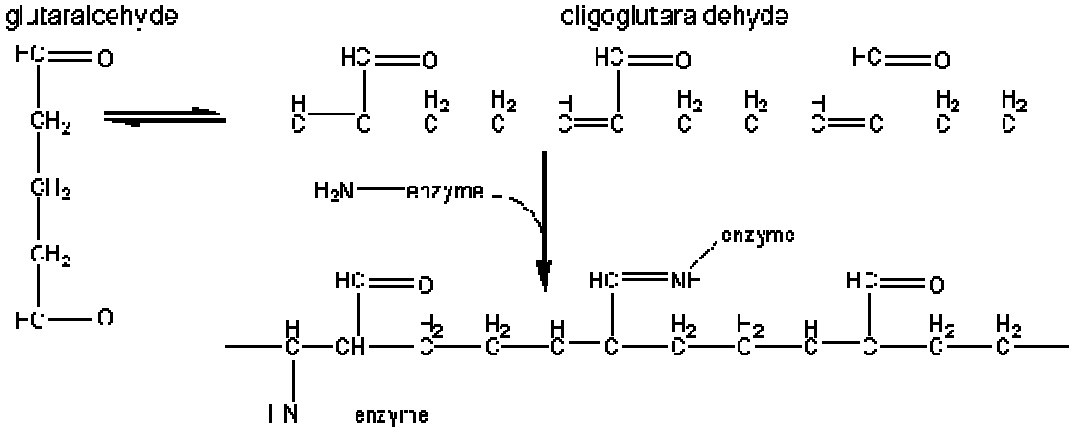
[3.3b]

(c) carbodiimide

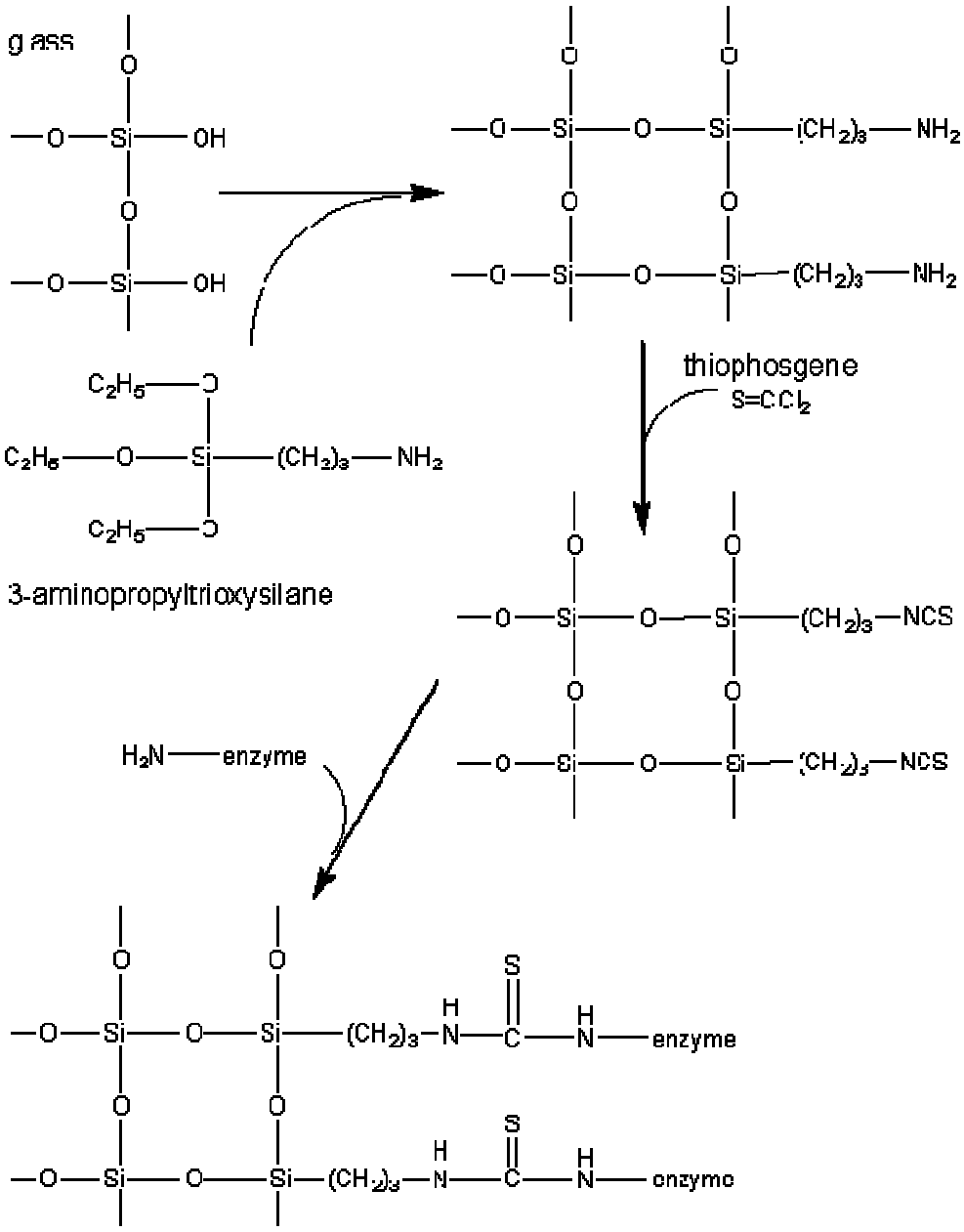


(3.3c)

(d) glutaraldehyde



3.3d



(e) 3-aminopropyltriethoxysilane

[3.3e]

Figure 3.3. Commonly used methods for the covalent immobilisation of enzymes. (a) Activation of Sepharose by cyanogen bromide. Conditions are chosen to minimise the formation of the inert carbamate. (b) Chloroformates may be used to produce similar intermediates to those produced by cyanogen bromide but without its inherent toxicity. (c) Carbodiimides may be used to attach amino groups on the enzyme to carboxylate

groups on the support or carboxylate groups on the enzyme to amino groups on the support. Conditions are chosen to minimise the formation of the inert substituted urea. (d) Glutaraldehyde is used to cross-link enzymes or link them to supports. It usually consists of an equilibrium mixture of monomer and oligomers. The product of the condensation of enzyme and glutaraldehyde may be stabilised against dissociation by reduction with sodium borohydride. (e) The use of trialkoxysilane to derivatise glass. The reactive glass may be linked to enzymes by a number of methods including the use thiophosgene, as shown.

Precautions during covalent immobilization

It is clearly important that the immobilised enzyme retains as much catalytic activity as possible after reaction. This can, in part, be ensured by reducing the amount of enzyme bound in non-catalytic conformations (fig3.4). Immobilisation of the enzyme in the presence of saturating concentrations of substrate, product or a competitive inhibitor ensures that the active site remains unreacted during the covalent coupling and reduces the occurrence of binding in unproductive conformations. The activity of the immobilised enzyme is then simply restored by washing the immobilised enzyme to remove these molecules

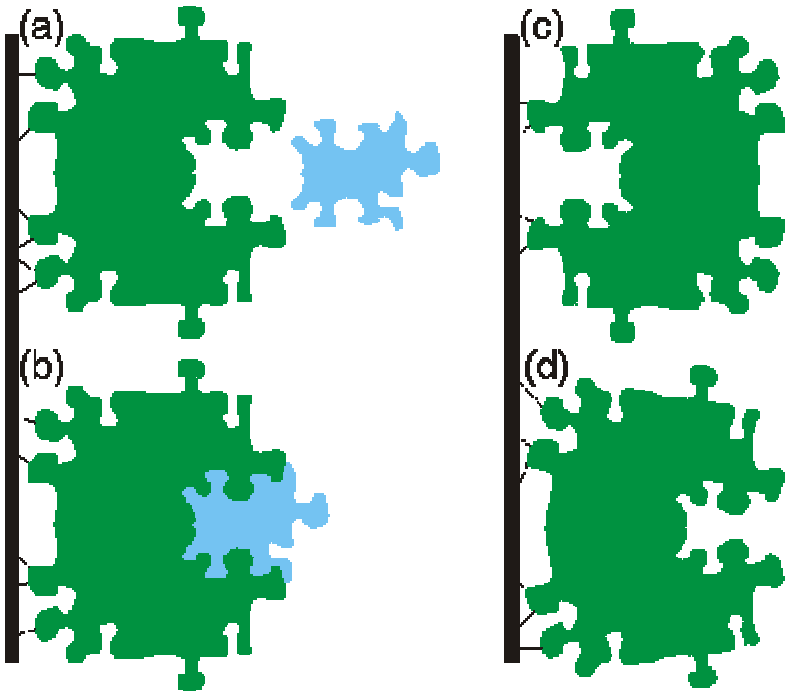


Figure 3.4. The effect of covalent coupling on the expressed activity of an immobilised enzyme. (a) Immobilised enzyme (E) with its active site unchanged and ready to accept the substrate molecule (S), as shown in (b). (c) Enzyme bound in a non-productive mode

due to the inaccessibility of the active site. (d) Distortion of the active site produces an inactive immobilised enzyme. Non-productive modes are best prevented by the use of large molecules reversibly bound in or near the active site. Distortion can be prevented by use of molecules which can sit in the active site during the coupling process, or by the use of a freely reversible method for the coupling which encourages binding to the most energetically stable (i.e. native) form of the enzyme. Both (c) and (d) may be reduced by use of 'spacer' groups between the enzyme and support, effectively displacing the enzyme away from the steric influence of the surface

4.3.3.4.c Entrapment:

Entrapment of enzymes within gels or fibres is a convenient method for use in processes involving low molecular weight substrates and products. Amounts in excess of 1 g of enzyme per gram of gel or fibre may be entrapped. However, the difficulty which large molecules have in approaching the catalytic sites of entrapped enzymes precludes the use of entrapped enzymes with high molecular weight substrates. The entrapment process may be a purely physical caging or involve covalent binding. As an example of this latter method, the enzymes' surface lysine residues may be derivatised by reaction with acryloyl chloride (CH_2=CH-CO-Cl) to give the acryloyl amides. This product may then be copolymerised and cross-linked with acrylamide (CH_2=CH-CO-NH_2) and bisacrylamide (H_2N-CO-CH=CH-CH=CH-CO-NH_2) to form a gel. Enzymes may be entrapped in cellulose acetate fibres by, for example, making up an emulsion of the enzyme plus cellulose acetate in methylene chloride, followed by extrusion through a spinneret into a solution of an aqueous precipitant. Entrapment is the method of choice for the immobilisation of microbial, animal and plant cells, where calcium alginate is widely used.

4.3.3.4.d Membrane confinement:

Membrane confinement of enzymes may be achieved by a number of quite different methods, all of which depend for their utility on the semipermeable nature of the membrane. This must confine the enzyme whilst allowing free passage for the reaction products and, in most configurations, the substrates. The simplest of these methods is achieved by placing the enzyme on one side of the semipermeable membrane whilst the reactant and product stream is present on the other side. Hollow fibre membrane units are available commercially with large surface areas relative to their contained volumes (> 20 m^2 l^{-1}) and permeable only to substances of molecular weight substantially less than the enzymes. Although costly, these are very easy to use for a wide variety of enzymes (including regenerating coenzyme systems. without the additional research and development costs associated with other immobilisation methods. Enzymes, encapsulated within small membrane-bound droplets or liposomes, may also be used within such reactors. As an example of the former, the enzyme is dissolved in an aqueous solution of 1,6-diaminohexane. This is then dispersed in a solution of hexanedioic acid in the immiscible solvent, chloroform. The resultant reaction forms a thin polymeric (Nylon-6,6) shell around the aqueous droplets which traps the enzyme. Liposomes are concentric spheres of lipid membranes, surrounding the soluble enzyme. They are formed by the addition of phospholipid to enzyme solutions. The micro-capsules and liposomes are washed free of non-confined enzyme and transferred back to aqueous solution before use.

4.3.3.5 Comparison

Table 3.3 presents a comparison of the more important general characteristics of these methods.

Table 3.3 :

Characteristics	Adsorption	Covalent binding	Entrapment	Membrane confinement
Preparation	Simple	Difficult	Difficult	Simple
Cost	Low	High	Moderate	High
Binding force	Variable	Strong	Weak	Strong
Enzyme leakage	Yes	No	Yes	No
Applicability	Wide	Selective	Wide	Very wide
Running Problems	High	Low	High	High
Matrix effects	Yes	Yes	Yes	No
Large diffusional barriers	No	No	Yes	Yes
Microbial protection	No	No	Yes	Yes

4.3.3.6 Applications Of Immobilised-enzymes

1.Immobilise -enzyme systems are used where they offer cost advantages to users on the basis of total manufacturing costs.

2.The plant size needed for continuous processes is two orders of magnitude smaller than that required for batch processes using free enzymes. The capital costs are, therefore, considerably smaller and the plant may be prefabricated cheaply off-site.

3. Immobilised enzymes offer greatly increased productivity on an enzyme weight basis and also often provide process advantages.

4.Currently used immobilised-enzyme processes are given inTable 3.4.

Table 3.4

Enzyme	**EC number**	**Product**
Aminoacylase	3.5.1.14	L-Amino acids
Aspartate ammonia-lyase	4.3.1.1	L-Aspartic acid
Aspartate 4-decarboxylase	4.1.1.12	L-Alanine
Cyanidase	3.5.5.x	Formic acid (from waste cyanide)
Glucoamylase	3.2.1.3	D-Glucose
Glucose isomerase	5.3.1.5	High -fructose corn syrup
Histidine ammonia-lyase	4.3.1.3	Urocanic acid
Hydantoinase[a]	3.5.2.2	D- and L-amino acids
Invertase	3.2.1.26	Invert sugar
Lactase	3.2.1.23	Lactose-free milk and whey
Lipase	3.1.1.3	Cocoa butter substitutes

Nitrile hydratase	4.2.I.x	Acrylamide
Penicillin amidases	3.5.1.11	Penicillins
Raffinase	3.2.1.22	Raffinose-free solutions
Thermolysin	3.2.24.4	Aspartame

[a] Dihydropyrimidinase.

5.High-fructose corn syrups (HFCS) production using immobilized enzymes.

With the development of glucoamylase in the 1940s and 1950s it became a straightforward matter to produce high DE glucose syrups. However, these have shortcomings as objects of commerce: D-glucose has only about 70% of the sweetness of sucrose, on a weight basis, and is comparatively insoluble. Batches of 97 DE glucose syrup at the final commercial concentration (71% (w/w)) must be kept warm to prevent crystallisation or diluted to concentrations that are microbiologically insecure. Fructose is 30% sweeter than sucrose, on a weight basis, and twice as soluble as glucose at low temperatures so a 50% conversion of glucose to fructose overcomes both problems giving a stable syrup that is as sweet as a sucrose solution of the same concentration. The isomerisation is possible by chemical means but not economical, giving tiny yields and many by-products (e.g. 0.1 M glucose 'isomerised' with 1.22 M KOH at 5°C under nitrogen for 3.5 months gives a 5% yield of fructose but only 7% of the glucose remains unchanged, the majority being converted to various hydroxy acids).

One of the triumphs of enzyme technology so far has been the development of 'glucose isomerase'. Glucose is normally isomerised to fructose during glycolysis but both sugars are phosphorylated. The use of this phosphohexose isomerase may be ruled out as a commercial enzyme because of the cost of the ATP needed to activate the glucose and because two other enzymes (hexokinase and fructose-6-phosphatase) would be needed to complete the conversion. Only an isomerase that would use underivatised glucose as its substrate would be commercially useful but, until the late 1950s, the existence of such an enzyme was not suspected. At about this time, enzymes were found that catalyse the conversion of D-xylose to an equilibrium mixture of D-xylulose and D-xylose in bacteria. When supplied with cobalt ions, these xylose isomerases were found to isomerise □-D-glucopyranose to □-D –fructofuranose. equilibration from the more abundant □-D-glucopyranose and to the major product □-D-fructopyranose occurring naturally and non-enzymically. Now it is known that several genera of microbes, mainly bacteria, can produce such glucose isomerases: The commercial enzymes are produced by *Actinoplanes missouriensis*, *Bacillus coagulans* and various *Streptomyces* species; as they have specificities for glucose and fructose which are not much different from that for xylose and ways are being found to avoid the necessity of xylose as inducer, these should perhaps now no longer be considered as xylose isomerases. They are remarkably amenable enzymes in that they are resistant to thermal denaturation and will act at very high substrate concentrations, which have the additional benefit of substantially stabilising the enzymes at higher operational temperatures. The vast majority of glucose isomerases are retained within the cells that produce them but need not be separated and purified before use.

All glucose isomerases are used in immobilised forms. Although differerent immobilisation methods have been used for enzymes from differerent organisms, the principles of use are very similar. Immobilisation is generally by cross-linking with glutaraldehyde, plus in some cases a protein diluent, after cell lysis or homogenisation.

Originally, immobilised glucose isomerase was used in a batch process. This proved to be costly as the relative reactivity of fructose during the long residence times gave rise to significant by -product production. Also, difficulties were encountered in the removal of the added Mg^{2+} and Co^{2+} and the recovery of the catalyst. Nowadays most isomerisation is performed in PBRs (table 3.5). They are used with high substrate concentration (35-45% dry solids, 93-97% glucose) at 55-60°C. The pH is adjusted to 7.5-8.0 using sodium carbonate and magnesium sulphate is added to maintain enzyme activity (Mg^{2+} and Co^{2+} are cofactors). The Ca^{2+} concentration of the glucose feedstock is usually about 25 □m, left from previous processing, and this presents a problem. Ca^{2+} competes successfully for the Mg^{2+} binding site on the enzyme, causing inhibition. At this level the substrate stream is normally made 3 mM with respect to Mg^{2+}. At higher concentrations of calcium a Mg^{2+} : Ca^{2+} ratio of 12 is recommended. Excess Mg^{2+} is uneconomic as it adds to the purification as well as the isomerisation costs. The need for Co^{2+} has not been eliminated altogether, but the immobilisation methods now used fix the cobalt ions so that none needs to be added to the substrate streams.

Table 3.5. Comparison of glucose isomerisation methods

Parameter	**Batch (soluble GI)**	**Batch (immobilised Gl)**	**Continuous (PBR)**
Reactor volume (m^3)	1100	1100	15
Enzyme consumption (tonnes)	180	11	2
Activity, half-life (h)	30	300	1500
Active life, half-lives	0.7	2	3
Residence time (h)	20	20	0.5
Co^{2+} (tonnes)	2	1	0
Mg^{2+} (tonnes)	40	40	7
Temperature (°C)	65	65	60
pH	6.8	6.8	7.6
Colour formation (A_{420})	0.7	0.2	< 0.1
Product refining	Filtration C-treatment[a] Cation exchange Anion exchange	- C-treatment Cation exchange Anion exchange	- C -treatment - -
Capital, labour and energy costs, £ $tonne^{-1}$	5	5	1
Conversion cost, £ $tonne^{-1}$	500	30	5

All processes start with 45% (w/w) glucose syrup DE 97 and produce 10000 tonnes per month of 42% fructose dry syrup. Some of the improvement that may be seen for PBR productivity is due to the substantial development of this process.

[a] Treatment with activated carbon.

It is essential for efficient use of immobilised glucose isomerase that the substrate solution is adequately purified so that it is free of insoluble material and other impurities that might inactivate the enzyme by chemical (inhibitory) or physical (pore-blocking) means. In effect, this means that glucose produced by acid hydrolysis cannot

be used, as its low quality necessitates extensive and costly purification. Insoluble material is removed by filtration, sometimes after treatment with flocculants, and soluble materials are removed by ion exchange resins and activated carbon beads. This done, there still remains the possibility of inhibition due to oxidised by-products caused by molecular oxygen. This may be removed by vacuum de-aeration of the substrate at the isomerisation temperature or by the addition of low concentrations (< 50 ppm) of sulphite.

At equilibrium at 60°C about 51 % of the glucose in the reaction mixture is converted to fructose. However, because of the excessive time taken for equilibrium to be attained and the presence of oligosaccharides in the substrate stream, most manufacturers adjust flow rates so as to produce 42-46% (w/w) fructose (leaving 47-51 % (w/w) glucose). To produce 100 tonnes (dry substance) of 42% HFCS per day, an enzyme bed volume of about 4 m^3 is needed. Activity decreases, following a first-order decay equation. The half-life of most enzyme preparations is between 50 and 100 days at 55°C. Typically a batch of enzyme is discarded when the activity has fallen to an eighth of the initial value (i.e. after three half -lives). To maintain a constant fructose content in the product, the feed flow rate is adjusted according to the enzyme activity. Several reactors containing enzyme preparations of different ages are needed to maintain overall uniform production by the plant (fig3. 5). In its lifetime 1 kg of immobilised glucose isomerase (exemplified by Novo's Sweetzyme T) will produce 10 -11 tonnes of 42% fructose syrup (dry substance).

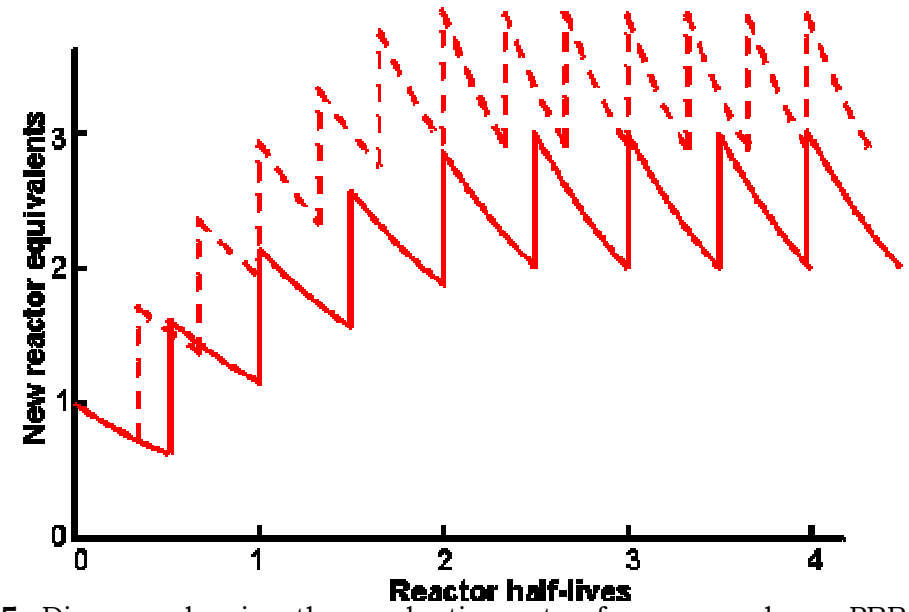


Figure3. 5. Diagram showing the production rate of a seven-column PBR facility on start -up, assuming exponential decay of reactor activity. The columns are brought into use one at a time. At any time a maximum of six PBRs are operating in parallel, whilst the seventh, exhausted, reactor is being refilled with fresh biocatalyst. ——— PBR activities allowed to decay through three half-lives (to 12.5% initial activity) before replacement. The final average productivity is 2.51 times the initial productivity of one

column. - - -- - - - PBR activities allowed to decay through two half-lives (to 25% initial activity) before replacement. The final average productivity is 3.23 times the initial productivity of one column. It may be seen that the final average production rate is higher when the PBRs are individually operated for shorter periods but this 29% increase in productivity is achieved at a cost of 50% more enzyme, due to the more rapid replacement of the biocatalyst in the PBRs. A shorter PBR operating time also results in a briefer start-up period and a more uniform productivity.
After isomerisation, the pH of the syrup is lowered to 4 - 5 and it is purified by ion-exchange chromatography and treatment with activated carbon. Then, it is normally concentrated by evaporation to about 70% dry solids.

For many purposes a 42% fructose syrup is perfectly satisfactory for use but it does not match the exacting criteria of the quality soft drink manufacturers as a replacement for sucrose in acidic soft drinks. For use in the better colas, 55% fructose is required. This is produced by using vast chromatographic columns of zeolites or the calcium salts of cation exchange resins to adsorb and separate the fructose from the other components. The fractionation process, although basically very simple, is only economic if run continuously. The fructose stream (90% (w/w) fructose, 9% glucose) is blended with 42% fructose syrups to give the 55% fructose (42% glucose) product required. The glucose-rich 'raffinate' stream may be recycled but if this is done undesirable oligosaccharides build up in the system. Immobilised glucoamylase is used in some plants to hydrolyse oligosaccharides in the raffinate; here the substrate concentration is comparatively low (around 20% dry solids) so the formation of isomaltose by the enzyme is insignificant.

Clearly the need for a second large fructose enrichment plant in addition to the glucose isomerase plant is undesirable and attention is being paid to means of producing 55% fructose syrups using only the enzyme. The thermodynamics of the system favour fructose production at higher temperatures and 55% fructose syrups could be produced directly if the enzyme reactors were operated at around 95°C. The use of miscible organic co -solvents may also produce the desired effect. Both these alternatives present a more than considerable challenge to enzyme technology!
The present world market for HFCS is over 5 million tonnes of which about 60% is for 55% fructose syrup with most of the remainder for 42% fructose syrup. This market is still expanding and ensures that HFCS production is the major application for immobilised-enzyme technology.
The high-fructose syrups can be used to replace sucrose where sucrose is used in solution but they are inadequate to replace crystalline sucrose. Another ambition of the corn syrup industry is to produce sucrose from starch. This can be done using a combination of the enzymes phosphorylase (EC 2.4.1.1), glucose isomerase and sucrose phosphorylase (EC 2.4.1.7), but the thermodynamics do not favour the conversion so means must be found of removing sucrose from the system as soon as it is formed. This will not be easy but is achievable if the commercial pull (i.e. money available) is sufficient:

1

$$\text{starch } (G_n) + \text{orthophosphate} \xrightleftharpoons{\text{phosphorylase}} \text{starch } (G_{n-1}) + \alpha\text{-glucose-1 phosphate} \quad [3.6]$$

2.

$$\text{glucose} \xrightleftharpoons{\text{glucose isomerase}} \text{fructose} \quad [3.7]$$

3.

$$\alpha\text{-glucose-1 -phosphate} + \text{fructose} \xrightleftharpoons{\text{sucrose hosphorylase}} \text{sucrose} + \text{orthophosphate} \quad [3.8]$$

A further possible approach to producing sucrose from glucose is to supply glucose at high concentrations to microbes whose response to osmotic stress is to accumulate sucrose intracellularly. Provided they are able to release sucrose without hydrolysis when the stress is released, such microbes may be the basis of totally novel processes.

6.Use of immobilised raffinase

The development of a raffinase (α-D-galactosidase) suitable for commercial use is another triumph of enzyme technology. Plainly, it would be totally unacceptable to use an enzyme preparation containing invertase to remove this material during sucrose production . It has been necessary to find an organism capable of producing an α-galactosidase but not an invertase. A mould, *Mortierella vinacea* var. *raffinoseutilizer*, fills the requirements. This is grown in a particulate form and the particles harvested, dried and used directly as the immobilised-enzyme preparation. It is stirred with the sugar beet juice in batch stirred tank reactors. When the removal of raffinose is complete, stirring is stopped and the juice pumped off the settled bed of enzyme. Enzyme, lost by physical attrition, is replaced by new enzyme added with the next batch of juice. The galactose released is destroyed in the alkaline conditions of the first stages of juice purification and does not cause any further problems while the sucrose is recovered. This process results in a 3% increase in productivity and a significant reduction in the costs of the disposal of waste molasses.

Immobilised raffinase may also be used to remove the raffinose and stachyose from soybean milk. These sugars are responsible for the flatulence that may be caused when soybean milk is used as a milk substitute in special diets.

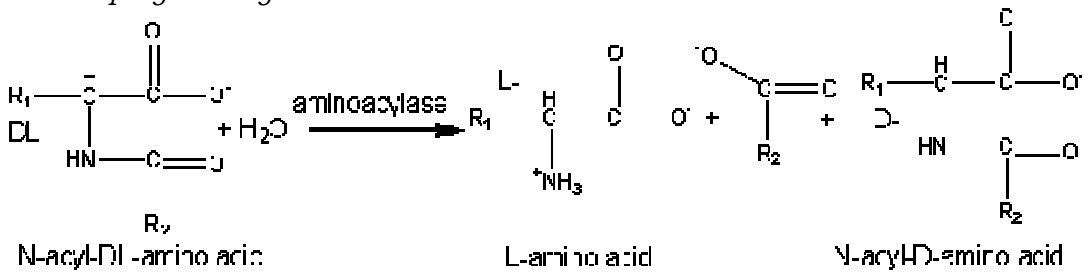
7.Use of immobilised invertase**.**

Invertase was probably the first enzyme to be used on a large scale in an immobilised form (by Tate & Lyle). In the period 1941 -1946 the acid, previously used in the manufacture of Golden Syrup, was unavailable, so yeast invertase was used instead. Yeast cells were autolysed and the autolysate clarified by adjustment to pH 4.7, followed by filtration through a bed of calcium sulphate and adsorption into bone char. A layer of the bone char containing invertase was included in the bed of bone char already used for decolourising the syrup. The scale used was large, the bed of invertase-char being 2 ft (60 cm) deep in a bed of char 20 ft (610 cm) deep. The preparation was very stable, the limiting factors being microbial contamination or loss of decolourising power rather than loss of enzymic activity. The process was cost-effective but, not

surprisingly, the product did not have the subtlety of flavour of the acid-hydrolysed material and the immobilised enzyme process was abandoned when the acid became available once again. Recently, however, it has been relaunched using Brimac™, where the invertase -char mix is stabilised by cross-linking and has a half-life of 90 days in use (pH 5.5, 50°C). The revival is due, in part, to the success of HFCS as a high-quality low-colour sweetener. It is impossible to produce inverted syrups of equivalent quality by acid hydrolysis. Enzymic inversion avoids the high-colour, high salt-ash, relatively low conversion and batch variability problems of acid hydrolysis. Although free invertase may be used (with residence times of about a day), the use of immobilised enzymes in a PBR (with residence time of about 15 min) makes the process competitive; the cost of 95% inversion (at 50% (w/w)) being no more than the final evaporation costs (to 75% (w/w)). A productivity of 16 tonnes of inverted syrup (dry weight) may be achieved using one litre of the granular enzyme.

8.Production of amino acids.

Another early application of an immobilised enzyme was the use of the aminoacylase from *Aspergillus oryzae* to resolve racemic mixtures of amino acids.

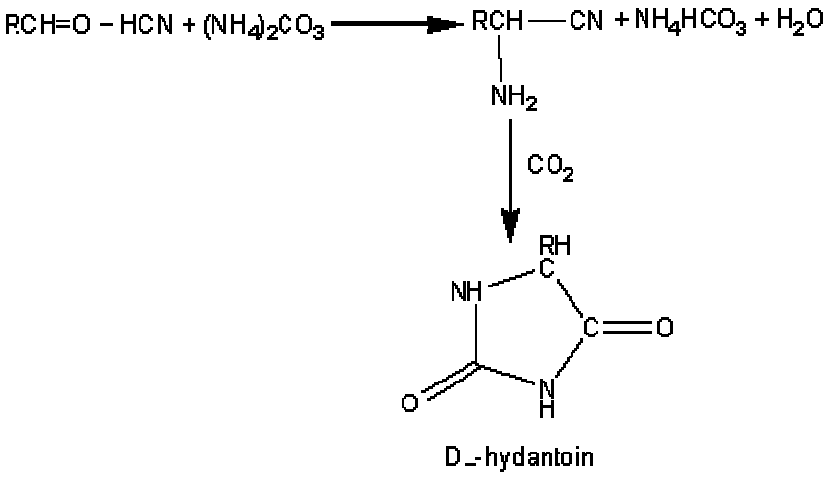


[3.9]

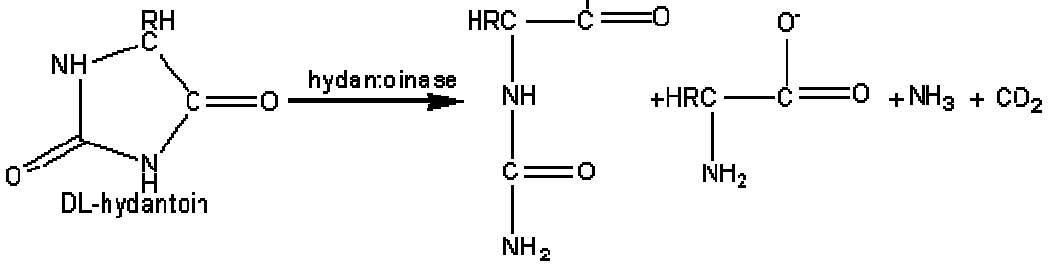
Chemically synthesised racemic N-acyl-DL-amino acids are hydrolysed at pH 8.5 to give the free L-amino acids plus the unhydrolysed N-acyl-D-amino acids. These products are easily separated by differential crystallisation and the N-acyl-D-amino acids racemised chemically (or enzymically) and reprocessed. The enzyme is immobilised by adsorption to anion exchange resins (e.g. DEAE-Sephadex) and has an operational half-life of about 65 days at 50°C in PBRs with residence times of about 30 min. The reactors may be re-activated *in situ* by simply adding more enzyme. The immobilised enzyme has proved a more economical process than the use of free enzyme mainly due to the more efficient use of the substrate and reductions in the cost of enzyme and labour.

Novel and natural L-amino acids can be produced by the chemical conversion of aldehydes through DL-amino nitrites to racemic DL-hydantoins (reaction scheme (3.7)) followed by enzymic hydrolysis with hydantoinase and a carbamoylase (reaction scheme(3.8)) at pH 8.5. Both enzymes may be obtained from *Arthrobacter* species.

D -Amino acids are important constituents in antibiotics and insecticides. They may be produced in a manner similar to the L-amino acids but using hydantoinases of differing specificity. The *Pseudomonas striata* enzyme is specific for D-hydantoins, allowing their specific hydrolysis to D-carbamoyl amino acids which can be converted to the D-amino acids by chemical treatment with nitrous acid. They remaining L-hydantoin may be simply racemised by base and the process repeated.



[3.10]



[3.11]

L -Aspartic acid is widely used in the food and pharmaceutical industries and is needed for the production of the low -calorific sweetener aspartame. It may be produced from fumaric acid by the use of the aspartate ammonia-lyase (aspartase) from *Escherichia coli*.

Aspartate ammonia-lyase

$^{-}OOCCH=CHCOO^{-} + NH_4^{+} \rightleftharpoons {}^{-}OOCCH_2CH(NH_3^{+})COO^{-}$ [3.12]

fumaric acid L-aspartic acid

A crude immobilised aspartate ammonia-lyase (50000 U g^{-1}) may be prepared by entrapping *Escherichia coli* cells in a □□-carageenan gel crosslinked with glutaraldehyde and hexamethylenediamine. The process is operated in a PBR at pH 8.5 using ammonium fumarate as the substrate, with a reported operational half-life of 680 days at 37°C.

Urocanic acid is a sun-screening agent which may be produced from L-histidine by the histidine ammonia -lyase (histidase) from *Achromobacter liquidum*. The organism cannot be used directly as it has urocanate hydratase activity, which removes the urocanic acid. However, a brief heat treatment (70°C, 30 min) inactivates this unwanted activity but has little effect on the histidine ammonia-lyase. A crude immobilised-enzyme preparation consisting of heat -treated cells entrapped in a polyacrylamide gel has been used to effect this conversion, showing a half-life of 180 days at 37°C.

9.Use of immobilised lactase**.**

Lactase is one of relatively few enzymes that have been used both free and immobilised in large-scale processes. The reasons for its utility has been given earlier .but the relatively high cost of the enzyme is an added incentive for its use in an immobilised state.

Immobilised lactases are important mainly in the treatment of whey, as the fats and proteins in the milk emulsion tend to coat the biocatalysts. This both reduces their apparent activity and increases the probability of microbial colonisation.

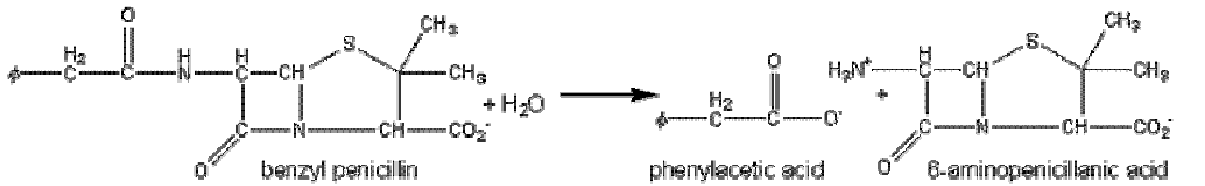
Yeast lactase has been immobilised by incorporation into cellulose triacetate fibres during wet spinning, a process developed by Snamprogetti S.p.A. in Italy. The fibres are cut up and used in a batchwise STR process at 5°C (*Kluyveromyces lactis*, pH optimum 6.4 -6.8, 90 U g^{-1}). Fungal lactases have been immobilised on 0.5 mm diameter porous silica (35 nm mean pore diameter) using glutaraldehyde and □-aminopropyltriethoxysilane (*Asperigillus niger*, pH optimum 3.0 -3.5, 500 U g^{-1}; *A. oryzae*, pH optimum 4.0 -1.5, 400 U g^{-1}). They are used in PBRs. Due to the different pH optima of fungal and yeast lactases, the yeast enzymes are useful at the neutral pH of both milk and sweet whey, whereas fungal enzymes are more useful with acid whey.

Immobilised lactases are particularly affected by two inherent short-comings. Product inhibition by galactose and unwanted oligosaccharide formation are both noticeable under the diffusion-controlled conditions usually prevalent. Both problems may be reduced by an increase in the effectiveness factor and a reduction in the degree of hydrolysis or initial lactose concentration, but such conditions also lead to a reduction in the economic return. The control of microbial contamination within the bioreactors is the most critical practical problem in these processes. To some extent, this may be overcome by the use of regular sanitation with basic detergent and a dilute protease solution.

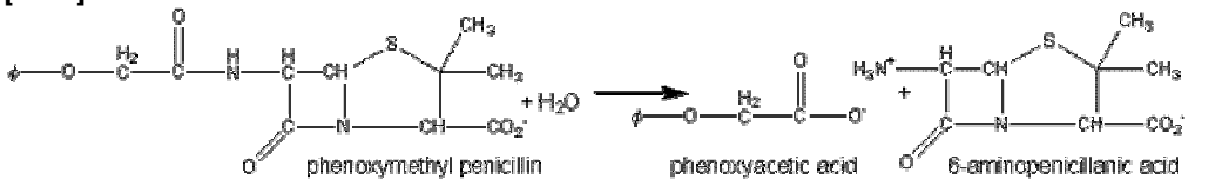
10.Production of antibiotics.

Benzylpenicillins and phenoxymethylpenicillins (penicillins G and V, respectively) are produced by fermentation and are the basic precursors of a wide range of semi-synthetic antibiotics, e.g. ampicillin. The amide link may be hydrolysed conventionally but the conditions necessary for its specific hydrolysis, whilst causing no hydrolysis of the intrinsically more labile but pharmacologically essential □-lactam ring, are difficult to attain. Such specific hydrolysis may be simply achieved by use of penicillin amidases (also called penicillin acylases). Different enzyme preparations are generally used for the hydrolysis of the penicillins G and V, pencillin-V-amidase being much more specific than pencillin-G-amidase.

Penicillin amidase may be obtained from *E. coli* and has been immobilised on a number of supports including cyanogen bromide-activated Sephadex G200. It represents one of the earliest successful processes involving immobilised enzymes and is generally used in batch or semicontinuous STR processes (40,000 Ukg^{-1}penicillin G, 35°C, pH 7.8, 2 h) where it may be reused over 100 times. It has also been used in PBRs, where it has an active life of over 100 days, producing about two tonnes of 6-aminopenicillanic acid $kg^{-1}$of immobolised enzyme.

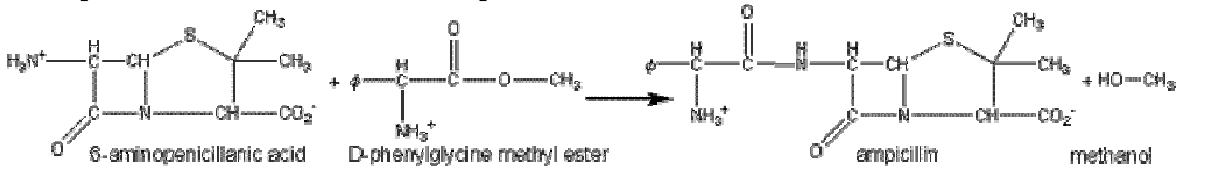


[3.11]



[3.12]

The penicillin-G-amidases may be used 'in reverse' to synthesise penicillin and cephalosporin antibiotics by non -equilibrium kinetically controlled reactions. Ampicillin has been produced by the use of penicillin-G-amidase immobilised by adsorption to DEAE -cellulose in a packed bed column:



[3.13]

Many other potential and proven antibiotics have been synthesised in this manner, using a variety of synthetic □-lactams and activated carboxylic acids.

11.Preparation of acrylamide

Acrylamide is an important monomer needed for the production of a range of economically useful polymeric materials. It may be produced by the addition of water to acrylonitrile.

$CH_2=CHCN + H_2O \longrightarrow CH_2=CHCONH_2$ **[3.14]**

This process may be achieved by the use of a reduced copper catalyst (Cu^+); however, the yield is poor, unwanted polymerisation or conversion to acrylic acid ($CH_2=CHCOOH$) may occur at the relatively high temperatures involved (80 -140°C) and the catalyst is difficult to regenerate. These problems may be overcome by the use of immobilised nitrile hydratase (often erroneously called a nitrilase). The enzyme from *Rhodococcus* has been used by the Nitto Chemical Industry Co. Ltd, as it contains only very low amidase activity which otherwise would produce unwanted acrylic acid from the acrylamide.

Immobilised nitrile hydratase is simply prepared by entrapping the intact cells in a cross-linked 10% (w/v) polyacrylamide/dimethylaminoethylmethacrylate gel and granulating the product. It is used at 10°C and pH 8.0-8.5 in a semibatchwise process, keeping the substrate acrylonitrile concentration below 3% (w/v). Using 1% (w/v) immobilised-enzyme concentration (about 50,000 U l^{-1}) the process takes about a day. Product concentrations of up to 20% (w/v) acrylamide have been achieved, containing negligible substrate and less than 0.02% (w/w) acrylic acid. Acrylamide production using this method is about 4000 tonnes per year.

The closely related enzymes cyanidase and cyanide hydratase see schemes 3.15 and [3.16], respectively) are used to remove cyanide from industrial waste and in the detoxification of feeds and foodstuffs containing amygdalin..

$HCN + 2H_2O \longrightarrow HCOO^- + NH_4^+$ **[3.15]**

$HCN + H_2O \longrightarrow HCONH_2$ **[3.16]**

4.3.3.7 Summary

a. Immobilisation of enzymes enables their efficient and continuous use. The rationale behind immobilisation is the easy separation of product from the biocatalyst.
b. Enzymes may be immobilised by adsorption, covalent binding, entrapment and membrane confinement, each method having its pros and cons. Adsorption is quick, simple and cheap but may be reversible. Covalent binding is permanent but expensive. Entrapment is generally applicable but may cause diffusional problems. Membrane confinement is a flexible method but expensive to set up.
c. Immobilisation of enzymes may have a considerable effect on their kinetics. This may be due to structural changes to the enzyme and the creation of a distinct microenvironment around the enzyme. The activity of an immobilised enzyme is governed by the physical conditions within this microenvironment not those prevalent in the bulk phase. The immobilisation matrix affects the partition of material between the product phase and the enzyme phase and imposes restrictions on the rate of diffusion of material.
d. Some effects of enzyme immobilisation are seen to be beneficial whilst others are detrimental to the economics of their use.
e. Immobilised enzymes are used in an increasing number o economically viable bioconversions, the most important of which is the isomerisation of glucose.

4.3.3.8. Modal questions

1.Define immobilization and explain various methods of immobilization.
2.Add a note on advantages of immobilizing an enzyme and Write about various applications of immobilization.

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Lesson 4.3.4.

BIOSENSORS

Structure

Objective

The main objective of this lesson is to bring the knowledge about the working principle of biosensors, types and their applications in various fields.

4.3.4.1 what are biosensors?

A biosensor is an analytical device, which converts a biological response into an electrical signal (Figure. 1). The term 'biosensor' is often used to cover sensor devices used in order to determine the concentration of substances and other parameters of biological interest even where they do not utilise a biological system directly.The emphasis of this Chapter concerns enzymes as the biologically responsive material, but it should be recognised that other biological systems may be utilised by biosensors, for example, whole cell metabolism, ligand binding and the antibody-antigen reaction.

Biosensors represent a rapidly expanding field, at the present time, with an estimated 60% annual growth rate; the major impetus coming from the health-care industry (e.g. 6% of the western world are diabetic and would benefit from the availability of a rapid, accurate and simple biosensor for glucose) but with some pressure from other areas, such as food quality appraisal and environmental monitoring. The estimated world analytical market is about £12,000,000,000 $year^{-1}$ of which 30% is in the health care area. There is clearly a vast market expansion potential as less than 0.1% of this market is currently using biosensors. Research and development in this field is wide and multidisciplinary, spanning biochemistry, bioreactor science, physical chemistry, electrochemistry, electronics and software engineering. Most of this current endeavour concerns potentiometric and amperometric biosensors and colorimetric paper enzyme strips. However, all the main transducer types are likely to be thoroughly examined, for use in biosensors, over the next few years.

A successful biosensor must possess at least some of the following beneficial features:

1. The biocatalyst must be highly specific for the purpose of the analyses, be stable under normal storage conditions and, except in the case of colorimetric enzyme strips and dipsticks (see later), show good stability over a large number of assays (i.e. much greater than 100).

2. The reaction should be as independent of such physical parameters as stirring, pH and temperature as is manageable. This would allow the analysis of samples with minimal pre-treatment. If the reaction involves cofactors or coenzymes these should, preferably, also be co-immobilised with the enzyme.
3. The response should be accurate, precise, reproducible and linear over the useful analytical range, without dilution or concentration. It should also be free from electrical noise.
4. If the biosensor is to be used for invasive monitoring in clinical situations, the probe must be tiny and biocompatible, having no toxic or antigenic effects. If it is to be used in fermenters it should be sterilisable. This is preferably performed by autoclaving but no biosensor enzymes can presently withstand such drastic wet-heat treatment. In either case, the biosensor should not be prone to fouling or proteolysis.
5. The complete biosensor should be cheap, small, portable and capable of being used by semi-skilled operators.
6. There should be a market for the biosensor. There is clearly little purpose developing a biosensor if other factors (e.g. government subsidies, the continued employment of skilled analysts, or poor customer perception) encourage the use of traditional methods and discourage the decentralisation of laboratory testing.

The biological response of the biosensor is determined by the biocatalytic membrane which accomplishes the conversion of reactant to product. Immobilised enzymes possess a number of advantageous features which makes them particularly applicable for use in such systems. They may be re-used, which ensures that the same catalytic activity is present for a series of analyses. This is an important factor in securing reproducible results and avoids the pitfalls associated with the replicate pipetting of free enzyme otherwise necessary in analytical protocols. Many such immobilised enzyme systems are re-usable up to 10,000 times over a period of several months. Clearly, this results in a considerable saving in terms of the enzymes' cost relative to the analytical usage of free soluble enzymes.

When the reaction, occurring at the immobilised enzyme membrane of a biosensor, is limited by the rate of external diffusion, the reaction process will possess a number of valuable analytical assets. It follows that the biocatalyst gives a proportional change in reaction rate in response to the reactant (substrate) concentration over a substantial linear range, several times the intrinsic K_m. This is very useful as analyte concentrations are often approximately equal to the K_ms of their appropriate enzymes which is roughly 10 times more concentrated than can be normally determined, without dilution, by use of the free enzyme in solution. The reaction rate is independent of pH, ionic strength, temperature and inhibitors. This simply avoids the tricky problems often encountered due to the variability of real analytical samples (e.g, fermentation broth, blood and urine) and external conditions. Control of biosensor response by the external diffusion of the analyte can be encouraged by the use of permeable membranes between the enzyme and the bulk solution. Even if total dependence on the external diffusional rate is not achieved (or achievable), any increase in the dependence of the reaction rate on external or internal diffusion will cause a reduction in the dependence on the pH, ionic strength, temperature and inhibitor concentrations.

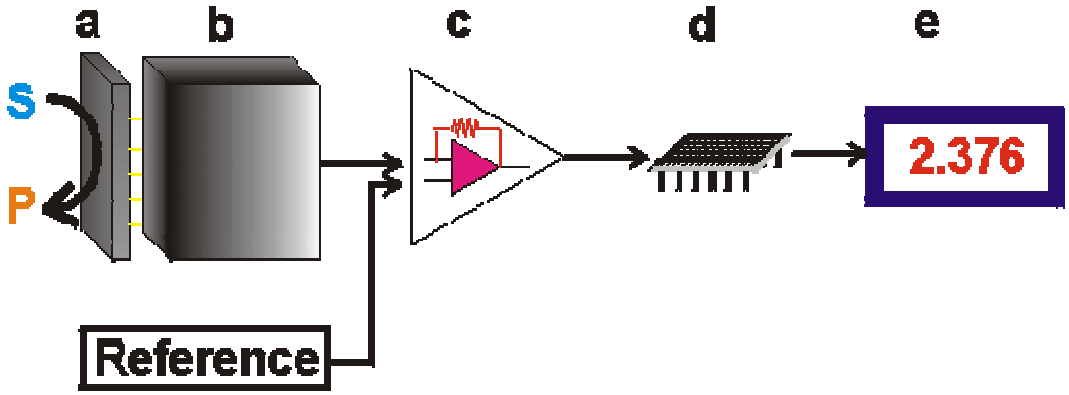


Figure 1. Schematic diagram showing the main components of a biosensor.
(a) The biocatalyst converts the substrate to product.
(b) This reaction is determined by the transducer which converts it to an electrical signal.
(c) The output from the transducer is amplified
(d) processer
(e). displayer

The key part of a biosensor is the transducer (shown as the 'black box' in Figure 1) which makes use of a physical change accompanying the reaction. This may be

1. the heat output (or absorbed) by the reaction (calorimetric biosensors),
2. changes in the distribution of charges causing an electrical potential to be produced (potentiometric biosensors),
3. movement of electrons produced in a redox reaction (amperometric biosensors),
4. light output during the reaction or a light absorbance difference between the reactants and products (optical biosensors), or
5. effects due to the mass of the reactants or products (piezo-electric biosensors).

There are three 'generations' of biosensors; First generation biosensors where the normal product of the reaction diffuses to the transducer and causes the electrical response, second generation biosensors which involve specific 'mediators' between the reaction and the transducer in order to generate improved response, and third generation biosensors where the reaction itself causes the response and no product or mediator diffusion is directly involved.

The electrical signal from the transducer is often low and superimposed upon a relatively high and noisy (i.e. containing a high frequency signal component of an apparently random nature, due to electrical interference or generated within the electronic components of the transducer) baseline. The signal processing normally involves subtracting a 'reference' baseline signal, derived from a similar transducer without any biocatalytic membrane, from the sample signal, amplifying the resultant signal difference and electronically filtering (smoothing) out the unwanted signal noise. The relatively slow nature of the biosensor response considerably eases the problem of electrical noise filtration. The analogue signal produced at this stage may be output directly but is usually converted to a digital signal and passed to a microprocessor stage where the data is processed, converted to concentration units and output to a display device or data store.

4.3.4.2 Calorimetric biosensors

Many enzyme catalysed reactions are exothermic, generating heat (Table 1) which may be used as a basis for measuring the rate of reaction and, hence, the analyte concentration. This represents the most generally applicable type of biosensor. The temperature changes are usually determined by means of thermistors at the entrance and exit of small packed bed columns containing immobilised enzymes within a constant temperature environment (Figure 2). Under such closely controlled conditions, up to 80% of the heat generated in the reaction may be registered as a temperature change in the sample stream. This may be simply calculated from the enthalpy change and the amount reacted. If a 1 mM reactant is completely converted to product in a reaction generating 100 kJ $mole^{-1}$ then each ml of solution generates 0.1 J of heat. At 80% efficiency, this will cause a change in temperature of the solution amounting to approximately 0.02°C. This is about the temperature change commonly encountered and necessitates a temperature resolution of 0.0001°C for the biosensor to be generally useful.

Table1. Heat output (molar enthalpies) of enzyme catalysed reactions.

Reactant	**Enzyme**	**Heat output -□H (kJ $mole^{-1}$)**
Cholesterol	Cholesterol oxidase	53
Esters	Chymotrypsin	4 - 16
Glucose	Glucose oxidase	80
Hydrogen peroxide	Catalase	100
Penicillin G	Penicillinase	67
Peptides	Trypsin	10 - 30
Starch	Amylase	8
Sucrose	Invertase	20
Urea	Urease	61
Uric acid	Uricase	49

Schematic diagram of a calorimetric biosensor is shown in figure below. The sample stream (a) passes through the outer insulated box (b) to the heat exchanger (c) within an aluminium block (d). From there, it flows past the reference thermistor (e) and into the packed bed bioreactor (f, 1ml volume), containing the biocatalyst, where the reaction occurs. The change in temperature is determined by the thermistor (g) and the solution passed to waste (h). External electronics (l) determines the difference in the resistance, and hence temperature, between the thermistors.

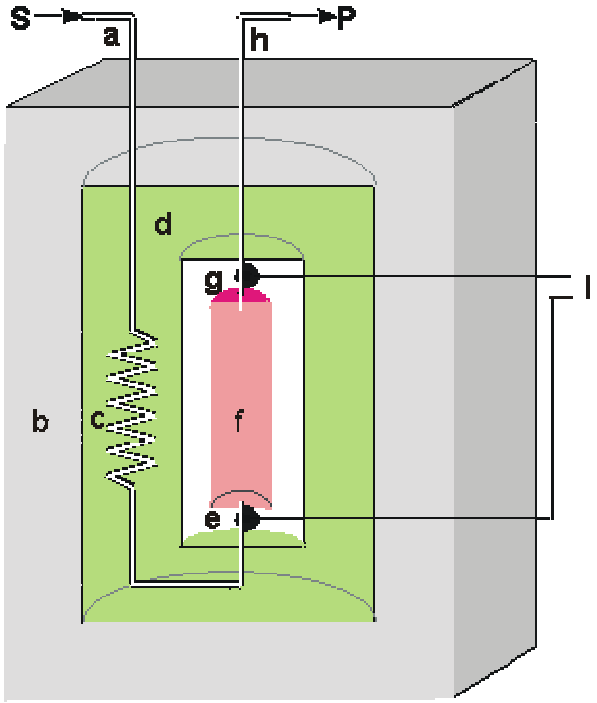


Figure 2 Schematic diagram of a calorimetric biosensor

The thermistors, used to detect the temperature change, function by changing their electrical resistance with the temperature, obeying the relationship

$$Ln\left(\frac{R_1}{R_2}\right) = B\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \qquad (6.2)$$

therefore:

$$\frac{R_1}{R_2} = e^{B\left(\frac{1}{T_1} - \frac{1}{T_2}\right)} \qquad (6.2b)$$

where R_1 and R_2 are the resistances of the thermistors at absolute temperatures T_1 and T_2 respectively and B is a characteristic temperature constant for the thermistor. When the temperature change is very small, as in the present case, $B(1/T_1) - (1/T_2)$ is very much smaller than one and this relationship may be substantially simplified using the approximation when $x<<1$ that $e^x \approx 1 + x$ (x here being $B(1/T_1) - (1/T_2)$),

$$R_1 = R_2\left\{1 + B\left(\frac{T_2 - T_1}{T_1 T_2}\right)\right\} \qquad (6.3)$$

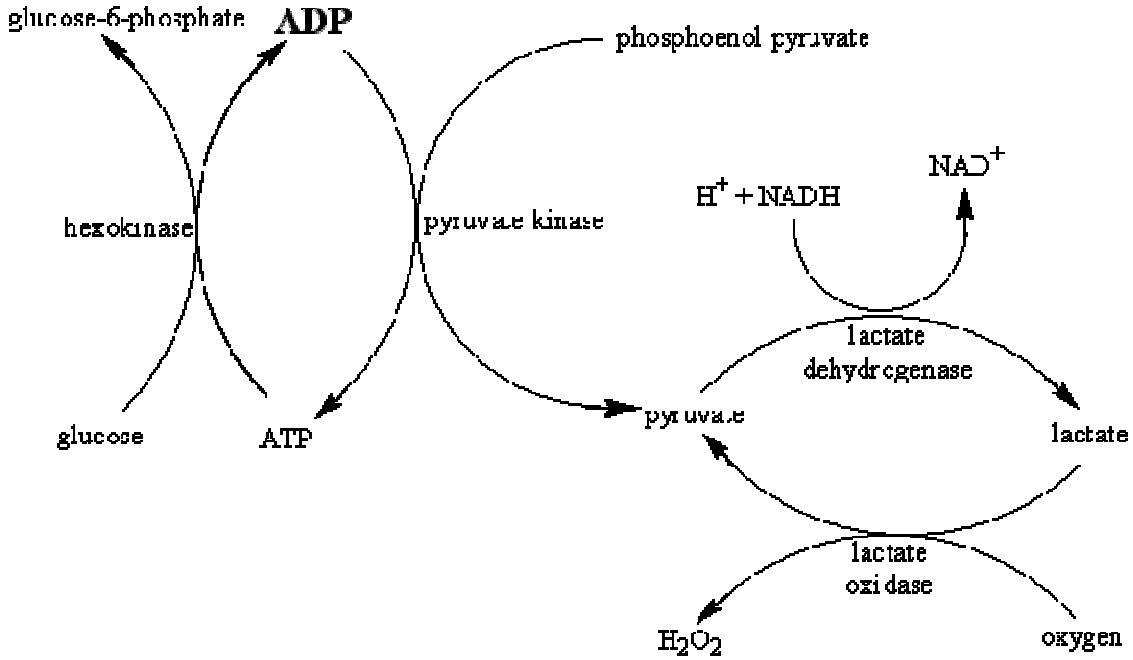
As $T_1 \approx T_2$, they both may be replaced in the denominator by T_1.

$$\frac{\Delta R}{R} = -\left(\frac{B}{T_1^2}\right)\Delta T \qquad (6.4)$$

The relative decrease in the electrical resistance ($\Delta R/R$) of the thermistor is proportional to the increase in temperature (ΔT). A typical proportionality constant ($-B/T_1^2$) is -4%°C^{-1}. The resistance change is converted to a proportional voltage change, using a balanced

Wheatstone bridge incorporating precision wire-wound resistors, before amplification. The expectation that there will be a linear correlation between the response and the enzyme activity has been found to be borne out in practice. A major problem with this biosensor is the difficulty encountered in closely matching the characteristic temperature constants of the measurement and reference thermistors. An equal movement of only 1°C in the background temperature of both thermistors commonly causes an apparent change in the relative resistances of the thermistors equivalent to 0.01°C and equal to the full-scale change due to the reaction. It is clearly of great importance that such environmental temperature changes are avoided, which accounts for inclusion of the well-insulated aluminium block in the biosensor design (see Figure 6.2).

The sensitivity (10^{-4} M) and range (10^{-4} - 10^{-2} M) of thermistor biosensors are both quite low for the majority of applications although greater sensitivity is possible using the more exothermic reactions (e.g. catalase). The low sensitivity of the system can be increased substantially by increasing the heat output by the reaction. In the simplest case this can be achieved by linking together several reactions in a reaction pathway, all of which contribute to the heat output. Thus the sensitivity of the glucose analysis using glucose oxidase can be more than doubled by the co-immobilisation of catalase within the column reactor in order to disproportionate the hydrogen peroxide produced. An extreme case of this amplification is shown in the following recycle scheme for the detection of ADP.



[6.2]

ADP is the added analyte and excess glucose, phosphoenol pyruvate, NADH and oxygen are present to ensure maximum reaction. Four enzymes (hexokinase, pyruvate kinase, lactate dehydrogenase and lactate oxidase) are co-immobilised within the packed bed reactor. In spite of the positive enthalpy of the pyruvate kinase reaction, the overall process results in a 1000 fold increase in sensitivity, primarily due to the recycling between pyruvate and lactate. Reaction limitation due to low oxygen solubility may be

overcome by replacing it with benzoquinone, which is reduced to hydroquinone by flavo-enzymes. Such reaction systems do, however, have the serious disadvantage in that they increase the probability of the occurrence of interference in the determination of the analyte of interest. Reactions involving the generation of hydrogen ions can be made more sensitive by the inclusion of a base having a high heat of protonation. For example, the heat output by the penicillinase reaction may be almost doubled by the use of Tris (tris-(hydroxymethyl)aminomethane) as the buffer.In conclusion, the main advantages of the thermistor biosensor are its general applicability and the possibility for its use on turbid or strongly coloured solutions. The most important disadvantage is the difficulty in ensuring that the temperature of the sample stream remains constant (± 0.01°C).

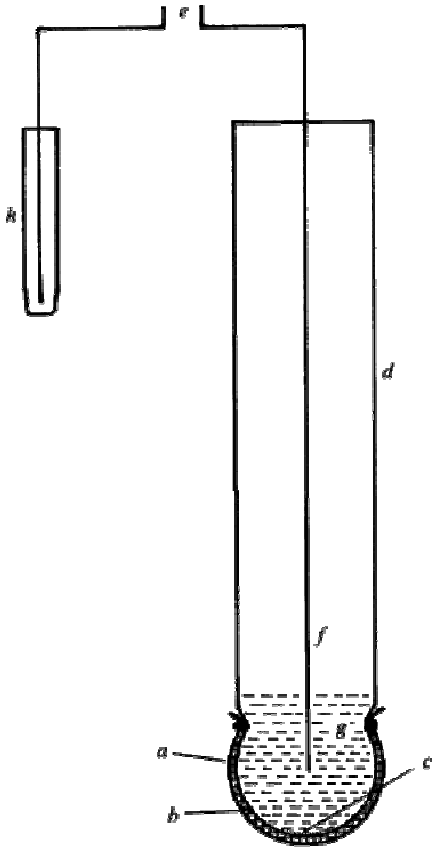
4.3.4.3 Potentiometric biosensors

Potentiometric biosensors make use of ion-selective electrodes in order to transduce the biological reaction into an electrical signal. In the simplest terms this consists of an immobilised enzyme membrane surrounding the probe from a pH-meter (Figure 6.3), where the catalysed reaction generates or absorbs hydrogen ions (Table 6.2). The reaction occurring next to the thin sensing glass membrane causes a change in pH which may be read directly from the pH-meter's display. Typical of the use of such electrodes is that the electrical potential is determined at very high impedance allowing effectively zero current flow and causing no interference with the reaction.

Figure 6.3. A simple potentiometric biosensor. A semi-permeable membrane (a) surrounds the biocatalyst (b) entrapped next to the active glass membrane (c) of a pH probe (d). The electrical potential (e) is generated between the internal Ag/AgCl electrode (f) bathed in dilute HCl (g) and an external reference electrode (h).

There are three types of ion-selective electrodes which are of use in biosensors:

1. Glass electrodes for cations (e.g. normal pH electrodes) in which the sensing element is a very thin hydrated glass membrane which generates a transverse electrical potential due to the concentration-dependent competition between the cations for specific binding sites. The selectivity of this membrane is determined by the composition of the glass. The sensitivity to H^+ is greater than that achievable for NH_4^+,



2. Glass pH electrodes coated with a gas-permeable membrane selective for CO_2, NH_3 or H_2S. The diffusion of the gas through this membrane causes a change in pH of a sensing solution between the membrane and the electrode which is then determined.
3. Solid-state electrodes where the glass membrane is replaced by a thin membrane of a specific ion conductor made from a mixture of silver sulphide and a silver halide. The iodide electrode is useful for the determination of I^- in the peroxidase reaction (Table 6.2c) and also responds to cyanide ions.

Table 6.2. Reactions involving the release or absorption of ions that may be utilised by potentiometric biosensors.

(a) H^+ cation,

$$\text{D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{D-glucono-1,5-lactone} + H_2O_2 \xrightarrow{H_2O} \text{D-gluconate} + H^+ \quad \textbf{[6.3]}$$

$$\text{penicillin} \xrightarrow{\text{penicillinase}} \text{penicilloic acid} + H^+ \quad \textbf{[6.4]}$$

$$H_2NCONH_2 + H_2O + 2H^+ \xrightarrow{\text{urease (pH 6.0)}^a} 2NH_4^+ + CO_2 \quad \textbf{[6.5]}$$

$$H_2NCONH_2 + 2H_2O \xrightarrow{\text{urease (pH 9.5)}^b} 2NH_3 + HCO_3^- + H^+ \quad \textbf{[6.6]}$$

$$\text{neutral lipids} + H_2O \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids} + H^+ \quad \textbf{[6.7]}$$

(b) NH_4^+ cation,

$$\text{L-amino acid} + O_2 + H_2O \xrightarrow{\text{L-amino acid oxidase}} \text{keto acid} + NH_4^+ + H_2O_2 \quad \textbf{[6.8]}$$

$$\text{L-asparagine} + H_2O \xrightarrow{\text{asparaginase}} \text{L-aspartate} + NH_4^+ \quad \textbf{[6.9]}$$

$$H_2NCONH_2 + 2H_2O + H^+ \xrightarrow{\text{urease (pH 7.5)}} 2NH_4^+ + HCO_3^- \quad \textbf{[6.10]}$$

(c) I^- anion,

$$H_2O_2 + 2H^+ + 2I^- \xrightarrow{\text{peroxidase}} I_2 + 2H_2O \quad \textbf{[6.11]}$$

(d) CN^- anion,

$$\text{amygdalin} + 2H_2O \xrightarrow{\beta\text{-glucosidase}} 2\text{glucose} + \text{benzaldehyde} + H^+ + CN^- \quad \textbf{[6.12]}$$

[a] Can also be used in NH_4^+ and CO_2 (gas) potentiometric biosensors.
[b] Can also be used in an NH_3 (gas) potentiometric biosensor.es80ll66bp
The response of an ion-selective electrode is given by

$$E = E_0 + \frac{RT}{zF} Ln([i]) \qquad (6.5)$$

where E is the measured potential (in volts), E_0 is a characteristic constant for the ion-selective/external electrode system, R is the gas constant, T is the absolute temperature (K), z is the signed ionic charge, F is the Faraday, and [i] is the concentration of the free uncomplexed ionic species (strictly, [i] should be the activity of the ion but at the concentrations normally encountered in biosensors, this is effectively equal to the concentration). This means, for example, that there is an increase in the electrical potential of 59 mv for every decade increase in the concentration of H^+ at 25°C. The logarithmic dependence of the potential on the ionic concentration is responsible both for the wide analytical range and the low accuracy and precision of these sensors. Their normal range of detection is 10^{-4} - 10^{-2} M, although a minority are ten-fold more sensitive. Typical response time are between one and five minutes allowing up to 30 analyses every hour.

Biosensors which involve H^+ release or utilisation necessitate the use of very weakly buffered solutions (i.e. < 5 mM) if a significant change in potential is to be determined. The relationship between pH change and substrate concentration is complex, including other such non-linear effects as pH-activity variation and protein buffering. However, conditions can often be found where there is a linear relationship between the apparent change in pH and the substrate concentration. A recent development from ion-selective electrodes is the production of ion-selective field effect transistors (**ISFET**s) and their biosensor use as enzyme-linked field effect transistors (**ENFET**s, Figure 6.4). Enzyme membranes are coated on the ion-selective gates of these electronic devices, the biosensor responding to the electrical potential change via the current output. Thus, these are potentiometric devices although they directly produce changes in the electric current. The main advantage of such devices is their extremely small size (<< 0.1 mm^2) which allows cheap mass-produced fabrication using integrated circuit technology. As an example, a urea-sensitive FET (ENFET containing bound urease with a reference electrode containing bound glycine) has been shown to show only a 15% variation in response to urea (0.05 - 10.0 mg ml^{-1}) during its active lifetime of a month. Several

analytes may be determined by miniaturised biosensors containing arrays of ISFETs and ENFETs. The sensitivity of FETs, however, may be affected by the composition, ionic strength and concentrations of the solutions analysed.

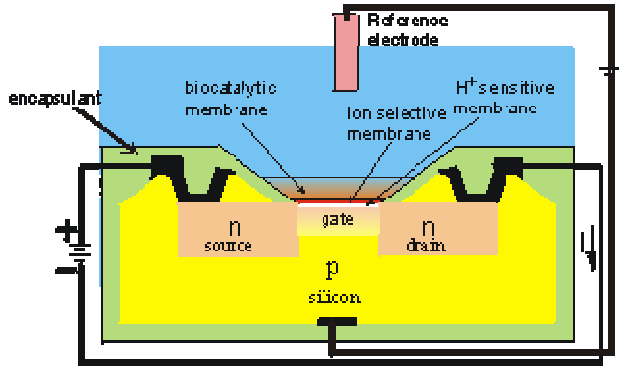


Figure 6.4. Schematic diagram of the section across the width of an ENFET. The actual dimensions of the active area is about 500 □m long by 50 □m wide by 300 □m thick. The main body of the biosensor is a p-type silicon chip with two n-type silicon areas; the negative source and the positive drain. The chip is insulated by a thin layer (0.1 □m thick) of silica (SiO_2) which forms the gate of the FET. Above this gate is an equally thin layer of H^+-sensitive material (e.g. tantalum oxide), a protective ion selective membrane, the biocatalyst and the analyte solution, which is separated from sensitive parts of the FET by an inert encapsulating polyimide photopolymer. When a potential is applied between the electrodes, a current flows through the FET dependent upon the positive potential detected at the ion-selective gate and its consequent attraction of electrons into the depletion layer. This current (I) is compared with that from a similar, but non-catalytic ISFET immersed in the same solution. (Note that the electric current is, by convention, in the opposite direction to the flow of electrons).

4.3.4.4 Amperometric biosensors

Amperometric biosensors function by the production of a current when a potential is applied between two electrodes. They generally have response times, dynamic ranges and sensitivities similar to the potentiometric biosensors. The simplest amperometric biosensors in common usage involve the Clark oxygen electrode (Figure 6.5). This consists of a platinum cathode at which oxygen is reduced and a silver/silver chloride reference electrode. When a potential of -0.6 V, relative to the Ag/AgCl electrode is applied to the platinum cathode, a current proportional to the oxygen concentration is produced. Normally both electrodes are bathed in a solution of saturated potassium chloride and separated from the bulk solution by an oxygen-permeable plastic membrane (e.g. Teflon, polytetrafluoroethylene). The following reactions occur:

Ag anode $4Ag^0 + 4Cl^- \longrightarrow 4AgCl + 4e^-$ **[6.13]**

Pt cathode $O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$ **[6.14]**

The efficient reduction of oxygen at the surface of the cathode causes the oxygen concentration there to be effectively zero. The rate of this electrochemical reduction therefore depends on the rate of diffusion of the oxygen from the bulk solution, which is dependent on the concentration gradient and hence the bulk oxygen concentration (see, for example, equation 3.13). It is clear that a small, but significant, proportion of the

oxygen present in the bulk is consumed by this process; the oxygen electrode measuring the rate of a process which is far from equilibrium, whereas ion-selective electrodes are used close to equilibrium conditions. This causes the oxygen electrode to be much more sensitive to changes in the temperature than potentiometric sensors. A typical application for this simple type of biosensor is the determination of glucose concentrations by the use of an immobilised glucose oxidase membrane. The reaction (see reaction scheme **[1.1]**) results in a reduction of the oxygen concentration as it diffuses through the biocatalytic membrane to the cathode, this being detected by a reduction in the current between the electrodes (Figure 6.6). Other oxidases may be used in a similar manner for the analysis of their substrates (e.g. alcohol oxidase, D- and L-amino acid oxidases, cholesterol oxidase, galactose oxidase, and urate oxidase)

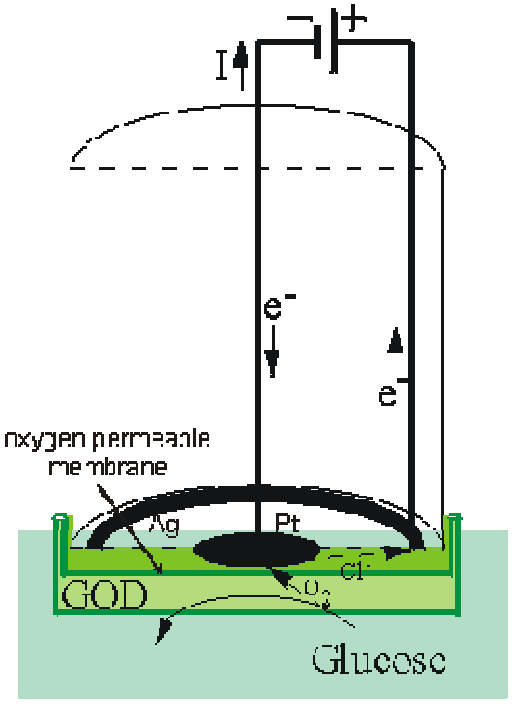


Figure 6.5. Schematic diagram of a simple amperometric biosensor. A potential is applied between the central platinum cathode and the annular silver anode. This generates a current (I) which is carried between the electrodes by means of a saturated solution of KCl. This electrode compartment is separated from the biocatalyst (here shown glucose oxidase, GOD) by a thin plastic membrane, permeable only to oxygen. The analyte solution is separated from the biocatalyst by another membrane, permeable to the substrate(s) and product(s). This biosensor is normally about 1 cm in diameter but has been scaled down to 0.25 mm diameter using a Pt wire cathode within a silver plated steel needle anode and utilising dip-coated membranes.

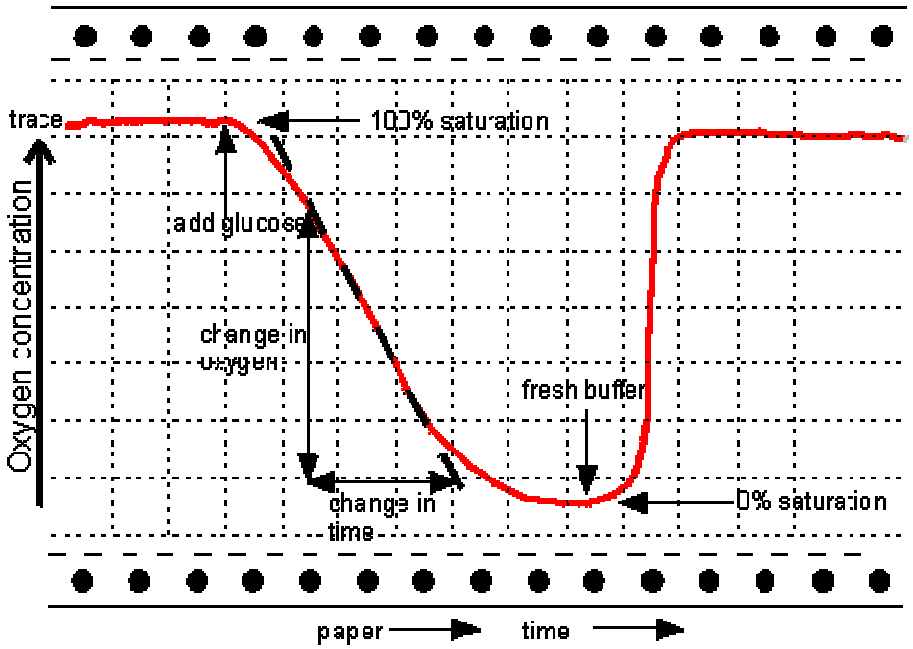


Figure 6.6. The response of an amperometric biosensor utilising glucose oxidase to the presence of glucose solutions. Between analyses the biosensor is placed in oxygenated buffer devoid of glucose. The steady rates of oxygen depletion may be used to generate standard response curves and determine unknown samples. The time required for an assay can be considerably reduced if only the initial transient (curved) part of the response need be used, via a suitable model and software. The wash-out time, which roughly equals the time the electrode spends in the sample solution, is also reduced significantly by this process.

An alternative method for determining the rate of this reaction is to measure the production of hydrogen peroxide directly by applying a potential of +0.68 V to the platinum electrode, relative to the Ag/AgCl electrode, and causing the reactions:

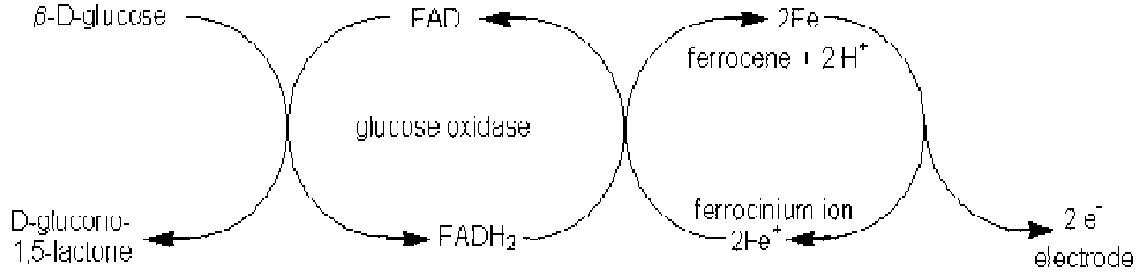
Pt anode $H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^-$ **[6.15]**

Ag cathode $2AgCl + 2e^- \longrightarrow 2Ag^0 + 2Cl^-$ **[6.16]**

The major problem with these biosensors is their dependence on the dissolved oxygen concentration. This may be overcome by the use of 'mediators' which transfer the electrons directly to the electrode bypassing the reduction of the oxygen co-substrate. In order to be generally applicable these mediators must possess a number of useful properties.

1. They must react rapidly with the reduced form of the enzyme.
2. They must be sufficiently soluble, in both the oxidised and reduced forms, to be able to rapidly diffuse between the active site of the enzyme and the electrode surface. This solubility should, however, not be so great as to cause significant loss of the mediator from the biosensor's microenvironment to the bulk of the solution. However soluble, the mediator should generally be non-toxic.
3. The overpotential for the regeneration of the oxidised mediator, at the electrode, should be low and independent of pH.
4. The reduced form of the mediator should not readily react with oxygen.

The ferrocenes represent a commonly used family of mediators (Figure 6.7a). Their reactions may be represented as follows,



[6.17]

Electrodes have now been developed which can remove the electrons directly from the reduced enzymes, without the necessity for such mediators. They utilise a coating of electrically conducting organic salts, such as N-methylphenazinium cation (NMP^+, Figure 6.7b) with tetracyanoquinodimethane radical anion ($TCNQ^{\cdot-}$ Figure 6.7c). Many flavo-enzymes are strongly adsorbed by such organic conductors due to the formation of salt links, utilising the alternate positive and negative charges, within their hydrophobic environment. Such enzyme electrodes can be prepared by simply dipping the electrode into a solution of the enzyme and they may remain stable for several months. These electrodes can also be used for reactions involving $NAD(P)^+$-dependent dehydrogenases as they also allow the electrochemical oxidation of the reduced forms of these coenzymes. The three types of amperometric biosensor utilising product, mediator or organic conductors represent the three generations in biosensor development (Figure 6.8). The reduction in oxidation potential, found when mediators are used, greatly reduces the problem of interference by extraneous material.

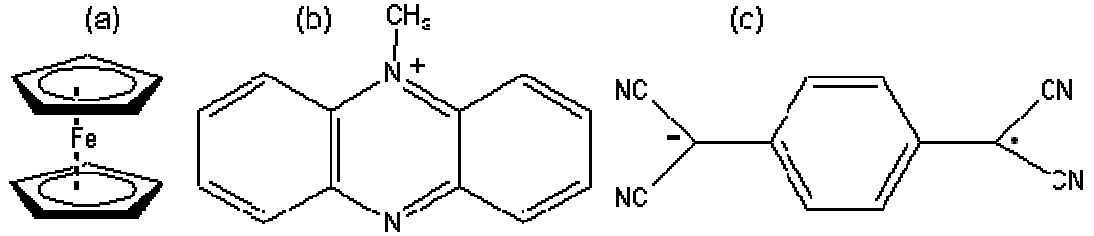


Figure 6.7. (a) Ferrocene (□5-bis-cyclopentadienyl iron), the parent compound of a number of mediators. (b) TMP^+, the cationic part of conducting organic crystals. (c) $TCNQ^{\cdot-}$, the anionic part of conducting organic crystals. It is a resonance-stabilised radical formed by the one-electron oxidation of $TCNQH_2$.

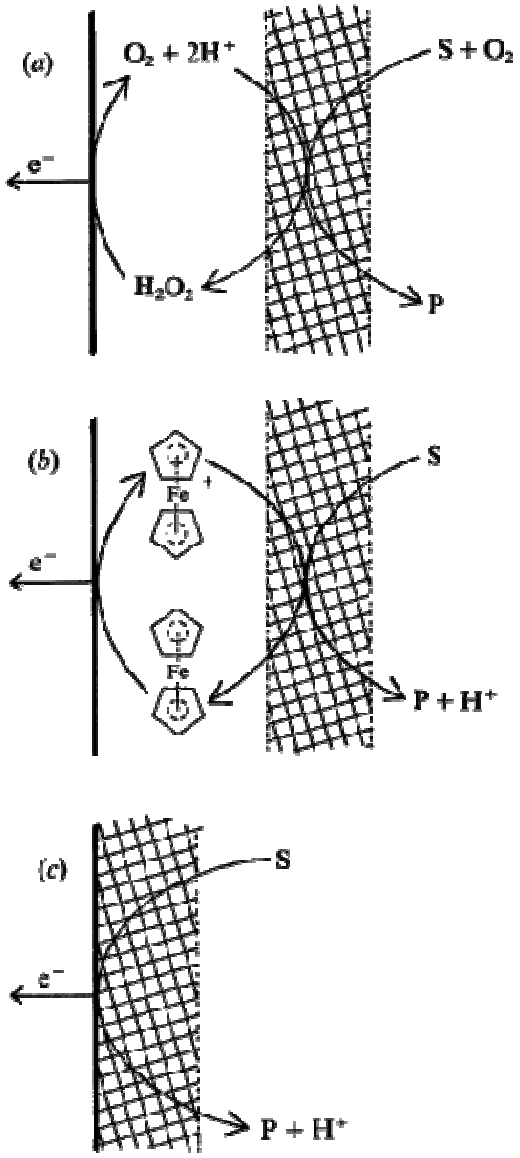


Figure 6.8. Amperometric biosensors for flavo-oxidase enzymes illustrating the three generations in the development of a biosensor. The biocatalyst is shown schematically by the cross-hatching. (a) First generation electrode utilising the H_2O_2 produced by the reaction. (E_0 = +0.68 V). (b) Second generation electrode utilising a mediator (ferrocene) to transfer the electrons, produced by the reaction, to the electrode. (E_0 = +0.19 V). (c) Third generation electrode directly utilising the electrons produced by the reaction. (E_0 = +0.10 V). All electrode potentials (E_0) are relative to the $Cl^-/AgCl,Ag^0$ electrode. The following reaction occurs at the enzyme in all three biosensors:

Substrate(2H) + FAD-oxidase ⟶ Product + $FADH_2$-oxidasefi **[6.18]**

This is followed by the processes:

(a)
biocatalyst

$$FADH_2\text{-oxidase} + O_2 \longrightarrow FAD\text{-oxidase} + H_2O_2 \quad \textbf{[6.19]}$$

electrode

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \quad \textbf{[6.20]}$$

(b)
biocatalyst

$$FADH_2\text{-oxidase} + 2\ Ferricinium^+ \longrightarrow FAD\text{-oxidase} + 2\ Ferrocene + 2H^+ \quad \textbf{[6.21]}$$

electrode

$$2\ Ferrocene \longrightarrow 2\ Ferricinium^+ + 2e^- \quad \textbf{[6.22]}$$

(c)
biocatalyst/electrode

$$FADH_2\text{-oxidase} \longrightarrow FAD\text{-oxidase} + 2H^+ + 2e^- \quad \textbf{[6.23]}$$

The current (i) produced by such amperometric biosensors is related to the rate of reaction (v_A) by the expression:

$$i = nFAv_A \qquad (6.6)$$

where n represents the number of electrons transferred, A is the electrode area, and F is the Faraday. Usually the rate of reaction is made diffusionally controlled (see equation 3.27) by use of external membranes. Under these circumstances the electric current produced is proportional to the analyte concentration and independent both of the enzyme and electrochemical kinetics.

4.3.4.5 Optical biosensors

There are two main areas of development in optical biosensors. These involve determining changes in light absorption between the reactants and products of a reaction, or measuring the light output by a luminescent process. The former usually involve the widely established, if rather low technology, use of colorimetric test strips. These are disposable single-use cellulose pads impregnated with enzyme and reagents. The most common use of this technology is for whole-blood monitoring in diabetes control. In this case, the strips include glucose oxidase, horseradish peroxidase (EC 1.11.1.7) and a chromogen (e.g. *o*-toluidine or 3,3',5,5'-tetramethylbenzidine). The hydrogen peroxide, produced by the aerobic oxidation of glucose (see reaction scheme **[1.1]**), oxidising the weakly coloured chromogen to a highly coloured dye.

$$\text{chromogen(2H)} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{dye} + 2H_2O \quad \textbf{[6.24]}$$

The evaluation of the dyed strips is best achieved by the use of portable reflectance meters, although direct visual comparison with a coloured chart is often used. A wide variety of test strips involving other enzymes are commercially available at the present time.A most promising biosensor involving luminescence uses firefly luciferase (*Photinus*-luciferin 4-monooxygenase (ATP-hydrolysing), EC 1.13.12.7) to detect the presence of bacteria in food or clinical samples. Bacteria are specifically lysed and the ATP released (roughly proportional to the number of bacteria present) reacted with D-luciferin and oxygen in a reaction which produces yellow light in high quantum yield.

$$ATP + \text{D-luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{oxyluciferin} + AMP + \text{pyrophosphate} + CO_2 + \text{light (562 nm)} \quad \textbf{[6.25]}$$

The light produced may be detected photometrically by use of high-voltage, and expensive, photomultiplier tubes or low-voltage cheap photodiode systems. The sensitivity of the photomultiplier-containing systems is, at present, somewhat greater (< 10^4 cells ml^{-1}, < 10^{-12} M ATP) than the simpler photon detectors which use photodiodes. Firefly luciferase is a very expensive enzyme, only obtainable from the tails of wild fireflies. Use of immobilised luciferase greatly reduces the cost of these analyses.

4.3.4.6 Piezo-electric biosensors

Piezo-electric crystals (e.g. quartz) vibrate under the influence of an electric field. The frequency of this oscillation (f) depends on their thickness and cut, each crystal having a characteristic resonant frequency. This resonant frequency changes as molecules adsorb or desorb from the surface of the crystal, obeying the relationships

$$\Delta f = \frac{Kf^2 \Delta m}{A} \qquad (6.7)$$

where □f is the change in resonant frequency (Hz), □m is the change in mass of adsorbed material (g), K is a constant for the particular crystal dependent on such factors as its density and cut, and A is the adsorbing surface area (cm^2). For any piezo-electric crystal, the change in frequency is proportional to the mass of absorbed material, up to about a 2% change. This frequency change is easily detected by relatively unsophisticated electronic circuits. A simple use of such a transducer is a formaldehyde biosensor, utilising a formaldehyde dehydrogenase coating immobilised to a quartz crystal and sensitive to gaseous formaldehyde. The major drawback of these devices is the interference from atmospheric humidity and the difficulty in using them for the determination of material in solution. They are, however, inexpensive, small and robust, and capable of giving a rapid response.

4.3.4.7 Immunosensors

Biosensors may be used in conjunction with enzyme-linked immunosorbent assays (**ELISA**). The principles behind the ELISA technique is shown in Figure 6.9. ELISA is used to detect and amplify an antigen-antibody reaction; the amount of enzyme-linked antigen bound to the immobilised antibody being determined by the relative concentration of the free and conjugated antigen and quantified by the rate of enzymic reaction. Enzymes with high turnover numbers are used in order to achieve rapid response. The sensitivity of such assays may be further enhanced by utilising enzyme-catalysed reactions which give intrinsically greater response; for instance, those giving rise to highly coloured, fluorescent or bioluminescent products. Assay kits using this technique are now available for a vast range of analyses.

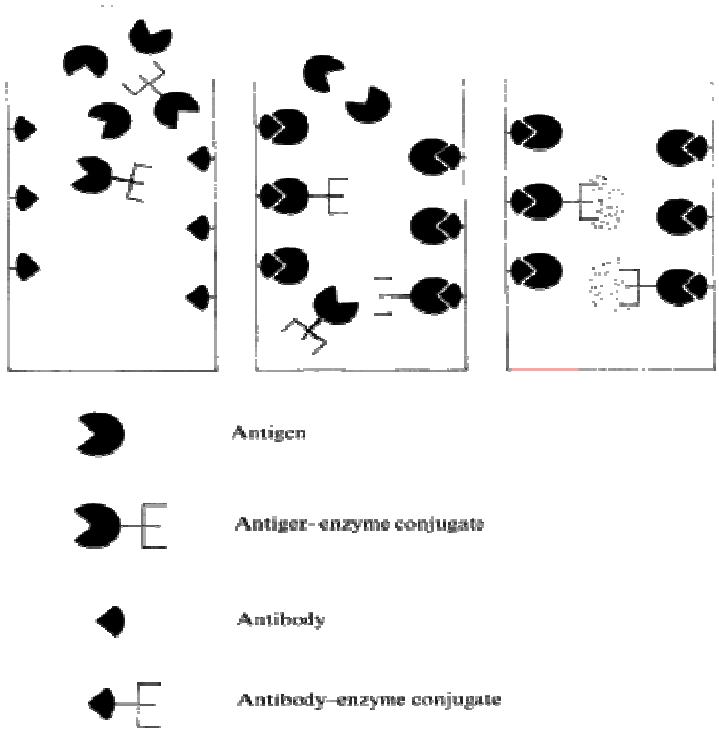


Figure 6.9. Principles of a direct competitive ELISA. (i) Antibody, specific for the antigen of interest is immobilised on the surface of a tube. A mixture of a known amount of antigen-enzyme conjugate plus unknown concentration of sample antigen is placed in the tube and allowed to equilibrate. (ii) After a suitable period the antigen and antigen-enzyme conjugate will be distributed between the bound and free states dependent upon their relative concentrations. (iii) Unbound material is washed off and discarded. The amount of antigen-enzyme conjugate that is bound may be determined by the rate of the subsequent enzymic reaction.

Recently ELISA techniques have been combined with biosensors, to form **immunosensors**, in order to increase their range, speed and sensitivity. A simple immunosensor configuration is shown in Figure 6.10 (a), where the biosensor merely replaces the traditional colorimetric detection system. However more advanced immunosensors are being developed (Figure 6.10 (b)) which rely on the direct detection of antigen bound to the antibody-coated surface of the biosensor. Piezoelectric and FET-based biosensors are particularly suited to such applications.

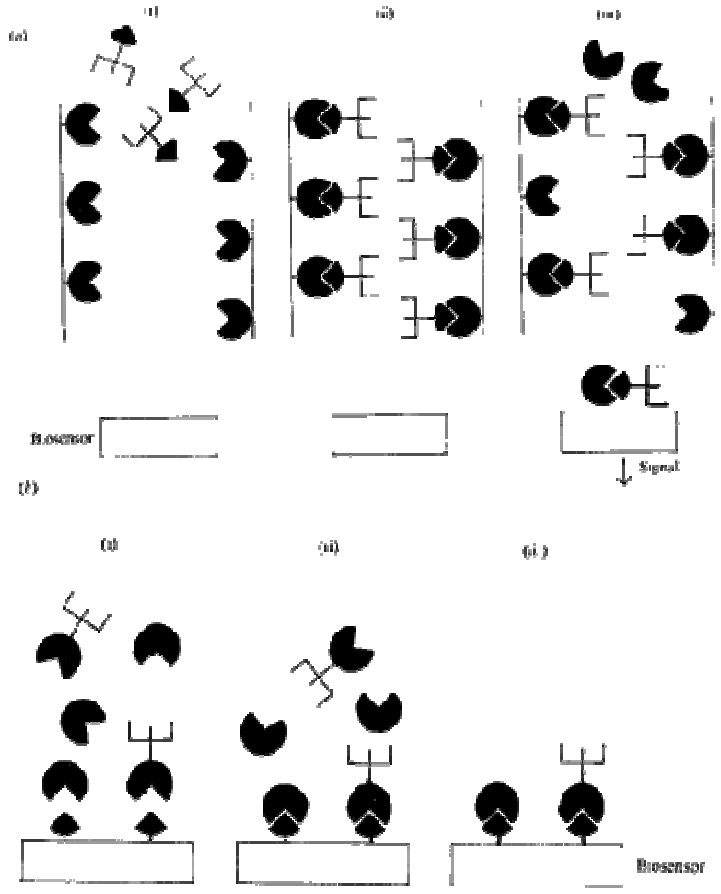


Figure 6.10. Principles of immunosensors. (a)(i) A tube is coated with (immobilised) antigen. An excess of specific antibody-enzyme conjugate is placed in the tube and allowed to bind. (a)(ii) After a suitable period any unbound material is washed off. (a)(iii) The analyte antigen solution is passed into the tube, binding and releasing some of the antibody-enzyme conjugate dependent upon the antigen's concentration. The amount of antibody-enzyme conjugate released is determined by the response from the biosensor. (b)(i) A transducer is coated with (immobilised) antibody, specific for the antigen of interest. The transducer is immersed in a solution containing a mixture of a known amount of antigen-enzyme conjugate plus unknown concentration of sample antigen. (b)(ii) After a suitable period the antigen and antigen-enzyme conjugate will be distributed between the bound and free states dependent upon their relative concentrations. (b)(iii) Unbound material is washed off and discarded. The amount of antigen-enzyme conjugate bound is determined directly from the transduced signal.

4.3.4.8 Summary

a. There is a vast potential market for biosensors which is only beginning to be expoited.
b. Biosensors generally are easy to operate, analyse over a wide range of useful analyte concentrations and give reproducible results.

c. The diffusional limitation of substrate(s) may be an asset to be encouraged in biosensor design due to the consequent reduction in the effects of analyte pH, temperature and inhibitors on biosensor response.

4.3.4.9 Model Questions

1. What are biosensors and explain the basic principle involved in working of biosensors
2. Write a note on Immunosensors?
3. Explain briefly Potentiometric biosensors and optical biosensors**?**

4.3.4.10 References and Bibliography

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Lesson 4.4.1

PRODUCTION OF ANTIBIOTICS

Structure
Objective
4.4.1.1 Introduction
4.4.1.2 the microbial groups involved in the production of antibiotics.
4.4.1.3. Applications of antibiotics.
4.4.1.4 Steps involved in production of antibiotics
4.4.1.5 Genetics of antibiotic production
4.4.1.6. Summary
4.4.1.7. Modal questions
4.4.1.8 References

Objective: the main objective of this lesson is to discus about various applications and Steps involved in production of antibiotics in general.

4.4.1.1. Introduction

Secondary metabolite products which inhibit the growth of other microbial species, even at low levels, are called antibiotics.

In the area of industrial microbiology screening of efficient strain, various molecular biological techniques and other microbial research continues for the effective production of antibiotics. Among large number of known antibiotics, only 100 are produced commercially via fermentation.

Production of antibiotics can be semisynthetic or in few cases they are produced synthetically eg: in case of chloramphenicol, phosphonomycin and pyrolnitrin.

4.4.1.2. The Microbial groups involved in production of antibiotics

Bacteria, actimonycetes and fungi are found to produce antibiotics. In fungi, aspergillaceae and moniliales produce important antibiotics. Only penicillins, cephalosporirn C, griciofulvin and fusidic acid are of clinical importance. In bacteria, many taxanomic groups produce antibiotics. Varied number of antibiotics are produced by the actinomycetes, of genus S*treptomyces*.Genus *Bacillus* can produce peptide antibiotics which are very important.

The significance of production of secondary metabolites by organism is not certainly clear.

1. Secondary metabolites are secreated by only few organisms.
2. They are essential for growth and reproduction.
3. Their production can be depending on environmental conditions.
4. Besides five phases of metabolism [i.e. intermediary, regulatory, transport, differentiation and morphogenesis) secondary metabolism is considered a “playing field” for the evolution of further biochemical developments, without damaging the primary metabolism.

1. β-lactum Atibiotics

(a) Penicillins	*Penicillium* sp *Aspergillus* sp
b) Cephalorpolins	*Cephalorposium acremonium*
(c) Clavulanic acid	*S.clavuligerus*
(d) Nocardins	*Norandia uniformis*
(e) Thienamycins	*S. cattleya*

2 .Amini acid & peptide

(a) Amphomycin	Streptomyces canus
(b) Bacitrain	Bacillus Licheniformis
(c) Granicidin A	B.Brevis
(d) Viomycin	S.floridae
(e) Nisin	Streptoccoccus cremories

3. Carbohydrate Antibiotics

(a) Nojirimycin	*Streptomyces reseochromogenes*
(b) Vancomycin	*S.orientalis*
(c) Lincrnycin	*S.Lincolensis*
(d) Meomycin	*S.banbergiensis*

4. Aminoglycoride Antibiotics

(a) Streptomycin	*S.griseus*
(b) Neomycin	*S.fradias*
[c] Kanrycin	*S.Kanamyceticus*
(d) Gentamycin	*Micromonorposa purpurea*

5.Macrolytic Lactone Antibiotics

(a) Carbomycin A	*S.haletedii*
(b) Erythromycin	*S.erythreus*

5ii. Polyene macrolides

(a)Amphotericin B	*S.molosus*
(b) Hamycin	*S.primprina*

5iii. Macrotetrolides

(a) Tetranactin	*S.flaveolus*

5iv. Typical macrolides

(a) Averunectin	*S.avermitilis*

6.Tetracyclins & Anthrocyclins

7-chlorotetracyclin (Aureomycin)	S.aureofociens
Daunormycin (daumosubicin)	S.peucetius
Adria mycin (doxorubicin)	

7.Nucleaside Antibiotics

Puromycin	S.aboniger

8.Aromatic antibiotics

Chlosamphenicol	*S.venezuelae, S.omiyaeneus etc.Streptamyces sp.*
Griseofulvin grieeofulvum	*Penicillium P.patulum,* other *P.sp.*
Novoliocin	*S.spheroids*, other sp.

9.Other cermercially produced

Mitomycin	S.caespitosus
Lasaloside	S.lasaliensis

4.4.1.3. Applications of Antibiotics

1. In the treatment of tumors:
   Many classes of antibiotics that belong to peptide, glycoside, anthrocyclin, and benzoquinone groups are used in the treatment of certain kinds of tumors.
   Eg: Chromomycin A_3, Mitomycin C
2. Antibiotics used in plant pathology:

Initially streptomycin is used against diseases caused by pseudomonas species and *Xanthamonas oryzae*.

- Blasticidin from *S. griseochromogenes* is used as a rice fungiride on *Piricularia oryzae* (Rice burn)
- Cycloheximide acts as leaf fungiside on warm blooded animals.
- Validomycin – fungiside against *Rhizoctonia solani* which causes leaf drop and stalk disease in rice .

3. Antibiotics are also used in veterinary medicine and as animal growth promoters. Animal feed with antibiotic additive has shown acceleration weight gain due to the effect on microbial flora in the GIT. Most of the therapeutically important antibioitics like penicillins, tetracyclins, bacitracin, erythomycin were also used in the feed. But due to the development of resistance these are stopped. Some antibiotics like. Enduramycin, Siomycin, Tylosin are used in animal feed which are only for nutritional purse.
4. Food industries also use antibiotics for preservation of food. Which has been controlled by regulatory agencies.
   Eg: Pimaricin used as fungicide, applied to the surface of foods.
   -Tylosin is effective against bacillus spores
   -Nisin against clostridium.
   -Chlorotetracyclins are used in fish, meat & poultry industries.
5. Some antibiotics like gentamicin are used in animal cell cultures to control contamination.
6. Antibiotics as selective inhibitors are also used in molecucular biology studies to understand cell functions, replication, transciption, translation, etc.
7. Many antibiotics are used therapeutically to cure human diseases.

-Penicillin is commonly given antibiotic against bacterial infections like “classical” pnemonia caused by streptococcus preumoniae.
-Streptomycin an aminoglycoside antibiotic is active against tuberculosis.

4.4.1.4 Steps Involved in Production of Antibiotics

Most antibiotics are still produced by fermentation or by the chemical modification of fermentation products. Therefore research is devoted mainly on improving the fermentation processes to have a significant beneficial impact.

Antibiotic production depends on the enzyme activity. Hence regulation of activity depends on understanding the physiology of the organism to maximize the production of antibiotics.

(a) Physiology of antibiotic production includes the following steps :

(a) Secondary Metabolism and its control
(b) Catabolic Repression
(c) Nitrogen and phosphate repression
(d) Feed back regulation
(e) Effects of precursors
(f) Conditions or fermentation

(a) Secondary metabolism and its control

Primary metabolites like ethanol and lysine for example are produced in the entire growth phase of a culture. Hence optimizing the conditions of growth is required

and continuous processes are feasible in such cases. In contrast secondary metabolite production is complex and poorly understood. Hence designing continuous fermentation is difficult as cultures do not produce antibiotics unless they are entering or are already in a stationary phase.

Molecular genetic studies showed some regulatory mechanisms that operate antibiotic production in prokaryotes. In Bacillus the end growth phase requires diffrential transcription of various types of promoters by several different sigma subunits of RNA polymerase.

(b) Catabolite Repression

In the presence of good carbon source like glucose, E. coli cells stop synthesizing the enzymes for the degradation of the preferable carbon sources, such as lactose. Similarly soil organisms, producing antibiotics, need to produce antibiotics and kill off their competitors only in a nutritionally poor environment and catabolite repression is useful for this arrangement.

In order to overcome catabolite repression, carbon source must be added in a careful manner in a small increments so that no large build-up of there compounds occur. Mutants which no longer effected by the presence of carbon source can be constructed.

(c) Nitrogen and phosphate repression

Presence of excess nitrogen or phosphate in the fermentation medium decreases antibiotic production. This causes an ecological advantage similar to catabolite repression. Phosphates are found to inhibit the transcription of some of the genes of antibiotic synthesis. Hence the concentrations of nitrogen and phosphate have to be regulated.

(d) Feedback regulation

It is suspected that the antibiotics themselves, as end products exert negative – feedback regulation on their synthesis. If feed back inhibition plays a role in regulating the production of an antibiotic, it must be possible to increase its production by promoting excretion of the antibiotic and thus lowering its intracellular concentration.

(e) Effects of precursors

Primary metabolites acts as precursors for secondary metabolite production for example α-aminoadipic acid is an intermediate in lysine biosynthesis. Therefore high levels of lysine, inhibits the first enzyme of the pathway which leads to shortage of all intermediates which in turn inhibit production of penicillin. In contract excess lysine stimulates the production of cephamycin C in Streptomyce.

(f) Conditions for fermentation

Most fermentation procedures run in two stages

(1) First stage: It starts from spores because antibiotic production is often unstable and can be lost if the stock is kept growing constantly though out culture. Hence organisms are grown under submerged conditions with sufficient aeration and nutrients so that they can attain maximal density in a short time.
(2) Second stage: In which culture reaches the stationary phase, stops growing and starts to produce antibiotics. Hence the concentrations of key nutrients

(carbon, phosphate and nitrogen) are to be controlled carefully by continuous feed processes.

4.4.1.5.Genetics of Antibiotic Production

The detailed knowledge of synthetic pathways, together with their relevant enzymes and genetic locations had a significant impact on the development of improved strains and optimization of productive fermentations.

(a) Strain improvement

Strain improvement is necessary as high concentrations of the antibiotics increase the volumetric productivity, increases the extraction efficiency, decreases the proportion of unwanted products, makes purification easier and thus reduce cost of the product.

The strain improvement programs involve forced creation of mutations in the DNA material of the micro-organism either by UV or chemical mutagens like nitro guanidine.

Traditional method of strain improvement depends entirely on random mutagenesis and screening of high procedures, unwanted mutations may be introduced into the organism, which may result weakened strain. These problems are overcome in classical genetics where desirable mutant alleles of various genes that affect antibiotic production are identified and recombined them into a single organism. This method is also replaced with back crossing of overproducing strains with parent to improve the vigor of the mutant strain. Though true sexual cycle is not found in penicillin a eukaryotic fungus, because of the identification of Para sexual cycle – resulting in the production of heterocaryons was and to improve the strain. Even strains of *Streptomyces* can also be improved by this proves.

Targeted mutagenesis is one of the classic approach in which mutations are introduced indiscriminately into all the genes. Then transductants that received the donor copy of a gene located next to the antibiotic synthesis gene complex are selected. This is a possible way to recover only those pieces of mutagenized DNA that contained the particular gene used for selection.

Grouping of genes involved in the production of a single antibiotic in closely linked clusters, several complete gene clusters have successfully been cloned into a single plasmid. This type of approach gave insights into the nature of antibiotic and helped to study polypeptide antibiotics like erythromycin A etc.

(b) Culture preservation and propagation aseptically

Attention has to be given to the correct preservation and consistant propagation of high producing strains. Repeated slant to slant transfer of high yielding strains can produce sub-populations with lower productivity and appearance of wild type morphology. Hence, stock cultures are maintained in liquid N_2 for a long time. There frozen cultures are further used to prepare working stock cultures.

New master cell lines are prepared though single cell or spore re -isolation and there are evaluated in shake flack and pilot fermentations to confirm superiority and stability before the culture is used in large scale.

Maintaining of aseptic conditions throughout the build-up of culture volumes is essential this is critical at the seed stage where the cultures are growing fast and scheduled tank transfer occurs before full status of asepsis is known. Impurity

profiling by gradient HPLC (high performance liquid chromatography) and MS (mass spectroscopy) is now standard practice in the evaluation of new strains, new media components and any engineering changes.

(c) Scale up

The conditions of shake flask are selected as close as possible to the stirred – tank large – scale fermentations. This may also have many compromises also like for example if the volume in shake – flask decrease by evaporation while in stirred tank volume may increase by sugar feed. Antifoaming agents are required in stirred tank where as in shake flack it is not needed.

Shake flack	Stirred – tank
1. Volume is less	Volume is more
2. Batch only	Batch & feed possible
3. Limited controls	Continuous controls : pH, Temp, dissolved oxygen, pressure
4. Slow metabolizing carbohydrates like lactose starch	Readily metabolized carbohydrates – glucose
5. Ambient pressures	Two atmospheric pressure possible
6. In processes sampling is difficult	In process sampling is easy & necessary
7. Solid growth of side walls	Very uniform growth
8. No control of dissolved oxygen	Control of dissolved oxygen

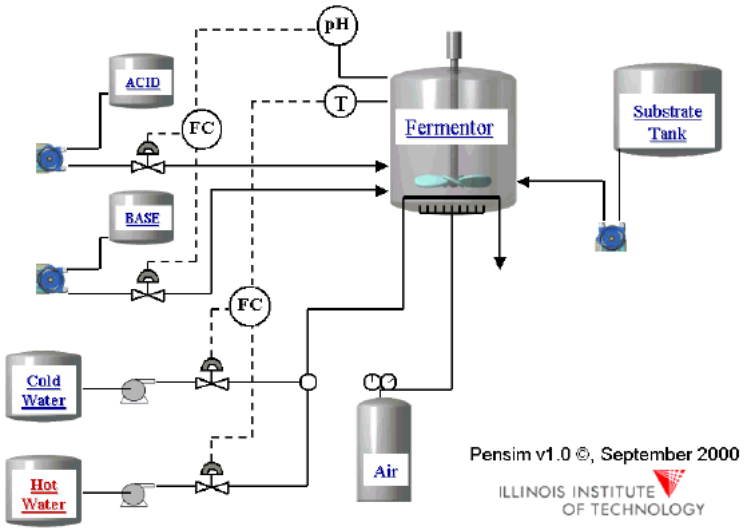
In scale-up interdisciplinary skills of the bio-engineers, microbiologists and biochemists can prove to be rewarding.

(d) Fermentation

Media for cell mass build-up are designed to provide fact growth with minimal changes in pH. The concentration of individual medium components need not be greater than 3.5%. Readily available carbon source like glucose or sucrose and soluble form of nitrogen like corn steep liquor or yeast extract are used. Calcium carbonate or phosphates can be added as buffers. Ammonium sulphate can be used to provide additional nitrogen.

[e]Components of seed media

Glucose/starch/sucrose	3 – 5%
Corn steep liquor	3 – 5%
Calcium carbonates	0.5 – 1.0%
Phosphate	0.1 – 0.5%
Ammonium sulphate	0.1 – 1.0%
Urea	0.1 – 0.5%
Oil	0.1 – 0.5%



General flow sheet for all antibiotic productions

Production Medias have been developed and modified according to the need. The media should be cost and performance effective. The most suitable media are there that are inexpensive raw materials and can maximize production.

Components of production media: - Shake flack

Glucose	0.2 – 1.0%
Starch	0.5 – 5.0%
Lactose	5.0 – 8.0%
Corn steep liquor	5.0 – 8.0%
Pharma media	1.0 – 5.0%
Soy flour	1.0 – 5.0%
Oil	0.5 – 5.0%
Ammonium Sulphate	0.5 – 1.0%
Calcium carbonate	0.5 – 1.0%
Phosphate	0.1 – 1.0%

In continuously producing large fermentation systems Ammonium sulphate, corn syrup and oil are fed continuously.

Final – stage fermentations are fed – batch, which can be controlled in a number of ways. Physically by temperature, aeration, agitation, pH. Bio chemically by addition of nutrients, precursors, inducers in some highly productive fermentation there is no clear separation of the primary (tropophasic) and secondary (idiophasic) stages, which mainly depends upon the state of the fermentation technology. In batch fermentation, clear primary and secondary stages can be seen, whereas in continuous system these differences are not always apparent.

Raw materials used in the initial batch have to provide immediate utilizable soluble nutrients as well as longer lasting and less soluble sources. Supplemented raw materials are soluble and rapidly utilized. Suitable carbon sources sucrose, glucose or enzyme hydrolyzed corn syrups can be used. Use of soluble, readily utilizable carbohydrates like glucose can prevent catabolite repression, as the concentrations of the sugar will always be very low.

Addition of oil has the benefit of controlling excessive framing and air hold-up. Oil as triacylglycerol can be lard oil, soy oil, palm oil, peanut oil or rapeseed oil. Other antifoams such as silicone based products or polypropylene glycol can also be used. Antifoam addition should be available on an as – need basis and not simply adding into the start medium due to toxic nature of some antifoams. As protenacious nature of raw materials cause foaming automated feed – back control of antifoaming agent has to be provided. Foam control and minimum air hold-up is important for maximum volumetric output. The final out put expected is 80 – 85%.

The pH of the broth can be controlled to within 0.1 pH units by the addition of acid like sulfuric acid or base ammonia or caustic. Using the cultures own metabolism of sugars can also control the pH. Excess feeding of sugar will produce acetic acid, which lowers pH.

Dissolved O_2 levels are also critical for maintaining the maximum rate of antibiotic production and culture viability. This is the reason why a fine balance has to be established between aeration and the agitation necessary to distribute the O_2 into the liquid phase, and the back pressure in the tank to increase O_2 solubility, the volume expansion of the fermentation broth, and the compounding of several of there effects on the dissolved CO_2 levels. The dissolved oxygen levels has to be maintained higher than 20% saturation at 1.5-2 atmospheres pressure throughout the fermentation, at air flow rates high enough to sweep out as much CO_2 as possible.

By the use of multiple probes for pH and dissolved oxygen; pressure and temperature fermentation process can be monitored. Aseptic nature of the fermentation can also be assessed by microbial examination of samples. All the probes have to be checked for this correct performance.

4.4.1.6.Summary:

Antibiotics are Secondary metabolite products that are mainly produced by many microbial species. Antibiotics have many applications in treatment of tumors, plant pathology, veterinary medicine, food industry; etc .the production can be studied under two headings, physiology and genetics. Physiology of antibiotic production includes the study of Secondary Metabolism and its control, Catabolic Repression, Nitrogen and phosphate repression, Feed back regulation, Effects of precursors, Conditions required for fermentation. Genetics of antibiotic production includes strain improvement, culture maintenance and scale up. Type of culture media used and optimum conditions, plays a major role in antibiotic production.

4.4.1.7.Modal questions

1.what are antibiotics? Write about various applications of antibiotics. And add a note on various types of antibiotics produced by microorganisms

2.Discus in detail about general antibiotic production protocol.

4.4.1.8.References:

1.Industrial microbiology by Casida

2.Atext book of industrial microbiology by wulf crueger et al.2nd edition.

3.Industrial microbiology by Samuel cate Prescott and eecil gardon dunn .

S.T.V.Raghavamma

Lesson 4.4.2

PRODUCTION OF β. LACTUM ANTIBIOTICS

Structure

Objective

Production of B. lactam Antibiotics

4.4.2.1 Introduction

The penicillins and the cephalosporins belong to the most effective of all therapeutic agents for the control of infections diseases.

Fleming in 1929 first described penicillin. It was isolated from surface cultures of *Penicillium notatum* in 1940 by a research group at oxford. Many fungi, particularly Penicillium and Aspergillus species, produce penicillins. Natural penicillins are effective against many gram – positive bacteria.

B-Lactum antibiotics are specific inhibitors of bacterial cell wall synthesis. By combining with penicillin – binding proteins (PBP) of the bacterial cell, inhibit the enzyme activity of this protein causing cell death.

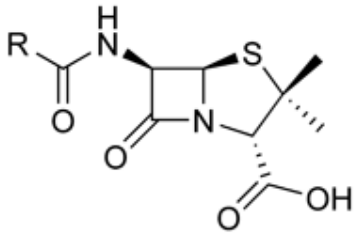
4.4.2.2 Chemical Structure

The basic structure of penicillin is 6 – amino penicillanic acid (6-APA) made of thiozolidine ring with a condensed B-lactum ring. This 6 – APA carries a variable acyl moiety in position 6. If fermentation involves no addition of side – chain precursors the natural penicillins are produced. Biosynthetic penicillins are produced by the controlled addition of side-chain precursors. In commercial scale, only penicillin G, penicillin V and very limited amounts of penicillin O are produced. By chemically or enzymatically splitting penicillin G or penicillin V to form 6-APA and recycling it chemically, semi synthetic penicillins can be produced. Because of broadened action spectrum of semi synthetic penicillins, they are some times compared with third generation cephalosporins.

Common β-lactam antibiotics

Penicillins

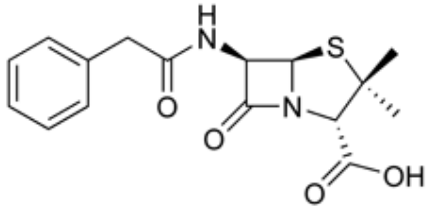
Main article: penicillin



main nucleus of penicillin

Narrow spectrum penicillins

- benzathine penicillin
- benzylpenicillin(penicillin G)
- phenoxymethylpenicillin(penicillin V)
- procaine penicillin



Penicillin G (Benzylpenicillin)

Narrow spectrum penicillinase-resistant penicillins

- methicillin
- dicloxacillin
- flucloxacillin

Moderate spectrum penicillins

- amoxicillin
- ampicillin

Broad spectrum penicillins

- co-amoxiclav (amoxycillin+clavulanic acid)

Extended Spectrum Penicillins

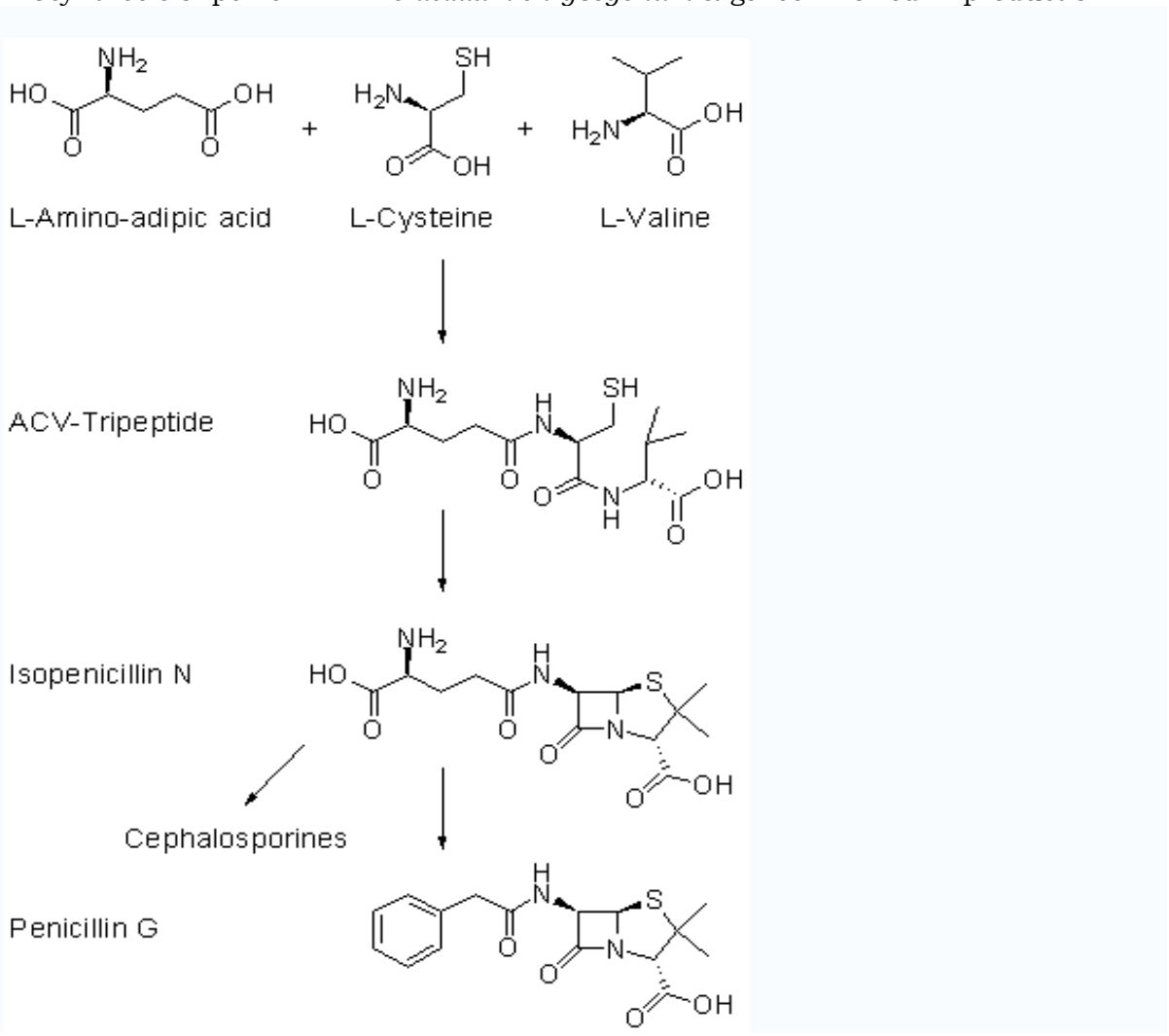
- piperacillin
- ticarcillin
- azlocillin
- carbenicillin

4.4.2.3 Biosynthesis & regulation

L-cysteine and L-valine construct the B-lactaum thiazolidine ring of penicillins. Biosynthesis occurs in a nonribosomal process by means of a dipeptide with L-α-aminoadipic acid (L-α-AAA) and L-cycteine as a breakdown product of cyetathionine. L-valine is subsequently connected via an epimerization reaction, resulting in the

formation of the tripeptide δ (L-α aminoadipyl) cysteinyl-D-valine. Isopenicillin N is the first product of the cyclization, but the biochemical reactions leading to this are unknown. By the exchange of L-α-AAA with activated phenylacetic acid Benzyl penicillin is produced. 6-AAA, which is not an intermediary product of biosynthesis, is excreted in the absence of side chain precursors.

Biosynthesis of penicillin in *Penicillium chrysogenum* & genes involved in production



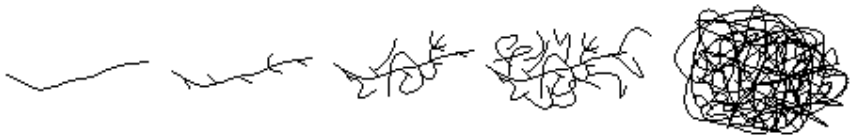
Penicillin biosynthesis is affected by phosphate concentration and show catabolite repression by glucose, in addition to a regulation by concentration of ammonium ion. Because of the glucose repression, penicillin fermentations were originally done only with the slowly metabolizable sugar lactose.

4.4.2.4 Strain development

Filamentous microorganisms (Figure 1) are used commercially for the production of secondary metabolites such as antibiotics.

Fig:penicillin production.

The formation of the target product, the antibiotic, is usually not associated with cell growth. For this reason, it is common practice to grow the microorganisms in a batch culture followed by a fed-batch operation to promote the synthesis of the antibiotic



Yield increase has been the main objective of strain development and other factors effecting fermentation.

Penicillium chrysogenum strain NRRL 1951 was better suited for submerged production than original strain *P.notatum*. Wis Q 176 was adopted by most penicillin manufactures and was used as the original strain for the various commercial strain improvement programs.

Until 1960's the widely used mutagens were X-rays, methyl bis – (β-chloro ethyl) amine (nitrogen mustard) and short – wave ultraviolet radiation. Nitrosoguanidine, alkylating agents, and nitrite are widely used.

The discovery of Para sexual cycle in *P.chrysogeum* provided a means of utilizing genetic recombination for strain development. The highest percentage of strains with increased penicillin production arose from haploid segregants of crosses between mutants of different strain lines. By the use of protoplast fusion technique, 8% increase of yield has been observed.

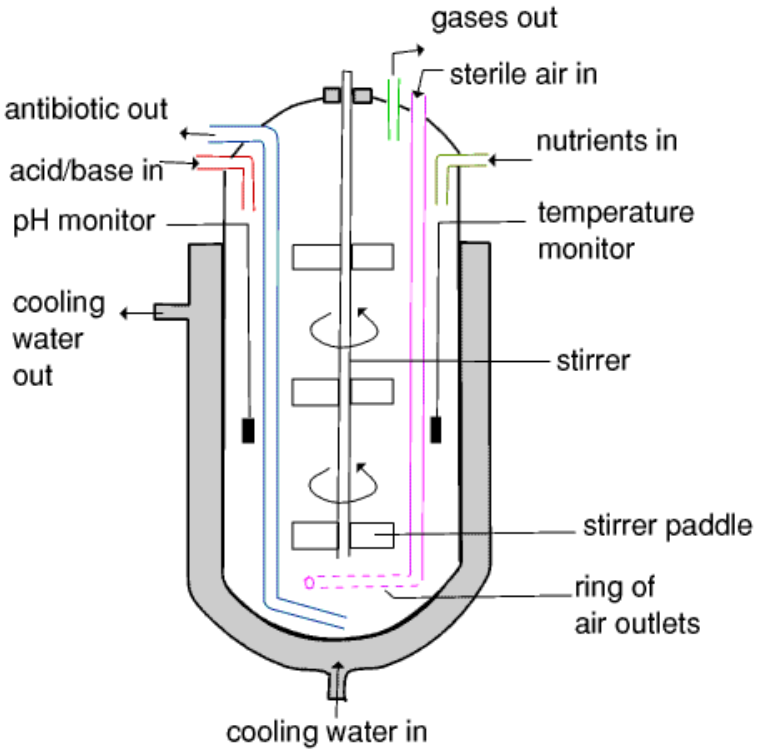
The use of recombinant DNA technology to increase the formation of rate – limiting enzymes through gene manipulation or improved transcription has not been possible in *P.chrysogenum*, due to the absence of precise biosynthetic data and host – vector systems. However, a gene bank was developed and a transformation system has been developed.

4.4.2.5 Production methods

Penicillin fermentation is an aerobic process with a volumetric oxygen absorption rate of 0.4 – 0.8 m M/1min the required aeration rate is between 0.5 – 1.0 vum

depending on type of strain, on the bioreactor, and on the impeller system. Different turbine impellers are used for mixing (120 – 150 rpm).

The type of fermenter used includes waldhof fermenters or Air – lift fermenters. There are basically used for mutants that generate low viscosity. Depending on the strain used the optimal temperature range between 25 – 27^{O}C. Penicillin G & V, are produced using submerged processes.



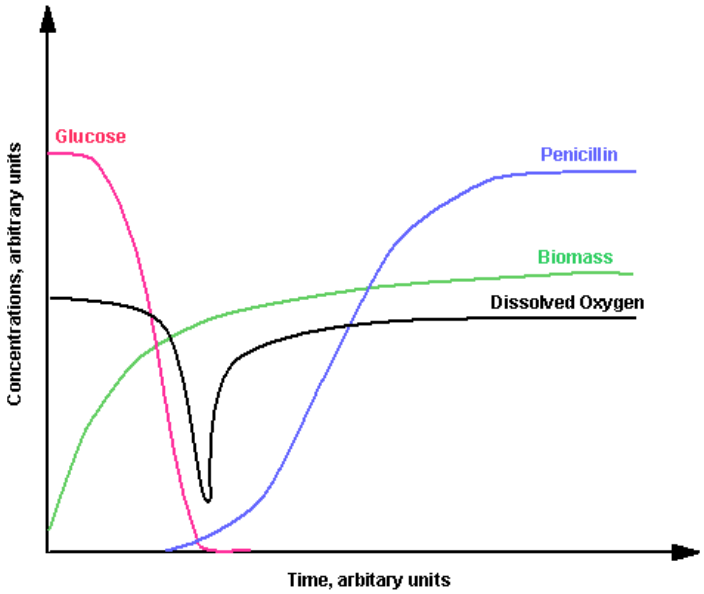
frmenter used in penicillin production

(a) The Inoculum

The starter is in the lyophilized spore form. Because of high variability of strains, maintenance of strains should be carefully done. Spore concentration is 5 x 10^3/mL. Pellet formation is crucial because optimal penicillin formation rate can be achieved when pellets are grown in a look form and not as compact balls.

In typical penicillin fermentation, the growth phase is about 40 hr, with doubling time of 6 hours. During this time greatest part of cell mass is formed. During this phase due to increased viscosity oxygen transfer is hindered. Hence use of mutant strains, which cause less viscosity, has to be used. After growth phase, comes the penicillin production phase. If penicillin production is very high, growth phase is reduced. By feeding with different culture medium components the production phase can be extended to 120 – 180 hours.

Shows time profiles of glucose, penicillin, biomass and oxygen for a typical run.



(b) Medium

In fed – batch culture, depending on strain medium consists of corn steep liquor (4-5% dry weight). It can be replaced with other nitrogen sources, an additional nitrogen source, such as soy meal, yeast extract, or whey.

Carbon sourse like lactose and various buffers. The pH is kept constant at 6.5. Phenyl acetic acid or phenoxy acetic acid is fed continuously as a precursor (0.5 – 0.8% of the total). Glucose or molasses feeding at a rate of 1.0 2.5 Kg $m^{-3}h^{-1}$ with a glucose concentration of 500 $Kg.m^{-3+}$ are also successful. About 65% of the metabolized carbon source is used for maintenance energy, 25% for growth and only 10% for penicillin production. The control of carbon metabolism offers optimization of production process. 25% increase in yield is seen when glucose and acetic acid were added. Critical parameters in fed – batch system are the rate of sugar utilization and oxygen supply.

Continuous penicillin fermentation is difficult due to the instability of the production strain. A "Batch fill & draw" system has been suggested as an alternative. In this procedure, 20-40% of the fermentation contents is drawn off and replaced with fresh nutrients & this is repeated up to 10 times without yield reduction.

4.4.2.6 Recovery

Penicillin is recovered by solvent extraction at an acidic pH at temperatures below $10^{o}C$ to minimize both chemical and enzymatic penicillin breakdown. Solvents used are n-butyl acetate, or methyl isobutyl ketone. After extraction of penicillin into the medium less than 1% remains mycelium – bound. Recovery after separation of mycelium involves two – stage continuous countercurrent extraction of the fermented broth at 0 – $3^{o}C$ & pH 2.5 – 3.0. Depending on the nature of the mycelium the solids can be removed by stirring filters, pre-coated diatomaceous filters or by ultra filtration. Separated mycelium can be treated, dried and used as a soil conditioner or it can be added back to the residual liquid waste for connectional anaerobic /aerobic digestion.

The penicillin rich solvent can be treated with activated carbon to remove pigments and other impurities. The penicillin is then recovered as the potassium or sodium salt by adding potassium or sodium acetate to the solvent. Further, impurities can be removed by washing the recovered salts with a dry solvent such as isopropanol or n-butanol.

Penicillin V is stable to acid and can be precipitated directly from clear filtrates at a pH of 2. Impurities in the penicillin can be removed by dissolution of the acid in organic solvent to allow treatment with activated carbon. Direct precipitation reduces the use of organic solvents, which can have positive cost and environmental impacts.

Technology is being developed to by pass the precipitation of the penicillin salt, and eliminate mother liquor losses, which can produce 6-APA, by the enzyme hydrolysis of penicillin salt using immobilized penicillin amidase system.

4.4.2.7 Cephalosporin production Structure & classification

Cephalosporins are β-lactum antibiotics containing a dihydrothiazine ring with D-α-aminoadipic acid as acyl moiety.

4.4.2.8 Introduction

Any of a group of broad-spectrum derived from species of fungi of the genus Cephalosporium and is related to the penicillins in both structure and mode of action but relatively penicillinase-resistant antibiotics. These antibiotics have low toxicity for the host, considering their broad antibacterial spectrum. They have the active nucleus of beta-lactam ring, which results in a variety of antibacterial and pharmacologic characteristics when modified mainly by substitution at 3 and 7 positions. Their antibacterial activities result from the inhibition of mucopeptide synthesis in the cell wall. They are widely used to treat gonorrhea, meningitis, pneumococcal, staphylococcal and streptococcal infections. The cephalosporin class of antibiotics is usually divided into generations by their antimicrobial properties. Three generations of cephalosporins are recognized and the fourth has been grouped. Each newer generation of cephalosporins has broader range of activity against gram-negative organisms but a narrower range of activity against gram-positive organisms than the preceding generation. The newer agents have much longer half-lives resulting in the decrease of dosing frequency. Accordingly, the third-generation cephalosporins can penetrate into tissues well, and thus antibiotic levels are good in various body fluids.

Cephalosporins are made from Cephalosporins C a fermented product of *Acremonium chrysogenum* which after extraction is hydrolyzed, either enzymatically or chemically, to the active nucleus, 7-amino Cephalosporinic acid (7-ACA), which serves as substrate for the chemical synthesis of injectable, semi synthetic Cephalosporins.

Other fungi like Emericellopis & Paecilomyces also produce the Cephalosporin antibiotics. Cephamycins (7-methoxy – cephalosporins) were produced from various streptamyces species like *S.lipmanii, S.Clavuligerus*, or *Nocardia Lactumdurans*.

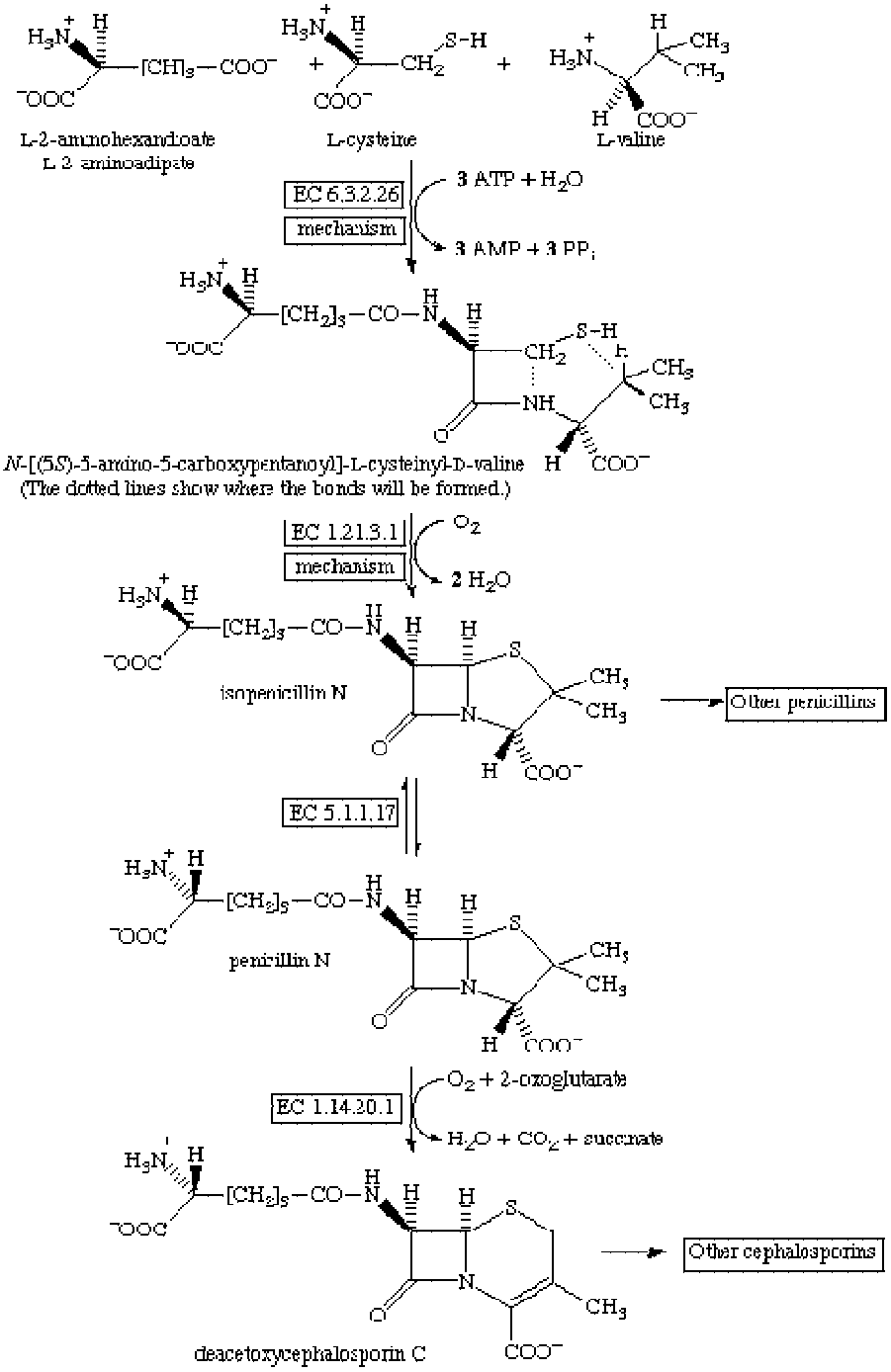
4.4.2.9 Biosynthesis & Regulation

Like Benzyl penicillins, δ – (α-amino adipyl) L-cyeteinyl –D- valine converts to isopenicillin N, in the 1st stage. In next stage L-α-AAA side chain is transformed to D-form by the action of racemase to produce penicillin N. After ring expansion to de acetoxy cephalosporin C by expandase hydroxylation via a dioxygenase to de acetyl – cephalosporin C occurs. The acetylation of cephalosporin C by an acetyl – CoA dependent transferase is the end point of biosynthesis in fungi. In streptamyces, further transformation occurs. Cephalosporin C is converted into 7-methoxy cephalosporin or cephamycin C by two-step reaction with molecular oxygen and S-adenosyl methionine.

Regulation of cephalosporin production is by phosphate regulation, nitrogen regulation and carbohydrate catabolite regulation. Rapidly metabolized carbon sources, such as glucose, maltose, or glycerol reduce the formation of cephalosporin. Repression of expandase also has significant effect. The addition of lysine in low concentrations promotes cephalosporin production. Methionine stimulates cephalosporin synthesis in C*ephalosporium acremonium*, but not in the streptomycetes.

In the biosynthesis of cephalosporins; by streptomyces, α-AAA is not the precursor of L-Lysine, because streptomyces synthesizes lysine via di aminopimellic acid pathway.

Fig: Di aminopimellic acid pathway.



Strain development has been conducted with original strain *C.acremonium* (M 149137]. by mutations, selection and para sexual techniques. The gene for isopenicillin N synthetase from *C.acremonium* has been cloned in E.coli & effective transformation system has been developed.

4.4.2.10 Production

The fermentation of cephalosporins is similar to that of penicillin. In the growth phase a high aeration rate is necessary, but during production phase (48 – 160 hr) oxygen consumption decreases. Complex media with corn steep liquor, meat meal, sucrose, glucose and ammonium acetate are used. Methionine is used both as a source of sulphur and as an inducer of morphological changes. Fermentations are carried out at as fed – batch processes with semi continuous addition of nutrients at pH 6.0 – 7.0. The temperature used ranges from 24 – 28^{O}C.

4.4.2.11 Recovery

Cephalosporin C is recovered from broth filtrates by a variety of hydrophobic and ion exchange resins. Cephalosporin C can be separated from related intermediates by the column chromatography. The rich fractions are either treated with zinc acetate to precipitate the low solubility zinc salt or with sodium or potassium acetate followed by a water miscible solvent to precipitate the salt complex.

4.4.2.12 Summary

β. Lactum antibiotics are specific inhibitors of bacterial cell wall, which includes penicillins and cephalosporins. For the production of these antibiotics various strategies are followed. They include the knowledge of biosynthetic pathways, strain improvement, type of inoculum, medium used and recovery procedure. The flow sheet for the production can be used for all the production methods.

4.4.2.13 Model questions

1.Discuss in detail about various steps involved in the production of penicillins.
2.what are cephalosporins? Write in detail about their production procedure.

4.4.2.14 References

1.Industrial microbiology by Casida
2.Atext book of industrial microbiology by wulf crueger et al.2nd edition.
3.Industrial microbiology by Samuel cate Prescott and eecil gardon dunn

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Lesson 4.4.3

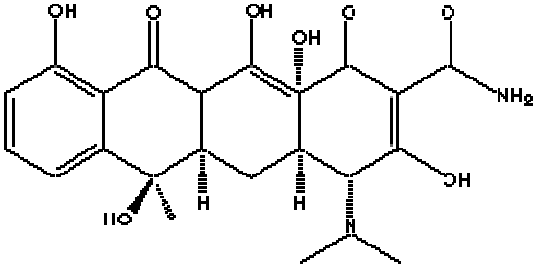
TETRACYCLINE PRODUCTION

Structure

Objective:
The main objective of this lesson is to make familiar with the steps involved in the production of tetracycline.

4.4.3.1 Introduction:

Tetracycline is a member of the tetracycline family of broad antimicrobial action. It is chiefly used in treating infections caused by streptococci, staphylococci, Gram-negative bacilli, rickettsiae, and certain protozoans and viruses. Tetracycline can be produced biosynthetically by fermentation with a strain of Streptomyces aureofaciens (or certain other species) or chemically by hydrogenolysis of chlortetracycline. Tetracyclines inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit. tetracyclines act as a inhibitor of a growth (bacteriostatic) rather than killer of the infectious agent (bacteriocidal) and is only effective against multiplying microorganisms.



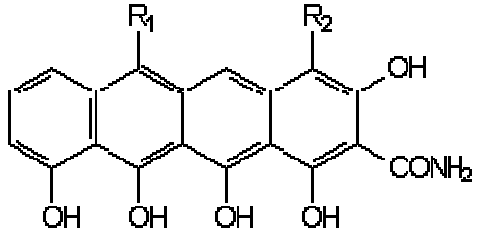
Tetracycline antibiotics include:

- Chlortetracycline
- Demeclocycline Hydrochloride
- Demethylchlortetracycline
- Dihydrostreptomycin Sesquisulfate
- Doxycycline
- Duramycin
- Meclocycline Sulfosalicylate
- Methacycline Hydrochloride
- Minocycline
- Neomycin
- Oxytetracycline
- Streptomycin

- Tetracycline)
- Vancomycin

4.4.3.2 Biosynthesis

A biosynthesis of tetracycline, particularly in its latter stages, has been studied with the use of mutant cultures. It has been established that the so-called **pretetronids**:



are intermediates, convertable by non-mutated *Streptomyces aureofaciens* into tetracyclines . Cosynthesis, by use of blocked mutants, has demonstrated that 4-hydroxy-6-methylpretetranid (R_1 = Me, R_2 = OH) is a transformable intermediate and the precursor to the 6-methyltetracyclines. The complete biosynthesis is as follows

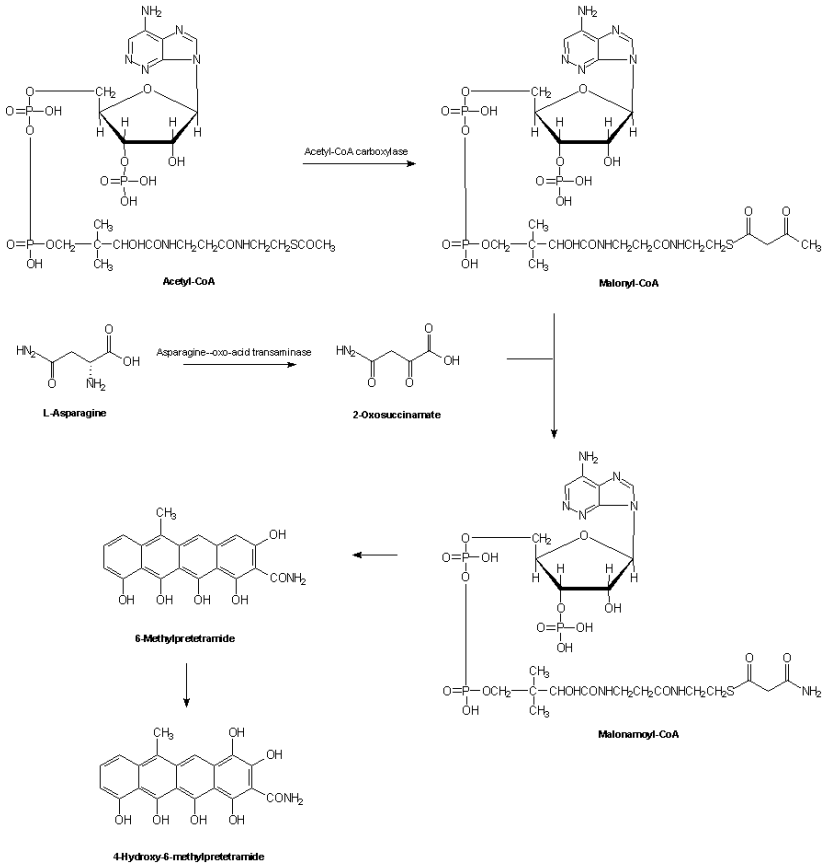


fig: the biosynthetic pathway of tetracyclins

4.4.3.3 Production procedure:

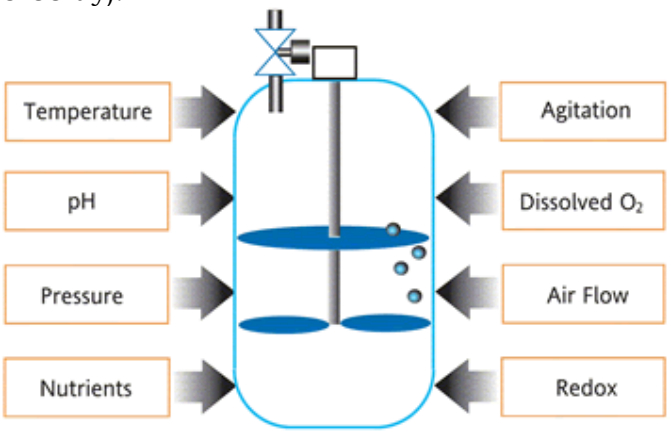
Microorganisms involved in production.

Streptomycin aureofaciens and *streptomycin rimosus* are used for the production of natural tetracycline by commercial fermentation. The former produces a mixture of chlortetracycline and tetracycline. The propagation of which can be manipulated by including chloride ion or bromide ion in the fermentation. Biosynthesis of tetracycline and its chlorinated derivatives by *S.aureofaciens* takes place in parallel. The chloro group is added mid way through the biosynthesis .it is therefore impossible to produce chlortetracycline exclusively as some of the biosynthetic intermediates avade the the chlorinated step and eventually are biosynthesized to tetracycline.

Medium composition:

A new composition of the nutrient medium for cultivation of the tetracycline-producing organism was developed with the fermentative hydrolysate of the tetracycline production mycelial waste as a source of nitrogen: 0.02 to 0.04 g/l by amino nitrogen. The use of the medium made it possible to increase the tetracycline yield by 5 to 25 per cent, to exclude cornsteep liquor from the medium composition, to provide a more efficient recovery of the waste and to significantly decrease the environment pollution.

For saving energy in antibiotic production and reducing the amount of agricultural wastes, solid state fermentation was also used to produce tetracycline with sweet potato residue by Streptomyces viridifaciens ATCC 11989. It was found that the optimal media for tetracycline production were sweet potato residue 100 g, organic nitrogen (rice bran, wheat bran, or peanut meal) 20 g, (NH4)2SO4 2.4 g, KH2PO4 0.4 g, CaCO3 1.8 g, NaCl 0.6 g, MgCl2 0.8 g, soluble starch 10 g, methionine 0.2 g, histidine 0.8 g, and monosodium glutamate 1.6 g with initial moisture content 68-72%, and initial pH 5.8-6.0. Each gram of dry weight substrate was inoculated with 1.0 × 108 conidia and incubated at 26°C for 5-7 days, producing 4720 g of total tetracycline equivalent potency. at 26°C with the initial moisture content 68%, the conidia in solid media germinates on the second day, mycelia will grew abundantly on the third day and reaches a stationary phase on the sixth day. The antibiotic production was consistent with the morphogenesis of S. viridifaciens: activity could be detected on the third day, had the maximal potency on the sixth day, and decreased slightly on the tenth day. (11-3-88 tly).



Various installations required for a fermenter.

4.4.3.4 Summary

Which is effective against gram-negative bacteria. For the production of these antibiotics various strategies are followed. They include the knowledge of biosynthetic pathways, strain improvement, type of inoculums, medium used and recovery procedure.

4.4.3.5 Model questions

1. Discuss in detail about various steps involved in the production of tetracycline?

4.4.3.6 References

1.Industrial microbiology by Casida
2.Atext book of industrial microbiology by wulf crueger et al.2nd edition.
3.Industrial microbiology by Samuel cate Prescott and eecil gardon dunn

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Lesson 4.4.4

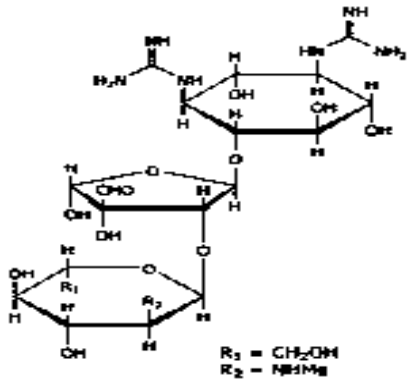
STREPTOMYCIN PRODUCTION

Structure
Objective
4.4.4.1 Introduction
4.4.4.2 Biosynthesis and regulation
4.4.4.3 Production
4.4.4.4 Recovery
4.4.4.5 Summary
4.4.4.6 Modal questions
4.4.4.7 Reference

Objective:
The main objective of this lesson is to make familiar about various steps involved in the production of streptomycin.

4.4.4.1 Introduction

Streptomycin is an amino glycoside antibiotic consisting of aminocyclohexanol moiety i.e .it is an streptamine derivative. Oraganism involved in producing streptomycin is S*treptomyes griseu*s.



Streptomycin

It is a broad – spectrum antibiotic that is primarily used to treat tuberculosis. All amino glycoside antibiotics are active against gram-negative bacteria. Dihydrostreptomycin, which is also a streptidine derivative, is produced by *Streptomyces humidu*s. (It can also be produced via chemical reduction of streptomycin).

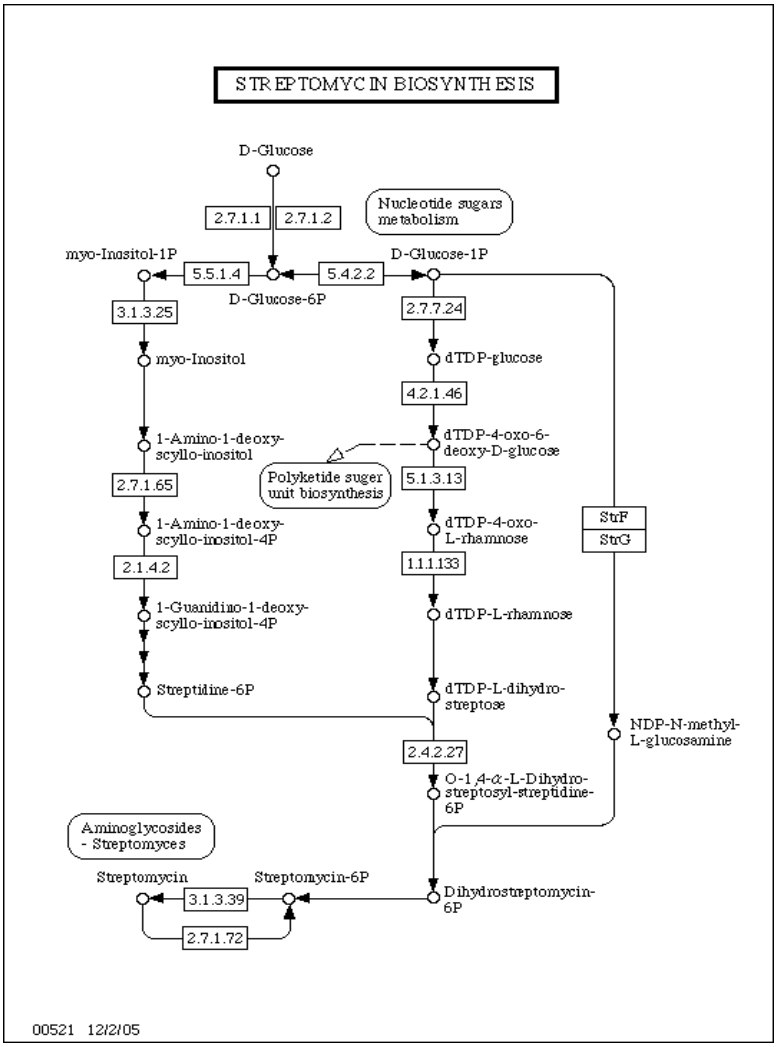
4.4.4.2 Biosynthesis and Regulation

All the amino glycoside antibiotics are glucose as a precursor. One of the principal components of streptomycin, streptidine, is synthesized from glucose – 6 –

phosphate via myo – inositol. Series of enzymatic reactions involving oxidation, amination, phosphorylation, carbamidinylation and dephosphorylation are involved in the formation of streptidine 6-P. the combination of 3-subunits is streptidine, streptose and N-methyl – L-glucosamine involves two steps. Removal of phosphate moiety in the last step gives biologically active streptomycin from the biologically inactive streptomycin phosphate.

Streptamycin – producing strains of *S.griseus* and *S. bikiniensis* show regulation via factor A, 2(s) – iso – capryloyl – 3(S) hydroxymethyl – γ – butyrolactone. This controls streptomycin biosynthesis, streptomycin resistance and sporulation.

Streptomycin yields have been increased from 100 – 200 μg/ml to about 15 mg/ml by strain improvement technique. Mutation and selection are the principal methods, which have been used in strain development. Cell fusion and recombinant DNA methods, protoplast fusion methods were also used.



2.7.1.1 hexokinase; 2.7.1.2.glucokinase;
5.5.1.4 inositol-3-phosphate synthase;

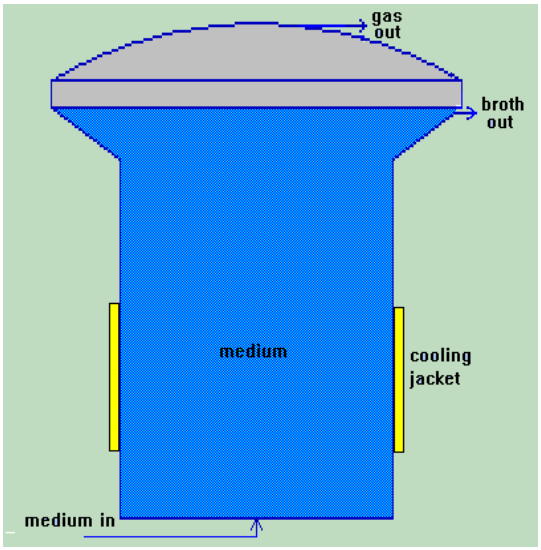
5.4.2.2 phosphoglucomutase;
3.1.3.25 inositol-phosphate phosphatase;
2.7.1.65 scyllo-inosamine 4-kinase
2.1.4.2 scyllo-inosamine-4-phosphate amidinotransferase
2.7.7.24 glucose-1-phosphate thymidylyltransferase
4.2.1.46 dTDP-glucose 4,6-dehydratase;
5.1.3.13 dTDP-4-dehydrorhamnose 3,5-epimerase
1.1.1.133 dTDP-4-dehydrorhamnose reductase;
2.4.2.27 dTDP-dihydrostreptose-streptidine-6-phosphate
3.1.3.39 streptomycin-6-phosphatase;
2.7.1.72 streptomycin 6-kinase

4.4.4.3 Production

Amino glycoside processes are typical secondary metabolite fermentations with tropho and idiophases.

Optimal oxygen supply is at rate of 0.5 to 1.0 uvm. Temperature is maintained between 28 – 30^{o}C with the pH in the neutral range. The length of fermentation is between 4 – 7 days, depending on the strain.

Media contains glucose in combination with starch or dextrin as carbon source. Starch is commonly used in the starting batch as streptomyces have poor amylase activity and the enzymatic release of glucose is slow and rate limiting. Alternatively, corn syrup can be fed at pre-determined rates. Soy meal is a source for nitrogen because of its slow catabolism. The necessary phosphate is obtained from organic sources like phytic acid. NaCl 1-3 g/l must be added to the streptomycin fermentation. Synthetic media for streptomycin fermentation were studied to determine which media gave highest yields of streptomycin. Substitution of amino acids for casein was examined. Of 17 amino acids tested, best results were obtained with sodium aspartate. Substitution of ammonium salts was tried, and an excellent streptomycin yield was obtained with a medium containing ammonium citrate.



Continuous fermentation system.

A pilot plant unit for continuous cultivation of hyphae forming micro-organisms, providing for a uniform flow velocity in fermentations on suspension media with a massive growth of mycelium, is used. For the maintenance of aseptic conditions, over-pressre of air is maintained in the whole equipment. Three-stage fermentation proved the most advantageous. The first stage serves for multiplication of the inoculums; the second and third for the formation of the antibiotic. The system was maintained for 300-400 h with yields amounting to 2,000-2,500 u. of streptomycin/ml without any signs of contamination or degeneration of the growing culture.

4.4.4.4 Recovery

Most of the amino glycoside antibiotics are excreted and are present in the culture supernatant. There are then removed by adsorption to ion exchange columns.

4.4.4.5 Summary

Streptomycin is an aminoglycoside antibiotic, which is effective against gram-negative bacteria. . Oraganism involved in producing streptomycin is *Streptomyes griseus*. For the production of these antibiotics various strategies are followed. They include the knowledge of biosynthetic pathways, strain improvement, type of inoculum, medium used and recovery procedure. The flow sheet for the production can be used for all the production methods.

4.4.4.6 Modal questions

1. Write about aminoglycoside antibiotics. Discuss in detail about various steps involved in the production of streptomycin.

4.4.4.7 References

1.Industrial microbiology by Casida
2.Atext book of industrial microbiology by wulf crueger et al.2nd edition.
3.Industrial microbiology by Samuel cate Prescott and eecil gardon dunn

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Lesson 4.5.1

PRODUCTION OF INSULIN, SOMATOTROPHIN AND VACCINES THROUGH GENETICALLY ENGINEERED MICROBES

Structure

4.5.1.1 Production of somatotrophin (human growth hormone

Somatrotrophin is required for the growth of the skeleton, and is secreted by the anterior lobe of the pituitary. Only the Growth hormone is effective for treatment of growth disorders. The extraction of hGH by classical methods is very time consuming and expensive. It required the pituitary glands from about 80 human bodies to produce a supply of hGH for one year's treatment. Long-term treatment for dwarfism lasts for a minimum of 8-10 years.

Synthesis of hGH by genetic engineering is by a combination of artificial gene synthesis & cDNA cloning. The mRNA for hGH present in the pituitary consists of 26 codons specifying a signal peptide and 191 codons for the amino acids of the hormone. If the hGH gene is inserted into in bacterial cells, it codes for the entire polypeptide, including the 26 aminoacid signal region. Such a protein is non-functional if weled for treatment.

If the signal sequence is cut off, the rest of the DNA sequence would code for the active hormone polypeptide. However, no restriction enzyme is known that can exactly cut off the Dna between signal peptide and the active hormone coding region. The restriction enzyme Hae III cuts off 50 bases from cDNA. This region includes the coding region for the unwanted 26 amino acids of the hormone. The missing 24 aminoacid coding region is artificially synthesized and joined to the truncated hGH gene.

To this complete gene is added a bacterial control gene and the resulting sequence is inserted into a plasmid. The recombinant plasmid is cloned into bacteria, which can synthesize the complete hGH without the signal sequence. The first 24 of 191 amino acids of the expressed gene are synthesized chemically, while amino acids 25-191 are derived from a CDNA copy of the hGH mRNA.

4.5.1.2 Production of insulin

Since Banting and Best discovered the hormone, insulin in 1921. diabetic patients, whose elevated sugar levels (see fig. 1) are due to impaired insulin production, have been treated with insulin derived from the pancreas glands of abattoir animals. The hormone, produced and secreted by the beta cells of the pancreas' islets of Langerhans, regulates the use and storage of food, particularly carbohydrates.

Although bovine and porcine insulin are similar to human insulin, their composition is slightly different. Consequently, a number of patients' immune systems produce antibodies against it, neutralising its actions and resulting in inflammatory responses at injection sites. Added to these adverse effects of bovine and porcine insulin, were fears of long term complications ensuing from the regular injection of a foreign substance, as well as a projected decline in the production of animal derived insulin. These factors led researchers to consider synthesising Humulin by inserting the insulin gene into a suitable vector, the E. coli bacterial cell, to produce an insulin that is chemically identical to its naturally produced counterpart. This has been achieved using Recombinant DNA technology. This method (see fig. 2) is a more reliable and sustainable method than extracting and purifying the abattoir by-product.

The structure of insulin.

Chemically, insulin is a small, simple protein. It consists of 51 amino acid, 30 of which constitute one polypeptide chain, and 21 of which comprise a second chain. The two chains (fig. 1) are linked by a disulfide bond.

Figure 1

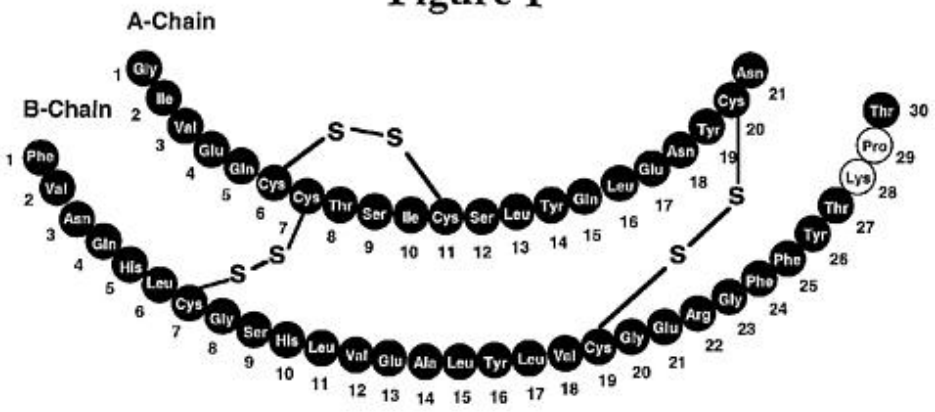
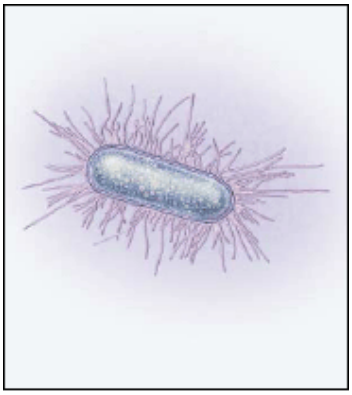


Fig 2: Structure of Insulin

The Vector (Gram negative E. coli).

A weakened strain of the common bacterium, Escherrichia coli (E. coli) (fig. 2), an inhabitant of the human digestive tract, is the 'factory' used in the genetic engineering of insulin



E. coli

Fig 2: The insulin is introduced into an E. coli cell such as this
When the bacterium reproduces, the insulin gene is replicated along with the plasmid, a circular section of DNA (fig. 3). E. coli produces enzymes that rapidly degrade foreign proteins such as insulin. By using mutant strains that lack these enzymes, the problem is avoided.

Fig 3 : Plasmid

Inside the genetic engineer's toolbox.
Restriction enzymes, naturally produced by bacteria, act like biological scalpels(fig.4), only recognising particular stretches of nucleotides, such as the one that codes for insulin.

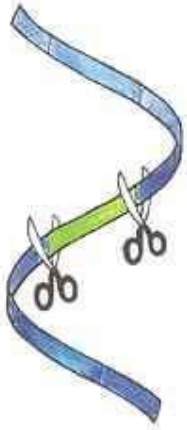


Fig 4

This makes it possible to sever certain nitrogen base pairs and remove the section of insulin coding DNA from one organism's chromosome so that it can manufacture insulin (fig. 5). DNA ligase is an enzyme which serves as a genetic glue, welding the sticky ends of exposed nucleotides together.

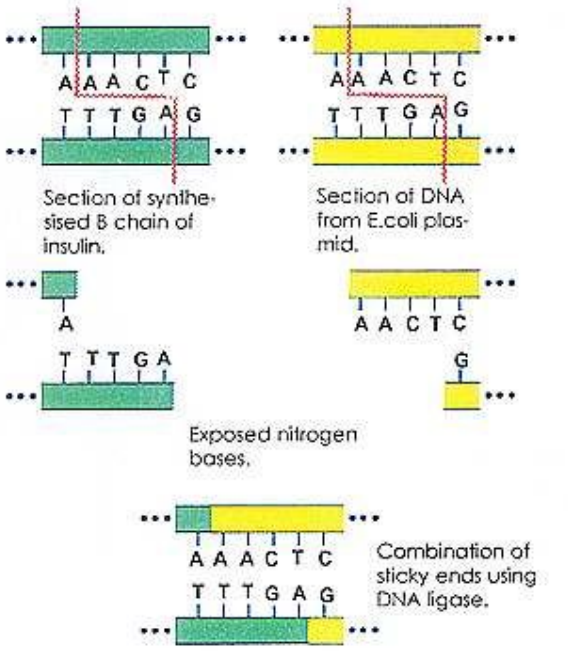


Fig.5
Source: Watson, J.D., Gilman, M., Witkovski., Zoller, M. - Recombinant DNA, pg 78.

Manufacturing Humulin.
The first step is to chemically synthesise the DNA chains that carry the specific nucleotide sequences characterising the A and B polypeptide chains of insulin.

The required DNA sequence can be determined because the amino acid compositions of both chains have been charted. Sixty three nucleotides are required for synthesising the A chain and ninety for the B chain, plus a codon at the end of each chain,signalling the termination of protein synthesis. An anti-codon, incorporating the amino acid, methionine, is then placed at the beginning of each chain which allows the removal of the insulin protein from the bacterial cell's amino acids. The synthetic A and B chain 'genes' (see fig. 6) are then separately inserted into the gene for a bacterial enzyme, B-galactosidase, which is carried in the vector's plasmid. At this stage, it is crucial to ensure that the codons of the synthetic gene are compatible with those of the B-galactosidase.

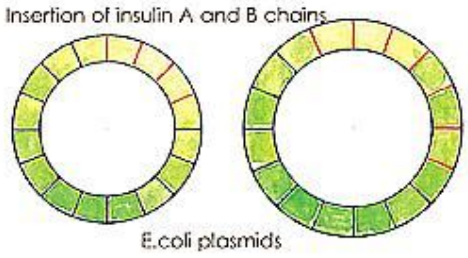


Fig.6
Source: Watson, J.D., Gilman, M.,Witkovski., Zoller, M. - Recombinant DNA, pg 456.

The recombinant plasmids are then introduced into E. coli cells. Practical use of Recombinant DNA technology in the synthesis of human insulin requires millions of copies of the bacteria whose plasmid has been combined with the insulin gene in order to yield insulin. The insulin gene is expressed as it replicates with the B-galactosidase in the cell undergoing mitosis (see fig. 7).

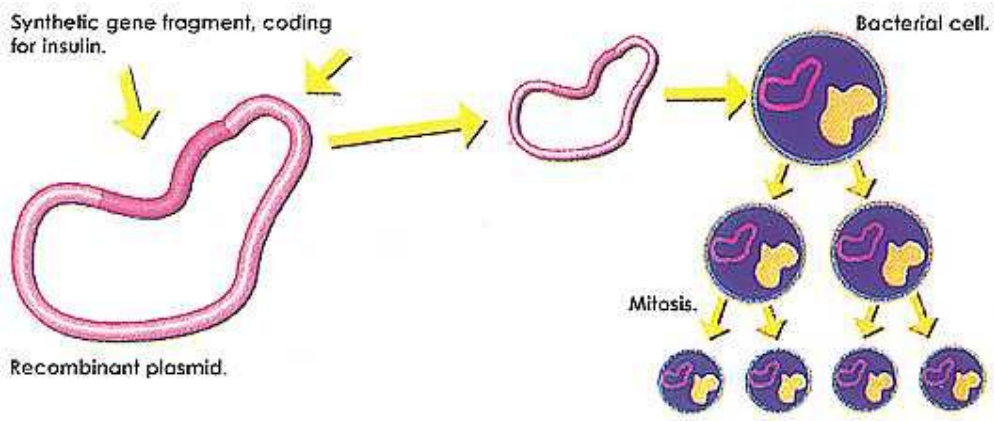


Fig.7
The process of mitosis. Source: Novo-Nordisk promotional brochure, pg 11.

The protein which is formed, consists partly of B-galactosidase, joined to either the A or B chain of insulin (see fig.8). The A and B chains are then extracted from the B-galactosidase fragment and purified.

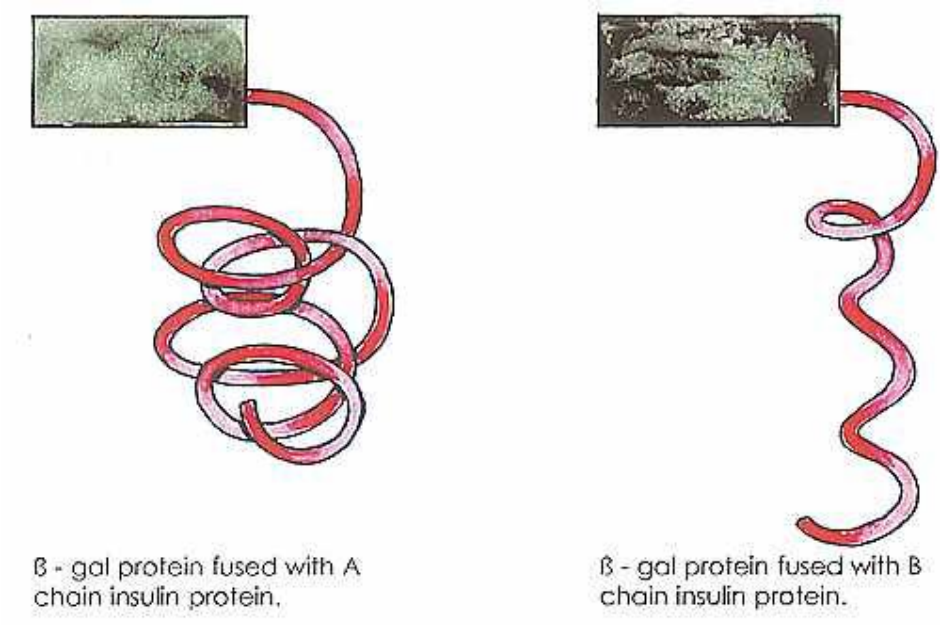


Fig. 8
Source: Watson, J.D., Gilman, M., Witkovski, J., Zoller, M. - Recombinant DNA, pg 456.

The two chains are mixed and reconnected in a reaction that forms the disulfide cross bridges, resulting in pure Humulin - synthetic human insulin (see fig. 9).

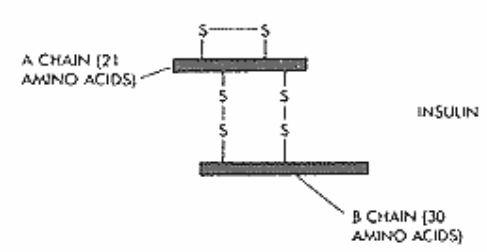


Fig. 9
Human insulin molecule. Source: Source: Watson, J.D., Gilman, M., Witkovski, J., Zoller, M. - Recombinant DNA, pg 456.

Biological implications of genetically engineered Recombinant human insulin.
Human insulin is the only animal protein to have been made in bacteria in such a way that its structure is absolutely identical to that of the natural molecule. This reduces the possibility of complications resulting from antibody production. In chemical and pharmacological studies, commercially available Recombinant DNA human insulin has proven indistinguishable from pancreatic human insulin. Initially the major difficulty encountered was the contamination of the final product by the host cells, increasing the risk of contamination in the fermentation broth. This danger was eradicated by the

introduction of purification processes. When the final insulin product is subjected to a battery of tests, including the finest radio-immuno assay techniques, no impurities can be detected. The entire procedure is now performed using yeast cells as a growth medium, as they secrete an almost complete human insulin molecule with perfect three dimensional structure. This minimises the need for complex and costly purification procedures.

The issue of hypoglycaemic complications in the administration of human insulin.
Since porcine insulin was phased out, and the majority of insulin dependent patients are now treated with genetically engineered recombinant human insulin, doctors and patients have become concerned about the increase in the number of hypoglycaemic episodes experienced. Although hypoglycaemia can be expected occasionally with any type of insulin, some people with diabetes claim that they are less cognisant of attacks of hypoglycaemia since switching from animal derived insulin to Recombinant DNA human insulin. In a British study, published in the 'Lancet", hypoglycaemia was induced in patients using either pork or human insulin, The researchers found "no significant difference in the frequency of signs of hypoglycaemia between users of the two different types of insulin."
An anecdotal report from a British patient who had been insulin dependent for thirty years, stated that she began experiencing recurring, unheralded hypoglycaemia only after substituting Recombinant DNA human insulin for animal derived insulin. After switching back to pork insulin to ease her mind, she hadn't experienced any unannounced hypoglycaemia. Eli Lilly and Co., a manufacturer of human insulin, noted that a third of people with diabetes, who have been insulin dependent for over ten years, "lose their hypoglycaemic warning signals, regardless of the type of insulin they are taking."
Dr Simon P. Wolff of the University College of London said in an issue of Nature , "As far as I can make out, there's no fault (with the human insulin)." He concluded, "I do think we need to have a study to examine the possible risk."

Although the production of human insulin is unarguable welcomed by the majority of insulin dependent patients, the existence of a minority of diabetics who are unhappy with the product cannot be ignored. Although not a new drug, the insulin derived from this new method of production must continue to be studied and evaluated, to ensure that all its users have the opportunity to enjoy a complication free existence.

4.5.1.3 Production of vaccines

Vaccines are antigenic preparations that on injection into the blood stream stimulate the immune system to synthesize antibodies that protect the body against subsequent infection.

i. Foot-and-mouth disease virus:

Genes for the proteins VP1 and VP3 of the food-and-mouth disease virus have been expressed, and their proteins obtained only VP3 is useful as vaccine. The gene coding for VP3 is identified and cloned into an expression vector. The recombinant DNA method of producing virus is preferred to the heating method, as inactivation is not 100% with heating.

ii. Recombinant vaccina viruses

The use of the vaccine virus as carries agent in rDNA technology has made vaccine production easies. The vaccinic virus has been used because of its large genome which can accommodate about 25,000 extra nucleotides, and because it is non-infections.

Vaccines are prepared by cloning genes for viral surface antigen that elicit a strong immune response. Viral pathogens have surface glycoproteins in their coat. These function as antigens and elicit the production of antibodies in the host.

The hepatitis B major surface antigen gene has been ligated into the vaccinia genome under the control of vaccinia promoter. Vaccinia particles containing the recombinant vaccinia genome were injected into the blood stream. New vaccinia particles were formed, and also the hepatitis B major surface antigen protein. This stimulates the immune system to synthesize antibodies against both vaccinia & hepatitis B. Therefore, immunity against both small pox & hepatitis results.

HEPATITIS B VIRUS VACCINE IS PRODUCED IN YEAST BY EXPRESSION OF A VIRAL SURFACE ANTIGEN

One of the successes of modern medicine is the development and implementation of vaccines against infectious diseases. Prior to the advent of recombinant DNA technology, two types of vaccines were used. Inactivated vaccines are chemically killed; derivatives; of he actual infectious agent. Attenuated vaccines are live viruses or bacteria altered so that they no longer multiply in the inoculated organism. Both types of vaccines work by presenting surface proteins (antigens) to B and T lymphocytes, which become primed to respond rapidly should the organism actually become infected, usually destroying the infectious agent before any damage is done. However, these; types of vaccines are protentially dangerous because they can be contaminated with infectious organisms. For example, a; small number of children each year contract polio from their polio vaccinations. Thus, one of the most promising applications of recombinant DNA technology is the production of subunit vaccines, consisting; solely of the surface protein to which the immune system responds. With a subunit vaccine, there is no risk of infection.

The first successful subunit vaccine was produced for hepatitis B virus (HBV), which infects the liver; and causes liver damage and, in some cases, cancer. The virus particle is coated with a surface antigen, BhsAg, and infected patients carry large aggregates of this protein in their blood. Early experiments; suggested that these aggregates would make a potent vaccine, but how could they be produced in quantities sufficient to vaccinate large populations against ;BHV? With the cloning of the HBV genome, the possibility of a subunit vaccine could be explored. Initial attempts to produce the BhsAg protein in E.coli failed, so researchers turned to yeast. The BhsAg gene was inserted into a high-copy yeast expression vector (Fig.13-3, page No.459) and engineered, in this case, so that it would not be secreted (Figure23-1 page No.459). Yeast transformed with this plasmid produced large quantities of the viral protein (about 1-2 present of the total yeast protein). By growing the yeast in large fermentors, it was possible to produce 50-100 mg of the protein per liter of culture. This recombinant protein closely resembled the natural viral protein; it even formed aggregates with properties similar to those of the immunogenic aggregates found in

infected patients. The yeast protein is now used commercially to vaccinate people against HBV infection.

Vaccines against many human and animal pathogens are currently in various stages of development. Recombinant DNA technology has provided a safe means to work with and to inoculate children and adults with only noninfectious parts of infectious agents.

4.5.1.6 Summary

Recombinant DNA technology has made a revolutionary impact in the area of healthcare by enabling mass production of safe, pure and more effective versions of various biochemicals used as therapeutics. It has helped in the production of new therapeutics as well as safer and/or effective versions of conventionally produced therapeutics. Further, since the recombinant therapeutics are identical to human proteins they do not induce unwanted immunological responses and are free from risk of infection as is commonly observed in case of similar products isolated from non-human sources.

Growth hormone is secreted by the anterior pituitary gland and exerts many effects on the human body. The effected target tissues of GH may include bone, immune cells, skeletal muscle, fat cells, and liver cells. An important function of GH is to stimulate growth in children who suffering with GH deficiency. The development of a biosynthetic replication of human growth hormone took place in 1985 , Synthetic Recombinant GH (rhGH) is originally used to help children with insufficient hormone levels for reaching their full height. However, it is an accessible item on the black market today. It is genuinely accepted that bodybuilders were the first amongst athletes to use rhGH to increase lypolysis. Since then, athletes who involve in track and field, weightlifting, and wrestling which require greater degrees of strength, power, and speed have pervasively used rhGH.

Hepatitis A is an acute, usually self-limiting disease of the liver caused by hepatitis A virus (HAV). HAV is transmitted from person to person, primarily by the faecal-oral route. The incidence of hepatitis A is closely related to socioeconomic development, and sero-epidemiological studies show that prevalence of anti-HAV antibodies in the general population varies from 15% to close to 100% in different parts of the world. An estimated 1.5 million clinical cases of hepatitis A occur each year. In young children HAV infection is usually asymptomatic whereas symptomatic disease occurs more commonly among adults. Infection with HAV induces lifelong immunity. hepatitis A usually occurs as single cases among persons in high-risk groups or as outbreaks involving a small number of persons. In areas of high endemicity most persons are infected with HAV without symptoms during childhood. This explains why clinical hepatitis A is uncommon. In countries of low and intermediate disease endemicity, adult disease is seen more often. Hepatitis A may represent a substantial medical and economic burden. Currently, four inactivated vaccines against HAV are internationally available. All four vaccines are safe and effective, with long-lasting protection. None of the vaccines are licensed for children less than one year of age.

Recombinant human insulin first entered clinical trials in humans in 1980. At that time, the A and B chains of the insulin molecule were produced separately and then

combined by chemical techniques. Since 1986, a different recombinant process has been used. The human genetic coding for proinsulin is inserted into Escherichia coli cells, which are then grown by fermentation to produce proinsulin. The connecting peptide is cleaved enzymatically from proinsulin to produce human insulin. Studies indicate that there are no important differences between pork insulin and human insulin in terms of therapeutic efficacy and disposition after intravenous administration. Recombinant human insulin has a faster onset of action and lower immunogenicity than pork or beef insulin. Diabetic patients may have an improvement in glucose concentrations when their therapy is switched from animal-source insulin to human insulin. Such a change usually requires a dosage adjustment, which must be determined by a physician. Pharmacists are responsible for educating patients concerning all insulin products and for preventing patients from interchanging insulin products. The availability of human insulin as the first pharmaceutical product manufactured through recombinant DNA technology, however, has had little effect on the pharmacist's role in the care of such patients. The production of human insulin through recombinant DNA technology represents an important advance in the treatment of patients with diabetes.

4.5.1.7 Model Questions

1. How is Recombinant insulin produced through genetically modified Microbes?
2. What is the importance of growth Hormone? How is it produced?
3. What is vaccine how is Recombinant Hepatitis B vaccine produced?

4.5.1.8 References

1. Recombinant DNA by Watson
2. Principles of Gene Manipulation By Old and Primrose

P. Sudhakar

Lesson 4.5.2

TRANSGENIC PLANTS

4.5.2.1 Objective : The objective of the lesson is to explain the basic methodology and production of Transgenic plants.

4.5.2.2 Introduction:

Genetic manipulation of plants has been practiced for many hundreds of years with great success by plant breeders & plant breeding has become a very Sophisticated branch of applied genetics. Breeders have developed elegant schemes for crossing plants to introduce and maintain desirable traits, in inbred lines & the yields of crops like maize & wheat have steadily increased over the past 60 years. However, the methods of classical plant breeding are slow & uncertain. To introduce a desired gene (or) set of genes by conventional methods requires a sexual cross between two lines, and then repeated back-crossing between the hybrid offspring & one of the parents until a plant with desired characteristics is obtained. This process, however is restricted to plants that can sexually hybridize & genes in addition to the desired gene will be transferred.

Recombinant DNA techniques promise to circumvent these limitations by enabling plant geneticists to identify & clone specific genes for desirable traits, such as resistance to an insect pest & to introduce these genes into already useful varieties of plants. Sexual compatibility becomes irrelevant & the process becomes faster because transgenic plants expressing the gene can be selected directly. Plants have a number of unique biological features that can be explored with recombinant DNA-techniques. These features include their patterns of growth, the means plants have devised to cope

with the challenges of a chaining environment from which they cannot escape and photosynthesis.

4.5.2.3 Plants have Advantages & Disadvantages for Genetic Engineering:

Plants present advantages & disadvantages for the genetic engineer. The long history of plant breeding means that plant geneticists have a wealth of strains carrying genetically characterized mutations that can be exploited at the molecular level. Plants are particularly amenable to genetic manipulation because many can be self-fertilized (or) selfed. When a plant heterozygous for a mutation is selfed, the progeny include wild-type plants, plants homozygous for the mutation & also heterozygotes in which the mutation is maintained.

Because plants produce very large numbers of progeny, rare mutations & recombination can be found. Genetic manipulation of some plants is particularly refined because of the many years scientists have spent analyzing plant transposable elements, which can be exploited as vectors & as insertion mutagens. Plant geneticists are also helped by the regenerative capabilities of plants.

Genetic manipulations can be performed on plant cells in culture & then with various degrees of difficulty, whole plants can be regenerated from single cells. The relative immobility of plant cells is important for experimental genetics, it is easy to observe clones of cells, because the descendants of a cell remain associated.

Disadvantages for the molecular geneticists are many plants have very large genomes often because of polyploidy. The presence of many genomes in the cell. Many groups of plants have polyploid species, for example about two-thirds of the grasses are ploypoid & species in the group that includes the potato have chromosome numbers ranging from 24 – 144. Ploidy may contribute to the phenomenon of somaclonal variation exhibited by plants cells in tissue culture.

Plants regenerated from single cells are not genetically homogeneous, for it appears that plant cells growing in tissue culture are genetically unstable. This is potentially serious problem in gene transfer – experiments. A final difficulty arises because of our preoccupation with plants like maize, rice & wheat which have great agricultural importance.

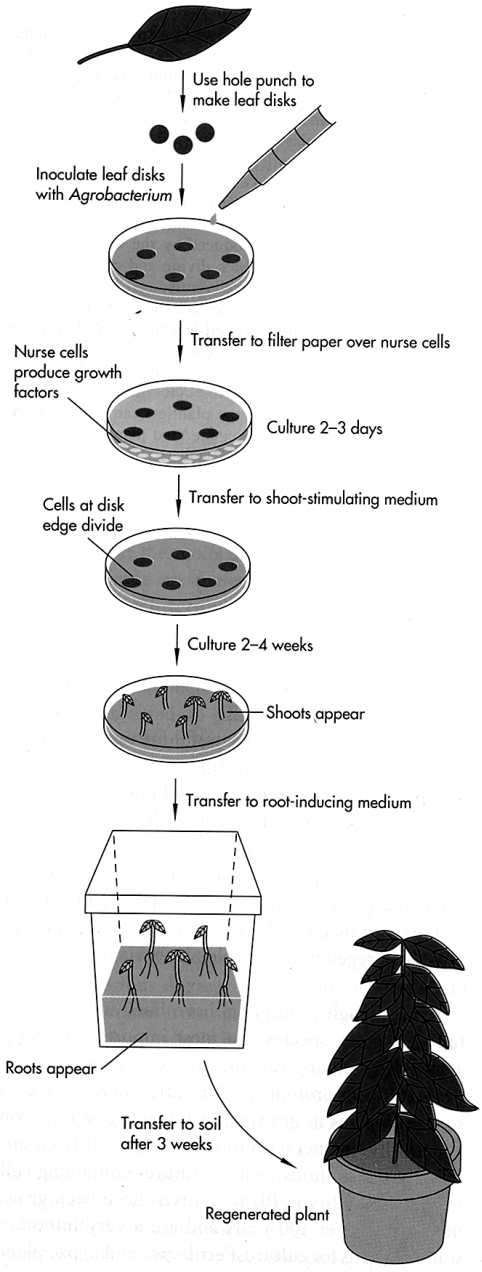
These are monocotyledonous plants (monocots) whose seeds have a single cotyledon – meaning “Seed Leaf”.

These monocots are proving to be very difficult to transform with the DNA vector systems that are very efficient with dicotyledonous plants (those with two cotyledons).

4.5.2.4 Whole Plants can be Grown from Single Cells:

An extraordinary phenomenon & one that is very useful to the geneticist is, that whole plants can be regenerated from single cells. When a plant is wounded mecharai cells, a patch of soft cells called a callus grows over the wound. If a piece of yound callus is removed & placed in a culture medium containing the appropriate nutrients & plant growth hormones, the cells will continue to grow & divide as a suspension culture. These cells can be plated out & they will grow to form new calli. It is some times necessary to use other cells as a nurse culture, equivalent to the feeder layers of cells sometimes used in mammalian cell culture. The callus will then redifferentiate into shoots & roots, ultimately a whole flowering plants will be produced.

Studies by Skoog & Miller showed that the differentiation of the cells in a callus depends on the relative concentrations of the plant hormones auxins & Cytokinins. If the ratio of auxins to cytokinins is high, then roots develop, Shoots develop when the ratio is low.



(Figure 15-1 Regenreration of plants from protoplasts.)

Leaf cells are characterized by a cytoplasmic compartment containing numerous chloroplasts, a large vacuole & a nucleus. These cells are not very useful for uptake of DNA because like all plant cells, they are surrounded by a cellulose wall. That can be removed by incubating pieces of plant tissue in a solution containing cellulase.

The resulting protoplast is enclosed only by a plasma membrane & is much more amenable to experimental manipulation. Protoplasts will take up macromolecules like DNA & they are capable of regenerating whole plants via the formation of calli.

Sugars & Salts are added to the solution to maintain Osmotic balance & prevent the protoplasts from lysing. Once the cell debris is removed, the protoplasts are placed on filter paper covering a layer of nurse cells. The filter paper is impervious to the cells out growth factors & other molecules produced by the nurse cells can diffuse in to the protoplasts which divide & grow to form microcolonies. The microcolonies are carefully transferred to a medium high in cytokinin & low in auxin. Shoots appear in about two to four weeks. Then the cultured cells are transferred to a container called a Magenta box. Which contains roo-inducing medium lacking cytokinin & low in auxin. Once the roots appear, the plantlets can be placed in soil, where they develop into regenerated plants.

Even though protoplasts have been used successfully for many species, the most important group of agricultural plants, cereals are very difficult to regenerate from protoplasts.

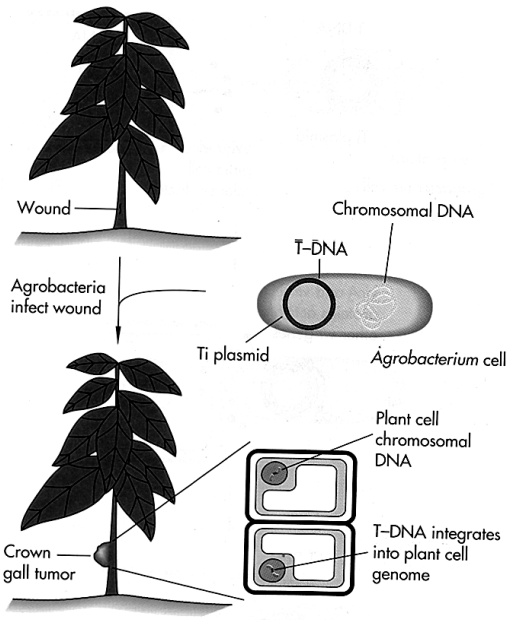
Recently, there have been some successes in growing both maize & rice from genetically engineered protoplasts derived from embryonic cell cultures.

4.5.2.5 Leaf Disks Are an Important Target for Gene Transfer:

Growing whole plants from protoplasts is not easy, even for the most amenable species of plants. A simple but very significant improvement came with the development of the leaf disk-technique. The technique is so important because it can be used with the most effective system for transferring genes into plants, a system using the Ti plasmid carried by the bacterium Agrobacterium tumefaciens. Plant cells must be wounded to be targets for Ti gene transfer & pieces of roots & stems have been used as targets.

Leaves are good source of regenerating cells, the cells coming from small disks cut from a leaf. Leave disks are cut out & placed in a shallow dish. A solution of Agrobacteria is added & after a few minutes the leaf disks are tranferred on to nurse cells medium. Wounded cells at the edge of the disk release factors that induce the agrobacteria to infect the cells.

The plant disks are cultured in a fashion similar to that described for protoplasts in a medium containing an antibiotic. Such as cefotaxime that kills Agrobacterium but does not harm plant cells, to yield a regenerated plant.



(figure 15.2 page no. 276)

The whole process, from cutting out the leaf disk to having rooted plants, takes between four & seven weeks. This process is extraordinarily fast compared with protoplast cultures. Further more, the technique is applicable to a wide variety of dicotyledons & is now used routinely.

4.5.2.6 Ti Plasmid of Agrobacterium causes Crown Gall Tumors:

Crown galls are tumors of plants that arise at the sits of infection by some species of the bacteria Agrobacterium. The cells of crown galls have acquired the properties of independent, unregulated growth. In culture, these cells grow in the absence of the plant hormones that are necessary for the culture of normal plant cells & the cells retain this phenotype even in the absence of the bacterium.

The tumor-inducing agent in Agrobacterium is a plasmid that integrated some of its DNA into the chromosome of its host plant cells. Ti plamids are large, circular double stranded DNA molecules of about 200 kb, and like other bacterial plamids, they exist in Agrobacterium cells as independently replicating genetic units.

When a wounded plant is infected by Agrobacterium, the agrobacteria cells donot enter the plant cell but transfer a DNA segment called the T-DNA from the circular extrachromosomal tunor inducing (Ti) plasmid. The T-DNA become stable incorporated into the plant cell chromosomal DNA. Genes with in T-DNA from natural Ti plamids are Expressed & their products stimulate the cells to divide uncontrollably. The structure formed by the rapidly dividing cells is called crown gall tumor.

Ti plamids are maintained in Agrobacterium because a part of the plasmid DNA, called T-DNA, carried the genes coding for the synthesis of unusual amino acids called opines. The infected plant cell is induced to synthesize these amino acids, but the plant can not utilize them. Instead, the Ti planmid is believed to carry genes coding for

enzymes that can degrade opines, so the opines may act as a nutrient for the Agrobacterium. This subversion of the plants metabolism could provide a selective advantage for Agrobacterium.

A second set of genes in T-Dna causes the unregulated growth of the plant cell. Two of these genes iaaM & iaa H, code for enzyems that lead to the production of an auxin.

The third gene, ipt Z codes for an enzyme that causes production of a seond phytohormone. These two hormone cause the infected plant cell to divide, they also affect the neighboring cells.

4.5.2.7 T-DNA part of the Ti Plasmid, is Tranferred to Plant Cells:

There are three components involved in Ti plasmid tumor induction. One is T-DNA, which is transferred to the host cell & is a form of mobile element. In addition genes called Vir (for virulence), present elsewhere on the Ti plasmid, are needed for the production of trans-acling proteins that are essential for, or at least enhance, plant cell transformation. A third set of genes is indirectly involved in transformation. These genes are carried on the Agrobacterium chromosome & are responsible for binding the bacterial cell to the plant.

The Virulence genes in Agrobacterium are switched on by chemcicals produced by wounded plant cells. Following Vir genes the T-DNA element is excised from the plasmid – DNA.

The T- DNA is flanked by Ti plasmid sequences, each 25 bp long. These flanking sequence are called borders and they are involved in excision of the T-DNA sequence. Excision is two-stage process in which the right-hand border is nicked between the third & fourth bases of the 25 bp repeat. A second nick in the left-hand border releases the T-DNA as a single strand. The process of transfer from the bacterial cell to the plant cell is analogous to the process of bacterial conjugation, it is as though the Agrobacterium is maling with a plant cell. The functions of the Vir proteins in the transfer process are still being explored.

By a mechanism that is still not completely worked out, the T-DNA molucule enters the plant cell, where it intigrates randomly into the chromosomal DNA. The singly stranded T – DNA region of the Ti plasmid is repaired by DNA replication, so the Agrobacterium has not lost any information by transferring DNA to plant cell.

4.5.2.8 T – DNA has been Modified to Act as a Gene Vector:

A method called cointegration was first used for gene transfer with the T-DNA, Ti-plasmid & Agrobacterium System. This method was developed to avoid the problems associated with manipulating large pieces of DNA the size of the Ti plasmid.

A cloned gene can be introduced into plant cells by first inserting it into the claning site of a plamid that can replicate in E.Coli & Contains a segment of T-DNA. The resulting intermediate shuttle vector is introduced into E.Coli cells & tranformants are selected by resistance to ampicillin, encoded with in the PBR 322 sequences. Next the plasmid is tranferred from the E.Coli cell to an Agrobacterium cell by mating. Once inside the Agrobacterium, the plasmid integrates into the Ti plasmid by means of

homologous recombination of the T-DNA sequences on the two plasmids. This process places the entire integrative plasmid between the left & right boundaries of the T-DNA. Plasmids that fail to integrate do not accumulate because they lack an origin of replication for Agrobacterium.

Agrobacteria containing the combinant Ti plasmid are selected & used to infect plant cells. Plant cells that have taken up the T-DNA are identified by the plant selectable market NPT II, which confers resistance to kanamycin. These cells also contain the cloned gene of interest.

The standard method for T-DNA tranfer is now the binary system. The binary Vector contains the 25 bp borders of the T-DNA that are needed for excision & integration. The Phytohormone genes of the T-DNA can be removed to create room for the insertion of foreign DNA, which will be transferred to the plant cell. At the same time deleting the phytohormone genes prevents the uncontrolled growth of the recipient cells.

The other essential genes are the vir genes of the Ti plasmid, & these can act in trans, if they are supplied on a separate plasmid, called the helper plasmid. A very important factor in the development of T-DNA based vectors is the availability of selectable markers such as neomycin phosphotranferase II (NPTII) & dihydrofolate reductase. These markers are included with in the 25 bp repeats of the binary vectors.

The vectors carry a second selectable market so that they can be mainpulated easily in E.Coli. Binary vectors differ from integrativevectors, in that the binary plasmid containing the DNA to be transferred to the plant cell is maintained as a separate replicating vector in Agrobacterium.

An example of the use of the binary system to introduce functional genes in plants cones from example experiments using antisense RNA to control plant gene expression. Polygalacturonase (PG) is an enzyme that solubilizes the walls of plant cells by digesting pectin. Reducing expression of polygalacturonase could lead to fruit that bruice less easily.

Transfer of an antisense gene for polygalacturonase into Tomato cells by using a binary vector. The T-DNA region in a binary vector has been detected to contain only the left (LB) & right border (RB) sequencess & the NPII gene has been inserted between them to allow selection in plant cells. The other partner of the binary system is a helper Ti plasmid, a modified Ti Plasmid that is missing its T-DNA but still contains the Vir genes. This plasmid is maintained in Agrobacterium. An experiment was performed to investigate the role the enzyme polygalacturonase (PG) plays in the senistivity of tomatoes to bruising. If the cellular levels of PG could be reduced, perhaps the fruit would be hardier.

A DNA fragment from the 5'end of the PG DNA was ligated in the antisense direction, to the constitutively active promoter from cauliflower mosaic virus (Ca MV) & then cloned into a binary vector between the sequences encoding the left (LB) & right (RB) T-DNA borders. The antisense plasmid transformed into E.Coli was then transferred by mating into a strain of Agrobacterium that contains the helper Ti-plasmid upon activation of the Agrobacterium by a wounded plant cell, the DNA between LB & RB on the binary vector was transferred into the plant cell. Plant cell transformants, which had this DNA integrated into their chromosomal DNA, were selected by Kanamycin resistance & used to regenerate fruit bearing tomato plants. Although tomatoes expressing the antisense RNA exhibited reduced PG activity, they were just as soft as normal tomatoes, presumably because PG is just one factor in the

process. Another factor is ethylene & successful inhibition of tomato ripening has now been achieved by expressing an antisense RNA for an enzyme in the metabolic pathway.

4.5.2.9 Viruses can be used as Vectors for whole Plants:

Viruses are attractive for use as vectors for introducing genes into plants. Their Principal advantage is that they are evolutionarily adapted to do just that, to distribute their own genome through out an infected plant. If the Viral genome includes aforeign gene, then that too should be spread systemically through out the plant. This methods has the potential for greatly simplifying delivery systems. Another advantage is that viral vectors amy circumvent the problems of delivering genes to monocotyledonous plants like maize. EG: gemini viruses have a wide host range & experiments have been performed to assess their potential as vectors.

Tomoto golden mosaic virus (TGMV) is composed of 2 single stranded DNA molecules of 2.5-kb pakced together in a protein coat. DNA-A encodes the Coat protein & replication functions. DNA-B is required for cell to cell infection. The naked DNA's are infections in plants. These DNA molecules have been engineered into plant expression vectors that transfer cloned DNA of interest into all cells of a plant by infection. This technique eliminates the need to regenerate plants from a transformed cell. T o this vector system, the NPTII gene for resistance to Kanamycin was ligated into a cloned fragment of the DNA-A molecule, replacing the Coat protein gene. This DNA fragment, positioned between T-DNA border sequences (LB & RB) in a binary vector, was then transferred to Agrobacterium.

The DNA was transferred to tobacco plants by injecting the Agrobacterium directly in to the stems. These plants had previously been transformed with T-DNA containing two copies of the geminivirus DNA-B. With in three weeks, the infection had spread through the plant. Singly-stromded DNA-B & DNA-A-NPTII recombinant molecules were detected in leaves from infected plants by southern blot DNA hybridization. Extracts prepared from infected leaves contained high levels of NPTII activity.

An alternative binary vector that has been developed contains both DNA-A & DNA-B sequences with this vector plants dono't have to have be transformed first with DNA-B & any plant capable of participating in Agrobacterium mediated transfer can be infected.

4.5.2.10 Guns & Electric Shocks Transfer DNA into Plant Cells:

The Agrobacterium Ti Plasmid Vector System is very effective for introducing DNA into dicotyledonous plants. Mammalian cells can be made to take up "naked" DNA presented as a calcium phophate-precipitate, (or) by electroporation. The calcium phosphate method works poorly for plant transformation but electroporation is being used successfully with plant cells. Typically a high concentration of plasmid DNA containing a cloned gene is added to a suspension of protoplasts & the mixture shocked with an electricalfield of 200 to 600 v/cm.

Direct transfer of DNA into plant cells by microprojectile bombardment – "Shot gun". A thin Coat of DNA is coated on to the surface of 1-μm – diameter tungsten (or)

gold particles by precipitation with calcium chloride. The beads are placed on the end of a plastic bulled in the barrel of particle gun designed especially for this purpose.

The target plant tissue (or) suspension cells are placed next to a small opeing at the end of the barrel. The macroprojectile is propelled toward the cells by an explosive charge & as it slams into the retaining plant, the particles it carries pass through the aperture & hit the cells. The barrel of the gun & the speciment chamber have to be evacuated otherwise the air resistance slows down the velocity of the microprojectiles.

Plant cells can with stand a vaccum for as long as two minutes. Follwing bombardment the cells are transferred to a nurse cell cultureplate & plants are regenerated. Examination of bombarded cells shows microparticles lodged inside the cytoplasm. New models of the gun use pressurized helium to propel the macro projectile down the barrel.

Bombardment has been used successfully to transform embryogenic maize cells with reporter genes & what is more interesting, with the bar gene for the enzyme phosphinothricin- acetyltranferase (PAT) . This enzyme inactivates phosphoinothricin (PPT) a component of herbicides & so protects the plant.

The gene-coated beads were shot into embryogenic cells, & transformed cells were selected by culturing them in medium containing PPT. Whole plant regenerated from these cells were resistant to a commercial herbicide applied directly to the leaves. So bomboardment has tremendous potential for performing recombinant DNA experiments on maize.

4.5.2.11 Bombardment with DNA-Coated Beads can produce Transgenic Organells:

Nuclear DNA is not the only DNA targeted by plant geneticists, for chloroplasts have their own DNA genome. Many of these genes are concerned with photosynthesis. If recombinant DNA techniques are to be used for genetic analysis of photosynthesis, then ways have to be devised for introducing DNA into chloroplasts. This can be done with microprojectiles was first shown by experiments performed with chlanydomonas, a unicellular alga with a singly, large chloroplast that occupies a large part of the cell.

Transformation of plant chloroplasts is a different matter, because plant cells have neumerous chloroplasts & these are very small. Nevertheless, transformation of tobacco plant chloroplasts has been achieved, using a gene for 16 Sr RNA with mutations conferring resistance to spectinomycin & streptomycin on the appropriate selection medium, plants carrying chloroplasts with spectionmycin resistance are green, where as non resistant plants are white.

Leaves were bombarded, cut into smaller pieces & cultured. Green, spectionomycin resistant calte on white leaves were selected, but only three transgenic clones were found. The investigators estimated that transformation of chloroplasts was about 100-fold less efficient than transformation of nuclear genomes.

Breeding experiments showed that seeds from selfed plants were all spectinomy – cin- resistant & as expected, resistance is maternally inherited through the ovum. Chloroplasts are small disk-shaped organelles, & the tungsten beads are probable too large to lodge in a chloroplast.

The efficiency of tranformation can be improved, this may be very important approach for genetic manipulation of chloroplasts.

Based on the above methodologies Transgen Plants canbe obtained

4.5.2.12 Plants Expressing a Viral Coat Protein Resist Infections:

Plant viruses are a serious problems for many of the major agricultural crops, results in reduced growth rate, copy yield, & quality. Through a standard genetic trick termed Cross-Protection, infection of a plant with a strain of virus that rpoduces only mild effects protects the plant against infection by more damaging strains. A particular viral-encoded protein is responsible for the protective effect.

The first experiment was performed on tobacco. The Tobacco mosaic virus (TMV) is an RNA virus about 6.5 kb in size. By cloning viral cDNA's, it was established that the TMV genome encodes four polypeptides, two replicase subunits, a coat protein & a protein important for cell to cell movement. A cloned cDNA encoding the coat protein from TMV was ligated into an integrative T-DNA vector with a cauliflower mosaic virus (CaMV) promoter.

The plasmid was transferred into tobacco by Agrobacterium – mediated DNA transfer into leaf disks. Regenerated trnsgenic tobacco plants were obtained that expressed various level of TMV coat protein in their leaves. Two transgenic platns, one expressing high levels of TMV coat protein & the other expressing undetectable levels, were infected with TMV, as was a nontransgenic control plant. After 2 weeks, the control plant & the nonexpressor exhibited symptoms of TMV infection. In contrast, the coat protein – expressing transgenic plant resisted infection. The protective agent was shown to be the viral coat protein itself, rather than the viral RNA.

Crops such as potato, alfalfa & tomato can also be achieved by CP expression. Other approaches that are being investigated include expression of ribozymes to cleave viral RNA's & transgenic expression of anti sense RNAs.

4.5.2.13 Insects fail to prey on Plants Expressing a Bacterial Toxin:

Plants are susceptible to damage by insects, to reduce the use of chemical pesticides, natural microbial pesticides, such as certain species of Bacillus thuringiensis (Bt) have been used in limited fashion for over 30 years. Upon sporulation, these bacteria produce a crystallized protein that is toxic to the larvae of a number of insects. The toxin protein doen not harm nonsusceptible insects & has no effect on vertebrates.

The crystal protein is normally expressed as a large, inactive pro-toxin about 1200 aminoacids in length, upon digestion by susceptible larva, proteases in the insects gut cleave the protein into an active 68,000- dalton fragment. The toxin acts by binding to receptors on the surface of midgut cells & blocking the functioning of these cells.

The 1178 aminoacid Bt crystal protein, an insect toxin, is encoded on a 75 – kb plasmid & is produced upon bacterial sporulation. The full-length crystal gene was poorly expressed in trangenicplants.

In order to improve expression, a truncated gene encoding the functional amino-termianl portion (aminoacids 1 to 615) was cloned into a T-DNA binary vector between LB & RB. To further increase expression in plants, a synthetic gene encoding aminoacids 1 to 453, containg codons preferred in plants, was substituted for the natural sequence. The synthetic sequence was ligated to a natural DNA fragment encoding amino acids 454 to 615. In addition, the T-DNA vector contained a duplicate CaMV promoter, which has been shown to increase transcription about five fold. The T-

DNA vector was transferred into cotton seedlings by Agrobacterium infection, & transgenic cotton plants were regenerated.

4.5.2.14 Herbicide – Tolerant Transgenic Plants allow more Effective Management of Weeds:

The presence of weeds in a field of crop plants care reduce yield by over 10 percent. It is difficult to find ways to destroy them without also affecting the crop plant. Weed killers, also called herbicides, are not very selective, with the ability to introduce DNA into plants, researchers are trying to create herbicide-tolerant crops by three strategies. Increasing the level of the target enzyme for a particular herbicide, expressing a mutant enzyme that is not affected by the compound, (or) expressing an enzyme that detoxified the herbicide. The third strategy for creating herbicide-tolerant plants is by transgenic expression of enzymes that convert the herbicide to a form that is not toxic to the plant. Some plants have developed their own detoxifying systems for certain herbicides however, these activities in plants are encoded by a complex set of genes that has not yet been fully characterized.

The enzyme 5 – enol pyruvyl shikinate 3 – phosphate synthase (ESPS) important for synthesis of aromatic amino acids in bacteria & plants, is inhibited by glycophosate, the active ingredient in the weed killer round up. The gene encoding a glycophosate-resistant EPSPS from bacteria was cloned into a T-DNA expression vector & introduced into tobacco by Agrobacterium-mediated gene transfer. Since EPSPS in plants is synthesized in the cytoplasm & them transferred into chloroplasts, a chimeric gene was constructed in which a segment encoding a 72 – amino acid transit peptide from petunia EPSPS was fused to the amino terminus of the bacterial EPSPS coding sequence.

Expression of the chimeric gene was controlled by the cauliflower nosaic virus (CaMV) 35 s promoter. Transgenic plants expressed both the endogenous plant EPSPS & the bacterial glyphosate – insensitive enzyme. Biochemical studies demonstrated that the transit peptide had properly targeted the bacterial enzyme to chloroplasts. When sprayed with glyphosate, wild plants are killed because EPSPS is inhibited the trangenic plant tolerated levels of glyphosate four times higher than that killed wild-type plants because the bacterial EPSPS still functions in the presence of the herbicide.

Researchers are searching for a bacterial enzyme that will degrade glyphosate.

4.5.2.15 The Potential use of Plants to Produce Proteins is of Commercial Importance:

Production of a protein drug in mammalian cell culture is an extremely expensive process requiring thousands of liters of cells to produce enough protein for commercial use one the other hand. Plants are cheap to grow, & huge quantities of protein could be obtained from a single field & : enkephalin, a human neuropeptide & serum albumin of human have been expressed in plants.

Another potential use is the expression of mouse monoclonal antibodies in plants. Antibodies against a plant toxin (or) herbicide could be produced in plants to protect the plant from these agents.

Alternatively, monoclonal antibodies for clinical therapy could be produced in plants. Plants as bioreactors to produce antibodies cloned cDNAs encoding the light &

heavy chains from a mouse monoclonal antibody were ligated into separate T-DNA vectors.

And placed under control of a constitutive CaMV promoter. The plasmid were tranferred separately into tobacco plants by Agrobacterium infection. Trangenic plants containing the light & heavy – chain genes were sexually crossed to produce progeny plants that contained both genes. Examination of protein extracted from leaves demonstrated the expression of functional antibody molecules in these progeny plants. Other experiments showed that the presence of a signal sequence was necessary for high-level expression. These results suggest that the plant secretion machinery can recognize the mouse signal peptide.

In addition, since of the cost for producing drug, such as tissue plasminagen activator, stems from post production purification, it remains to be seen whether plant bioreactors will be economically competitive.

4.5.2.16 summary

Transgenic plants are plants that have been genetically modified by inserting genes directly into a single plant cell. Transgenic crop plants modified for improved flavor, pest resistance, or some other useful property are being used increasingly.

Transgenic plants are unique in that they develop from only one plant cell. In normal sexual reproduction, plant offspring are created when a pollen cell and an ovule fuse. In a similar laboratory procedure, two plant cells that have had their cell walls removed can be fused to create an offspring

There are three general approaches that can be used to insert the DNA into a plant cell: **vector**-mediated transformation, particle-mediated transformation, and direct DNA absorption. With vector-mediated transformation, a plant cell is infected with a virus or bacterium that, as part of the infection process, inserts the DNA. The most commonly used vector is the crown-gall bacterium, *Agrobacterium tumefaciens*. With particle-mediated transformation (particle bombardment), using a tool referred to as a "gene gun," the DNA is carried into the cell by metal particles that have been accelerated, or "shot," into the cell. The particles are usually very fine gold pellets onto which the DNA has been stuck. With direct DNA absorption, a cell is bathed in the DNA, and an electric shock usually is applied ("electroporation") to the cell to stimulate DNA uptake.

No matter what gene insertion method is used, a series of events must occur to allow a whole genetically modified plant to be recovered from the genetically modified cell: The cell must incorporate the new DNA into its own chromosomes, the transformed cell must initiate division, the new cells need to organize themselves into all the tissues and organs of a normal plant ("regeneration"), and finally, the inserted gene must continue to work properly ("**gene expression**") in the regenerated plant.

To help ensure all this occurs, a "cassette" of genes is inserted during the initial transformation. In addition to the gene coding for the desired trait, other genes are added. Some of these genes promote the growth of only those plant cells that have successfully incorporated the inserted DNA. It might do this by providing the transformed cells with resistance to a normally toxic antibiotic that is added to the growth medium, for example. Other genes ("**promoters**") may be added to control the functioning of the trait gene by directing when and where in the transformed plant it will operate.

The genes put into plants using genetic engineering can come from any organism. Most genes used in the genetic engineering of plants have come from bacteria. However, as scientists learn more about the genetic makeup of plants ("plant genomics"), more plant-derived genes will be used.

The major use of plant genetic engineering has been to make crops easier to grow by decreasing the impact of pests. Insect resistance has been achieved by transforming a crop using a Bt gene. Bt genes were isolated from *Bacillus thuringiensis,* a common soil bacterium. They code for proteins that severely disrupt the digestive system of insects. Thus an insect eating the leaf of a plant expressing a Bt gene stops eating and dies of starvation. There are many Bt genes, each of which targets a particular group of insects. Some Bt genes, for example, target caterpillars. Others target beetles

An emerging major use of genetic engineering for crops is to alter the quality of the crop. Fresh fruits and vegetables begin to deteriorate immediately after being harvested. Delaying or preventing this deterioration not only preserves a produce's flavor, and appearance, but maintains the nutritional value of the produce. Genes that change the hormonal status of the harvested crops are the major targets for genetic engineering toward longer shelf-life.

For example, the plant hormone ethylene is associated with accelerated ripening, as well as leaf and flower deterioration, in fruits that are injured or harvested. Scientists insert genes that interfere with a plant's ability to synthesize or respond to ethylene, thereby extending postharvest quality for many fresh products, including tomatoes, lettuce, and cut flowers. Scientists are also using gene insertion to improve a plant's nutritional value and color

4.5.2.17 Model Questions

1.what is a transgenic plant ? explain the methods of gene delivery?
2. what are herbicide resistant plants ? how are they produced by Transgenic technology?
3. How are viral resistant plants produced?
4. What are the different Agrobacterium vectors used to deliver the genes?

4.5.2.18 References

1. Recombinent DNA by Watson
2. Principles of gene Manipulation by Old and Primrose

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Lesson 4.5.3

TRANSGENIC ANIMALS

Structure

4.5.3.1 Objective:

The main Objective of the lesson is to explain in detail the various methods of production of Transgenic animals and their applications in various fields.

4.5.3.2 Introduction:

Nowadays, breakthroughs in molecular biology are happening at an unprecedented rate. One of them is the ability to engineer transgenic animals, i.e., animals that carry genes from other species. The technology has already produced transgenic animals such as mice, rats, rabbits, pigs, sheep, and cows. Although there are many ethical issues surrounding transgenesis, But it is being applied on the basics of the technology and its applications in agriculture, medicine, and industry

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. In addition to a structural gene, the DNA usually includes other sequences to enable it

- to be incorporated into the DNA of the host and
- to be expressed correctly by the cells of the host.
- Transgenic sheep and goats have been produced that express foreign proteins in their milk.
- Transgenic chickens are now able to synthesize human proteins in the "white" of the eggs

What is a transgenic animal?

There are various definitions for the term *transgenic animal*. The Federation of European Laboratory Animal Associations defines the term as an animal in which there has been a deliberate modification of its *genome*, the genetic makeup of an organism responsible for inherited characteristics.[5]

The nucleus of all cells in every living organism contains genes made up of DNA. These genes store information that regulates how our bodies form and function. Genes can be altered artificially, so that some characteristics of an animal are changed. For example, an embryo can have an extra, functioning gene from another source artificially introduced into it, or a gene introduced which can knock out the functioning of another particular gene in the embryo. Animals that have their DNA manipulated in this way are knows as transgenic animals.[20]

The majority of transgenic animals produced so far are mice, the animal that pioneered the technology. The first successful transgenic animal was a mouse.[6] A few years later, it was followed by rabbits, pigs, sheep, and cattle.[8,14,15,16]

Why are these animals being produced? The two most common reasons are:

- Some transgenic animals are produced for specific economic traits. For example, transgenic cattle were created to produce milk containing particular human proteins, which may help in the treatment of human emphysema.
- Other transgenic animals are produced as disease models (animals genetically manipulated to exhibit disease symptoms so that effective treatment can be studied). For example, Harvard scientists made a major scientific breakthrough when they received a U.S. patent (the company DuPont holds exclusive rights to its use) for a genetically engineered mouse, called OncoMouse® or the Harvard mouse, carrying a gene that promotes the development of various human cancers

Since 1980, when the first successful gene-transfer experiment using DNA microinjection on a mouse was reported, a method has been established that allows the transfer of a single isolated gene. By using molecular biology methods already established in the 1970s, genes that are of interest for breeding purposes can be isolated, sequenced, recombined with regulatory elements and tested *in vivo* and *in vitro* for their functional ability. The implementation of this basic research in animal breeding programmes is only just beginning and it is expected that drastic changes will emerge in the future. . The possibilities of application of gene transfer have been highlighted

How are transgenic animals produced?
Since the discovery of the molecular structure of DNA by Watson and Crick in 1953, molecular biology research has gained momentum. Molecular biology technology combines techniques and expertise from biochemistry, genetics, cell biology, developmental biology, and microbiology.[2]
Scientists can now produce transgenic animals because, since Watson and Crick's discovery, there have been breakthroughs in:

- recombinant DNA (artificially-produced DNA)
- genetic cloning
- analysis of gene expression (the process by which a gene gives rise to a protein)
- genomic mapping

The underlying principle in the production of transgenic animals is the introduction of a foreign gene or genes into an animal (the inserted genes are called transgenes). The foreign genes "must be transmitted through the germ line, so that every cell, including germ cells, of the animal contains the same modified genetic material. (*Germ cells* are cells whose function is to transmit genes to an organism's offspring.)

4.5.3.3 Gene transfer techniques

Gene transfer is the transfer of *in vitro* recombined gene constructs into animals. When a gene construct is integrated into the genome of the animal it is described as a transgene. The coded protein produced by this transgene is the transgenic product.

Animals that contain transgenes are transgenic and, if the transgene is passed on to the offspring, transgenic lines or populations will be created. The target of the technology is the trait that is to be influenced by the transgenic product.

To date, there are three basic methods of producing transgenic animals:

- DNA microinjection
- Retrovirus-mediated gene transfer
- Embryonic stem cell-mediated gene transfer

Gene transfer by microinjection is the predominant method used to produce transgenic farm animals. Since the insertion of DNA results in a random process, transgenic animals are mated to ensure that their offspring acquire the desired transgene. However, the success rate of producing transgenic animals individually by these methods is very low and it may be more efficient to use cloning techniques to increase their numbers. For example, gene transfer studies revealed that only 0.6% of transgenic pigs were born with a desired gene after 7,000 eggs were injected with a specific transgene.

1). DNA Microinjection

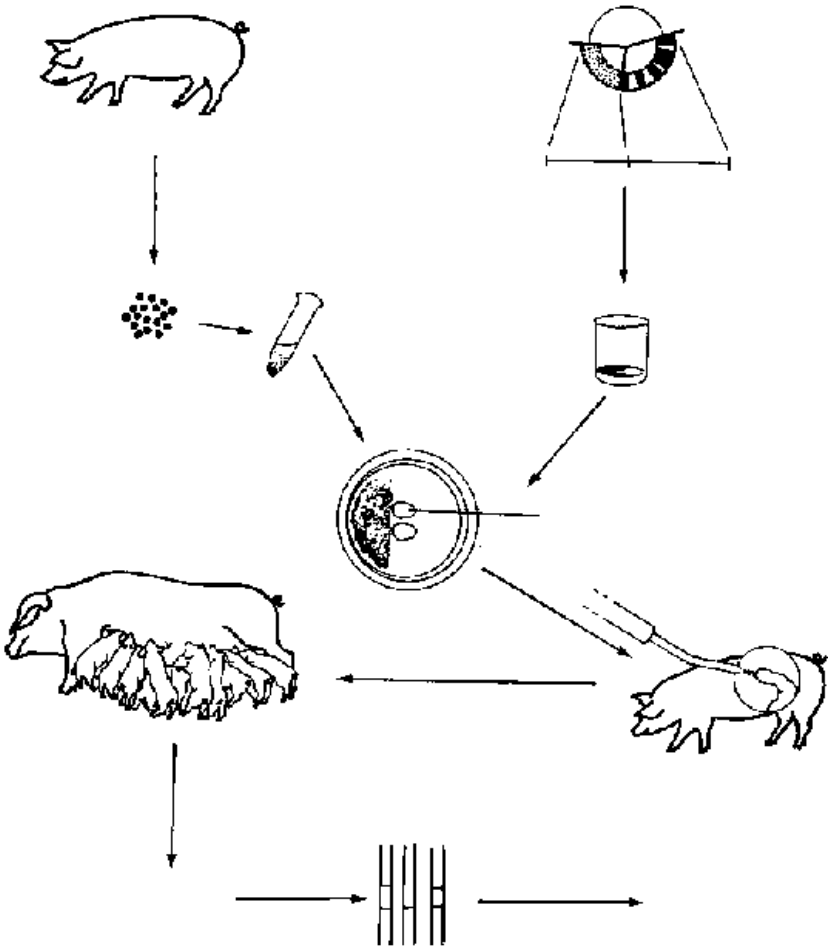


Fig 1 Gene transfer through direct DNA microinjection

Until now the microinjection of DNA into the pronuclei of zygotes is the only method used for gene transfer in domestic animals. The procedure consists of the following phases:

· cloning and recombination of a suitable gene construct;
· preparation of donors;
· recovery of zygotes (fertilized oocytes);
· visualization of pronuclei (necessary in some domestic animal species);
· preparation of the DNA solution to be injected;
· microinjection of DNA solution into the pronuclei of the zygotes;
· transfer of injected zygotes into the oviducts of synchronized recipients;
· investigation of newly born animals to ascertain whether they have integrated the gene construct (Dot - Southern - Blot).

Figure 1 shows the procedure of a gene transfer Programme with a pig as an example. The donor animals are super ovulated by means of gonadotropin injections according to the Programme established for each species and mated or inseminated twice. The recovery of the zygotes is performed 12-24 hours after fertilization through surgical flushing of the oviducts. Persisting cumulus cells are removed using hyaluronidase treatment. In almost all domestic species no nucleus structures are visible because of dark lipid-containing granule. Centrifuging is a useful method of making pronuclei visible.

Gene constructions needed for gene injection are recombined in plasmids or cosmids and then cloned. After splitting the recombined vectors with restriction endonuclease the gene construct is extracted, precipitated, washed and placed in an injection buffer. In order to avoid problems during injection, all solutions utilized during the preparation of the injection fluid must be sterile-filtered. The gene solution must be free of contamination and particles. The DNA solution is diluted so that one picolitre contains about 1 000 copies of the gene construct.
The equipment needed for microinjection (Figure 2) includes an invert microscope, two micromanipulators and injection equipment. Additional necessary items are an injection chamber and holding and injection pipettes. The injection pipette, with an outer diameter of 1-2 m m, is filled with DNA solution. During injection, the zygote is held with the holding pipette. The injection pipette is introduced into the pronucleus, passing through the zone pellucida, the cell membrane and the nucleus membrane. About one to two picolitres of the DNA solution are injected into the pronucleus, increasing its volume.

These injected zygotes are transferred, after brief *in vitro* culture, into the oviducts of synchronized recipient animals. Recipient animals are synchronized with the donors by means of hormonal treatment. After the birth of offspring from gene injection, high molecular DNA is isolated from tissue (blood or cells can be conveniently taken from the tail), to confirm successful integration. The integration of the injected DNA and the number of integrated copies can be determined after further processing with Southern Blot or Dot Blot hybridization. Integration sites in the chromosomes can be proved through hybridization of metaphase chromosomes, using the injected gene as a probe.
Transgenic animals are raised and mated. Offspring from these matings are tested to discover whether the transgene has been passed on. In mating hemizygous-transgenic F_1 *inter se* attempts are made to produce homozygous transgenic animals

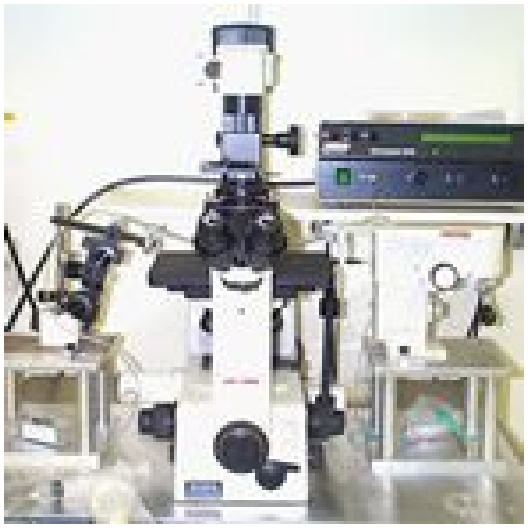


FIG 2

2. Retrovirus-Mediated Gene Transfer

A retrovirus is a virus that carries its genetic material in the form of RNA rather than DNA. This method involves

- retroviruses used as vectors to transfer genetic material into the host cell, resulting in a *chimera*, an organism consisting of tissues or parts of diverse genetic constitution
- chimeras are inbred for as many as 20 generations until homozygous (carrying the desired transgene in every cell) transgenic offspring are born

Gene transfer with retroviral-vector assistance.

Retroviruses have an RNA genome, which is transcribed through the virus' own reverse transcriptase into DNA in infected cells and subsequently integrated into the genome of the cell. Integration occurs accidentally. However, only one copy can generally be found in the provirus. Based on this cycle retroviral vectors can be used as vehicles for gene transfer.

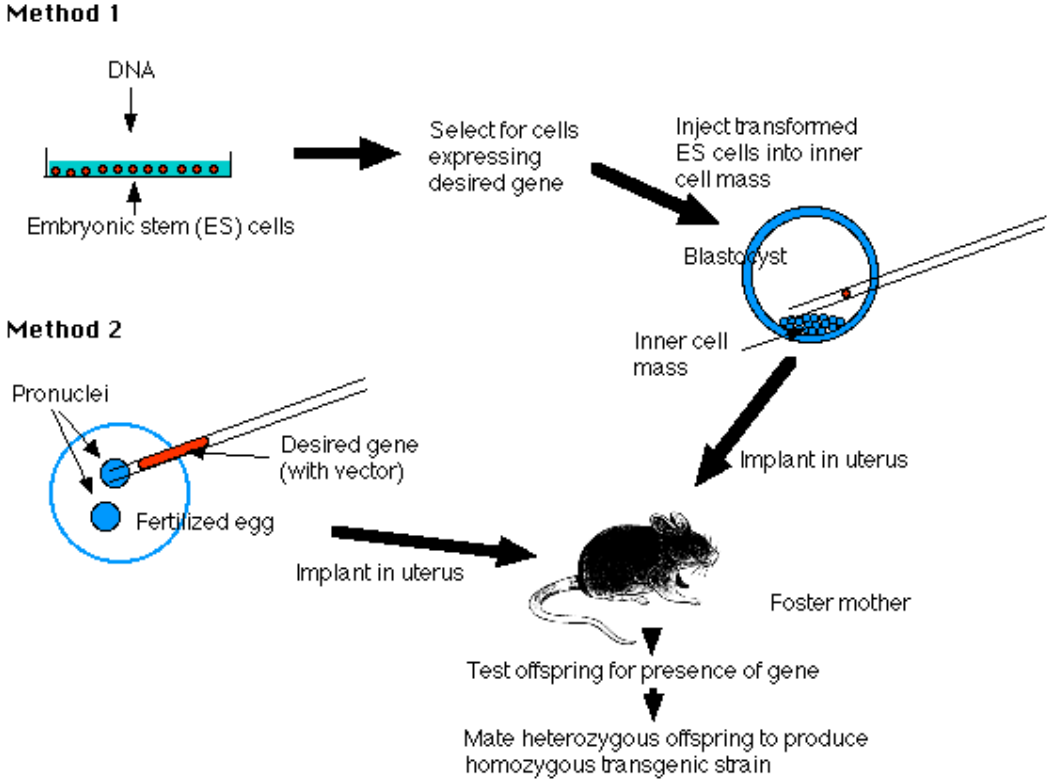
In 1974 it was shown for the first time that after injection of SV40 DNA into the blastocoel of mouse blastocysts, the DNA could then be found later in the cells of adult mice . The Mo-Mulv-provirus DNA used in these experiments which followed was integrated into the genome and passed on to offspring, thus building up stable lines.

For utilization as a vector, different parts of the viral genome are replaced by the gene construct which is to be transferred. Care has to be taken that the LTR and psi-region remain intact. The virus cover is provided by a second provirus, which lacks the coverage signal (psi-region) .

With the assistance of retrovirus vectors, the bacterial neo-gene was integrated into the germ line of mice and were able to prove their expression under the control of the TK promoter. Transgenic mice, carrying a mutant DHFR gene, were produced by infecting zone-free mouse embryos with recombinant defective retroviruses without viral

assistance. and were able to achieve integration of the provirus when co culturing denuded embryos with psi2-cells, transgenic mouse lines were produced through infection of pre-implantation embryos with recombined retroviruses containing the complete human beta-goblin gene including its own promoter. These mouse lines expressed the transgene in the haemopoetic system.
The successful utilization of retroviral vectors in domestic mammals has not yet been reported. In poultry DNA of interest have been injected in to the wild strain as well as the recombinant chicken-leucosis-virus into eggs before hatching. Some animals were found which showed integration.

3. Embryonic Stem Cell-Mediated Gene Transfer



This method involves:

- isolation of totipotent stem cells (stem cells that can develop into any type of specialized cell) from embryos
- the desired gene is inserted into these cells
- cells containing the desired DNA are incorporated into the host's embryo, resulting in a chimeric animal

Unlike the other two methods, which require live transgenic offspring to test for the presence of the desired transgene, this method allows testing for transgenes at the cell stage.
Gene transfer through production of germ-line chimeras
The procedure to utilize totipotent transformed stem cells to transfer recombined gene constructs into the germ line is receiving increasing attention, especially in respect of the mouse. Totipotent stem cells are isolated from *in vitro* cultured blastocysts. Through

aggregation with early embryonic division stages or through injection of these cells into blastocysts, chimeras can be produced. Up to 30 percent of the chimeras so produced are germ-line chimeras containing the genotype of the cell line. mentioned the possibility of using these stem cells as a vehicle for the introduction of new-genes.
Thet gene constructs that have been transformed into embryonic stem cells were expressed in the somatic tissues of the mouse chimeras produced. The percentage of mice born expressing chimerism was very high and in some cases it could also be proved that some of the offspring had inherited the transgene. Because no suitable stem cells are so far available for use in-farm animals, this technique has not been applied for the latter. Important efforts are being made to produce these cell lines.
The advantage of such gene transfer through transformed stem cells would be that integration and eventually expression of the transgene could already be tested in the cell line. Perhaps of even more importance is the fact that the-transfer could possibly be done through manipulating morulae and blastocysts. Since embryos at that stage, particularly in the bovine, can be obtained non-surgically, this would mean that gene transfer programmes would be greatly facilitated.
Another method, which could also be a gene transfer technique through manipulation of blastocysts, is the microinjection of DNA packed into liposome's have been injected these into bovine blastocysts, but positive results of this experiment are not yet available.

In 1989, the use of sperm-mediated gene transfer for the gene transfer for the generation of transgenic mice and pigs was published. This method is quite simple and, as shown by the authors, also efficient. Other scientists have not been able to repeat these results

Transgenic Animals
In the application of gene transfer in livestock production programmes, it is of key importance that the transferred gene be subsequently transferred to its progeny. This means that all, or at least some, of the germ cells of primary transgenic animals must contain the transgene Unfortunately little knowledge exists about the molecular biological processes that take place when the injected DNA is integrated in the genome. For example, the exact time of the integration is not known nor whether this is stable in all cells during the further development of the embryo. When transgenic animals have been checked after birth, particularly in trials to produce offspring, it has been shown that mosaics might occur. Mosaics are animals with different cell lines of different genotype that have, however, originated from one zygote. Transgenic mosaics contain both transgenic and non-transgenic cells and this sometimes can become a problem. It is obvious that the progeny cannot inherit the transgene from its transgenic parents if the transgene is not present in the gonads. From past experience, it appears that about 30 percent of the primary transgenic animals produced through microinjection are mosaics and therefore pass on the transgene to less than half or even to none of their progeny.

In animals that have integrated the injected gene construct in a stable way, inheritance follows that of a Mendel gene since, in general, integration takes place at one locus in the chromosome. These animals are called hemizygote transgenics. The term "heterozygous" is not appropriate since the allele corresponding to the transgene is

missing in the homologous non-transgenic chromosome. If the gonads are mosaics of transgenic and non-transgenic cells, the percentage of transgenic progeny depends on the participation of these two cell lines in the production of gametes and can be between 0 and 50 percent. Mosaics normally only occur in the F_0 generation. Offspring of transgenic F_1 and the following generations contain, if they are positive, the gene construct in all somatic and gonadal cells. It has been observed, however, that a transgene has seldom not been transmitted reliably through several generations. The reason why a transgene does not remain stably integrated but can be lost from the genome has sometimes not been satisfactorily explained. Even more frequent than the instability of integration is the large variability in the expression of the transgene. In subsequent generations, all different forms of changes from an increase to a reduction or even to a complete absence of the expression have occurred. Even within full-sibs, a significant variability of transgenic expression has been observed. The reasons for this have not yet been clarified but one possibility could be the different grade of methylization of the DNA.

Transgenic Mice

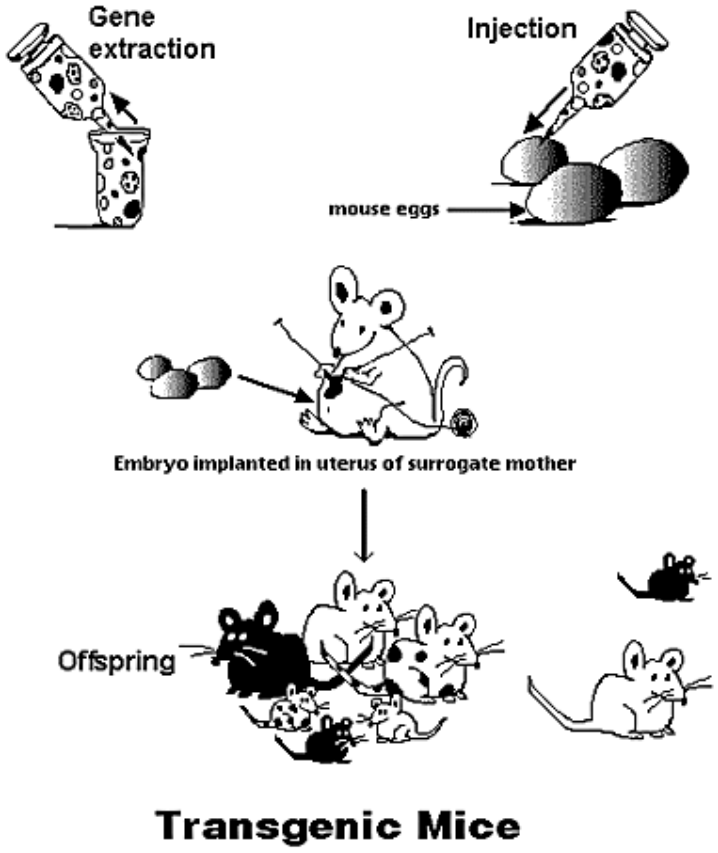
. A transgenic mouse (or any other species) is simply an organism that has had DNA introduced into one or more of its cells artificially. This is commonly done in one of two ways. DNA can be integrated in a random fashion by injecting it into the pronucleus of a fertilized ovum. In this case, the DNA can integrate anywhere in the genome, and multiple copies often integrate in a head-to-tail fashion. There is no need for homology between the injected DNA and the host genome.

Targeted insertion, the other common method of producing transgenic animals, is accomplished by introducing the DNA into embryonic stem (ES) cells and selecting for cells in which the DNA has undergone homologous recombination with matching genomic sequences. For this to occur, there must be several kilobases of homology between the exogenous and genomic DNA, and positive selectable markers (e.g., antibiotic resistance genes) must be included. In addition, negative selectable markers (e.g., "toxic" genes) are often used to select against cells that have incorporated DNA by non-homologous recombination (i.e., random insertion).

Pronuclear injection of DNA is often used to characterize the ability of a promoter to direct tissue-specific gene expression. For example, promoter/enhancer constructs may be used to drive expression of a reporter gene, such as LacZ, whose protein product, beta-galactosidase, is detected histochemically. Another major use for transgenic mice produced by pronuclear injection of DNA is to examine the effects of over expressing and misexpressing endogenous or foreign genes at specific times and locations in the animal.

Many factors influence whether a promoter/ transgene construct will express (produce the appropriate mRNA and protein) in transgenic mice. The promoters that are used must be known to function appropriately *in vivo* (*in vitro* function does not always guarantee this). . At many chromosomal locations, transgenes will be transcriptionally silent. At others they may express, but with a tissue- and temporal specificity that is not identical to what has previously been seen with the same promoter construct. The intrinsic ability of a promoter construct to drive transgene expression reliably and with

faithful tissue specificity also varies from promoter to promoter, for reasons that are not well understood. For these reasons, the TMF can guarantee to produce mice that have integrated the injected DNA, but we cannot make guarantees about transgene expression



Transgenic Mice

How do transgenic animals contribute to human welfare?
The benefits of these animals to human welfare can be grouped into areas:

- Agriculture
- Medicine
- Industry

The examples below are not intended to be complete but only to provide a sampling of the benefits.

1. Agricultural Applications

a)breeding

Farmers have always used selective breeding to produce animals that exhibit desired traits (e.g., increased milk production, high growth rate).[11,15,17] Traditional breeding is a time-consuming, difficult task. When technology using molecular biology was

developed, it became possible to develop traits in animals in a shorter time and with more precision. In addition, it offers the farmer an easy way to increase yields.
b)quality
Transgenic cows exist that produce more milk or milk with less lactose or cholesterol[12], pigs and cattle that have more meat on them[8,17], and sheep that grow more wool[18]. In the past, farmers used growth hormones to spur the development of animals but this technique was problematic, especially since residue of the hormones remained in the animal product

c) disease resistance
Scientists are attempting to produce disease-resistant animals, such as influenza-resistant pigs, but a very limited number of genes are currently known to be responsible for resistance to diseases in farm animals.

2. Medical Applications
a) xenotransplantation
Patients die every year for lack of a replacement heart, liver, or kidney. For example, about 5,000 organs are needed each year in the United Kingdom alone.[25] Transgenic pigs may provide the transplant organs needed to alleviate the shortfall.[9] Currently, xenotransplantation is hampered by a pig protein that can cause donor rejection but research is underway to remove the pig protein and replace it with a human protein.[25]

b) nutritional supplements and pharmaceuticals
Products such as insulin, growth hormone, and blood anti-clotting factors may soon be or have already been obtained from the milk of transgenic cows, sheep, or goats.[3,12,23] Research is also underway to manufacture milk through transgenesis for treatment of debilitating diseases such as phenylketonuria (PKU), hereditary emphysema, and cystic fibrosis

In 1997, the first transgenic cow, Rosie, produced human protein-enriched milk at 2.4 grams per litre. This transgenic milk is a more nutritionally balanced product than natural bovine milk and could be given to babies or the elderly with special nutritional or digestive needs.4,21,23 Rosie's milk contains the human gene alpha-lactalbumin

c) human gene therapy
Human gene therapy involves adding a normal copy of a gene (transgene) to the genome of a person carrying defective copies of the gene. The potential for treatments for the 5,000 named genetic diseases is huge and transgenic animals could play a role. For example, the A. I. Virtanen Institute in Finland produced a calf with a gene that makes the substance that promotes the growth of red cells in humans

3. Industrial Applications
In 2001, two scientists at Nexia Biotechnologies in Canada spliced spider genes into the cells of lactating goats. The goats began to manufacture silk along with their milk and secrete tiny silk strands from their body by the bucketful. By extracting polymer strands from the milk and weaving them into thread, the scientists can create a light, tough, flexible material that could be used in such applications as military uniforms, medical microsutures, and tennis racket strings.[1]

Toxicity-sensitive transgenic animals have been produced for chemical safety testing. Microorganisms have been engineered to produce a wide variety of proteins, which in turn can produce enzymes that can speed up industrial chemical reactions.[20]
What are the ethical concerns surrounding transgenesis?
This article focuses on the benefits of the technology; however, thoughtful ethical decision-making cannot be ignored by the biotechnology industry, scientists, policy-makers, and the public. These ethical issues, better served in their own article, include questions such as:

- Should there be universal protocols for transgenesis?
- Should such protocols demand that only the most promising research be permitted?
- Is human welfare the only consideration? What about the welfare of other life forms?
- Should scientists focus on *in vitro* (cultured in a lab) transgenic methods rather than, or before, using live animals to alleviate animal suffering?
- Will transgenic animals radically change the direction of evolution, which may result in drastic consequences for nature and humans alike?
- Should patents be allowed on transgenic animals, which may hamper the free exchange of scientific research?

4.5.3.4. Summary.

Interestingly, the creation of transgenic animals has resulted in a shift in the use of laboratory animals -- from the use of higher-order species such as dogs to lower-order species such as mice -- and has decreased the number of animals used in such experimentation,[26] especially in the development of disease models. This is certainly a good turn of events since transgenic technology holds great potential in many fields, including agriculture, medicine, and industry.

4.5.3.5. Model questions:

1. Describe different gene transfer methods for the production of transgenic animals?
2. Give out the applications of Transgenic animals in the field of Biotechnology?

4.5.3.6 Reference books

1)Principles of gene manipulations by OLD and PRIMROSE
2)Recombinant DNA technology by Watson

Lesson 4.5.4

TRANSGENIC PLANTS AND ANIMALS AND THEIR APPLICATIONS IN MEDICINE AND OTHER FIELDS

Structure

Objective

The lesson explains the applications of transgenic animals and plants in various fields for human welfare.

4.5.4.1 Introduction

Nowadays, breakthroughs in molecular biology are happening at an unprecedented rate. One of them is the ability to engineer transgenic animals, i.e., animals that carry genes from other species. The technology has already produced transgenic animals such as mice, rats, rabbits, pigs, sheep, and cows. Although there are many ethical issues surrounding transgenesis, this article focuses on the basics of the technology and its applications in agriculture, medicine, and industry.

4.5.4.2 What is a transgenic animal?

There are various definitions for the term *transgenic animal*. The Federation of European Laboratory Animal Associations defines the term as an animal in which there has been a deliberate modification of its *genome*, the genetic makeup of an organism responsible for inherited characteristics

The nucleus of all cells in every living organism contains genes made up of DNA. These genes store information that regulates how our bodies form and function. Genes can be altered artificially, so that some characteristics of an animal are changed. For example, an embryo can have an extra, functioning gene from another source artificially introduced into it, or a gene introduced which can knock out the functioning of another particular gene in the embryo. Animals that have their DNA manipulated in this way are knows as transgenic animals.

The majority of transgenic animals produced so far are mice, the animal that pioneered the technology. The first successful transgenic animal was a mouse. A few years later, it was followed by rabbits, pigs, sheep, and cattle

Why are these animals being produced? The two most common reasons are:

- Some transgenic animals are produced for specific economic traits. For example, transgenic cattle were created to produce milk containing particular human proteins, which may help in the treatment of human emphysema.

Other transgenic animals are produced as disease models (animals genetically manipulated to exhibit disease symptoms so that effective treatment can be studied. For example, Harvard scientists made a major scientific breakthrough when they received a U.S. patent (the company DuPont holds exclusive rights to its use) for a genetically engineered mouse, called OncoMouse or the Harvard mouse, carrying a gene that promotes the development of various human cancers.

4.5.4.3 How are transgenic animals produced?

Since the discovery of the molecular structure of DNA by Watson and Crick in 1953, molecular biology research has gained momentum. Molecular biology technology combines techniques and expertise from biochemistry, genetics, cell biology, developmental biology, and microbiology.

Scientists can now produce transgenic animals because, since Watson and Crick's discovery, there have been breakthroughs in:

- recombinant DNA (artificially-produced DNA)
- genetic cloning
- analysis of gene expression (the process by which a gene gives rise to a protein)
- genomic mapping

The underlying principle in the production of transgenic animals is the introduction of a foreign gene or genes into an animal (the inserted genes are called transgenes). The foreign genes "must be transmitted through the germ line, so that every cell, including germ cells, of the animal contain the same modified genetic material." (*Germ cells* are cells whose function is to transmit genes to an organism's offspring.)

To date, there are three basic methods of producing transgenic animals:

- DNA microinjection
- Retrovirus-mediated gene transfer
- Embryonic stem cell-mediated gene transfer

Gene transfer by microinjection is the predominant method used to produce transgenic farm animals. Since the insertion of DNA results in a random process, transgenic animals are mated to ensure that their offspring acquire the desired transgene. However, the success rate of producing transgenic animals individually by these methods is very low and it may be more efficient to use cloning techniques to increase their numbers. For example, gene transfer studies revealed that only 0.6% of transgenic pigs were born with a desired gene after 7,000 eggs were injected with a specific transgene.

1. DNA Microinjection

The mouse was the first animal to undergo successful gene transfer using DNA microinjection. This method involves:

- transfer of a desired gene construct (of a single gene or a combination of genes that are recombined and then cloned) from another member of the same species or from a different species into the pronucleus of a reproductive cell
- the manipulated cell, which first must be cultured *in vitro* (in a lab, not in a live animal) to develop to a specific embryonic phase, is then transferred to the recipient female

2. Retrovirus-Mediated Gene Transfer

A retrovirus is a virus that carries its genetic material in the form of RNA rather than DNA. This method involves:

- retroviruses used as vectors to transfer genetic material into the host cell, resulting in a *chimera*, an organism consisting of tissues or parts of diverse genetic constitution
- chimeras are inbred for as many as 20 generations until homozygous (carrying the desired transgene in every cell) transgenic offspring are born

The method was successfully used in 1974 when a simian virus was inserted into mice embryos, resulting in mice carrying this DNA.

3. Embryonic Stem Cell-Mediated Gene Transfer

This method involves

- isolation of totipotent stem cells (stem cells that can develop into any type of specialized cell) from embryos
- the desired gene is inserted into these cells
- cells containing the desired DNA are incorporated into the host's embryo, resulting in a chimeric animal

Unlike the other two methods, which require live transgenic offspring to test for the presence of the desired transgene, this method allows testing for transgenes at the cell stage.

4.5.4.4 Applications of transgenic animals

The benefits of these animals to human welfare can be grouped into areas:

- Agriculture
- Medicine
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The examples below are not intended to be complete but only to provide a sampling of the benefits.

1. Agricultural Applications

a) breeding

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b)quality

Transgenic cows exist that produce more milk or milk with less lactose or cholesterol, pigs and cattle that have more meat on them, and sheep that grow more wool. In the past, farmers used growth hormones to spur the development of animals but this technique was problematic, especially since residue of the hormones remained in the animal product.

c)Diseaseresistance

Scientists are attempting to produce disease-resistant animals, such as influenza-

resistant pigs, but a very limited number of genes are currently known to be responsible for resistance to diseases in farm animals.

2. Medical Applications

a)Xenotransplantation

Patients die every year for lack of a replacement heart, liver, or kidney. For example, about 5,000 organs are needed each year in the United Kingdom alone. Transgenic pigs may provide the transplant organs needed to alleviate the shortfall. Currently, xenotransplantation is hampered by a pig protein that can cause donor rejection but research is underway to remove the pig protein and replace it with a human protein.

b)Nutritional supplements and pharmaceuticals

Products such as insulin, growth hormone, and blood anti-clotting factors may soon be or have already been obtained from the milk of transgenic cows, sheep, or goats. Research is also underway to manufacture milk through transgenesis for treatment of debilitating diseases such as phenylketonuria (PKU), hereditary emphysema, and cystic fibrosis.

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c)Humangenetherapy

Human gene therapy involves adding a normal copy of a gene (transgene) to the genome of a person carrying defective copies of the gene. The potential for treatments for the 5,000 named genetic diseases is huge and transgenic animals could play a role. For example, the A. I. Virtanen Institute in Finland produced a calf with a gene that makes the substance that promotes the growth of red cells in humans.

3. Industrial Applications

In 2001, two scientists at Nexia Biotechnologies in Canada spliced spider genes into the cells of lactating goats. The goats began to manufacture silk along with their milk and secrete tiny silk strands from their body by the bucketful. By extracting polymer strands from the milk and weaving them into thread, the scientists can create a light, tough, flexible material that could be used in such applications as military uniforms, medical microsutures, and tennis racket strings.

Toxicity-sensitive transgenic animals have been produced for chemical safety testing. Microorganisms have been engineered to produce a wide variety of proteins, which in turn can produce enzymes that can speed up industrial chemical reactions.

4.5.4.5 Transgenic plants

Genetic engineering of plants is much easier than that of animals. There are several reasons for this: (1) there is a natural transformation system for plants (the bacterium *Agrobacterium tumefaciens*), (2) plant tissue can redifferentiate (a transformed piece of leaf may be regenerated to a whole plant), and (3) plant transformation and regeneration are relatively easy for a variety of plants.

The soil bacterium *Agrobacterium tumefaciens* ("tumefaciens" meaning tumor-making) can infect wounded plant tissue, transferring a large plasmid, the Ti plasmid, to the plant cell. Part of the Ti (tumor-inducing) plasmid apparently randomly integrates into the chromosome of the plant. The integrated part of the plasmid contains genes for the synthesis of (1) food for the bacterium, and (2) plant hormones. Genes from the Ti plasmid that are integrated in the plant chromosome are expressed at high levels in the plant. Overproduction of the plant hormones leads to continuous growth of the transformed cells, causing plant tumors. Rapid, cancerous growth of the transformed plant tissue obviously is advantageous to the bacterium: more food gets produced.
The Ti plasmid has been genetically modified ("disarmed") by deleting the genes involved in the production of bacterial food and of plant hormones, and inserting a gene that can be used as a selectable marker. Selectable marker genes generally are coding for proteins involved in breakdown of antibiotics, such as kanamycin. Any gene of interest can be inserted into the Ti plasmid as well. In principle, one can thus transform any plant tissue, and select transformants by screening for antibiotic resistance. However, unfortunately, there are some complications: (1) it has proven difficult to transform some monocots (grasses, etc.) by *Agrobacterium*, and (2) regeneration of plants from tissue culture or leaf discs is not always possible.

A number of genetically engineered plant varieties have been developed. Traits that have been introduced by transformation include herbicide resistance, increased virus tolerance, or decreased sensitivity to insect or pathogen attack. Traditionally, most of such genetically engineered plants were tobacco, petunia, or similar species with a relatively limited agricultural application. However, during the past decade it now has become possible to transform major staples such as corn and rice and to regenerate them to a fertile plant. Increasingly, the transformation procedures used do not depend on *Agrobacterium tumefaciens*. Instead, DNA can be delivered into the cells by small, µm-sized tungsten or gold bullets coated with the DNA. The bullets are fired from a device that works similar to a shotgun. The modernized device uses a sudden change in pressure of He gas to propel the particles, but the principle of "shooting" the DNA into the cell remains the same. This DNA-delivery device is nicknamed "gene gun", and has been shown to work for DNA delivery into chloroplasts as well. Over the last several years, use of the "gene gun" has become a very common method to transform plants, and has been shown to be applicable to virtually all species investigated. For example, transformation of rice by this method is now routine. This is a very important development as rice is the most important crop in the world in terms of the number of people critically dependent on it for a major part of their diet.
Another method to get foreign genes into cereals is by electroporation: a jolt of electricity is used to puncture self-repairing holes in protoplasts (i.e., the cell without the cell wall), and DNA can get in through these holes. However, it is often very difficult to regenerate fertile plants from protoplasts of cereals. Nonetheless, significant advances in overcoming these practical difficulties have been made over the years. Now even transgenic trees have been created: for example, the gene for a coat protein of the plum pox virus has been introduced into apricot. The plum pox virus leads to the feared Sharka disease, for which there is no cure. The resulting transgenic tree shows a markedly decreased sensitivity to this virus. The reason why continuous exposure of the tree to the viral coat protein leads to tolerance against viral infection is not yet understood, however.

Thus, now there are a number of different techniques to introduce foreign genes into plants. Essentially all major crop plants can be (and have been or are being) genetically engineered, the procedures are now routine and the frequency of success is very high. Even though genetically engineered crops are more costly than the usual ones, they have been rather readily accepted by US farmers provided that tangible benefits can be demonstrated. However, it is questionable whether the farmer in poorer countries can come up with the funds to "try out" and use the new crops. Another issue in this respect is how genetically engineered crops are perceived by the consumer. Even though in the US there is little resistance to such crops as long as the products can be shown to be safe and advantageous, in other countries (for example in sections of Europe) genetically modified foods are received poorly by the consumer. It is unlikely that there is a rationally sound basis for this rather hostile reaction of the consumer, as most of the crops are the result of human manipulation (such as centuries of breeding) and may have been treated with harmful herbicides and pesticides. Time and education will need to be invested to provide consumers and consumer advocates with a balanced opinion on the acceptability of the origin of their foods.

4.5.4.6 Applications of transgenic plants

Pharming" and "plantibodies"

An increasingly viable option is the production of highly valuable enzymes by plants and animals. In addition to production of human proteins in these organisms (see a subsequent section), other valuable proteins that are currently produced by microorganisms could very well be produced by higher organisms instead. Animal and plant "bioreactors" in some respects may be superior to recombinant bacterial systems, because eukaryotes glycosylate proteins. Whereas the glycosylation pattern may be species-specific, appropriate glycosylation is often required for protein function. Production through these organismal systems may also be cheaper than cell fermentation techniques.

Two examples of production of human proteins in plants include the production of human serum albumin in transgenic tobacco and potato, and production of human insulin by tobacco. In both cases, the produced protein appears to be fully effective in humans. Unfortunately, however, one cannot raise his/her insulin level by eating transgenic tobacco leaves, as the protein in most cases will be broken down to amino acids before it reaches the blood stream. Therefore, in these cases one cannot escape the practice of protein isolation and purification before transgenic leaves are converted into drugs.

Also antibodies are being produced in plants. Initially, the antibody's light and heavy chains were produced in different plants. But a subsequent cross of these two varieties resulted in progeny carrying assembled and functional antibodies. These "plantibodies" are now used for diagnostic and therapeutic purposes (see http://www.epicyte.com for the use of Lemna (a small aquatic plant), and http://www.moleculartarming.com/plantigens.html for some recent examples and information).

Reversible male sterility in plants

As has been indicated earlier, heterozygous individuals often are healthier and stronger than homozygous ones. The only way to guarantee heterozygoiscity in plants is to make

sure self-pollination cannot occur. For most crop plants it was very tedious or practically impossible to exclude selfing. To exclude self-pollination, it would be good to introduce male sterility in plants: progeny from such plants are then expected to be 100% heterozygous (assuming they were pollinated with pollen from an unrelated variety). To introduce male sterility, a promoter was identified that was turned on exclusively in tapetum cells (a tissue around the pollen sac that is essential for pollen production). This promoter then was linked up to a gene coding for a bacterial ribonuclease (named barnase). This ribonuclease selectively chops up ribonucleic acids. The promoter/ribonuclease construct was then introduced into plants (canola, tobacco, you name it). Because the promoter allows expression only in tapetum cells, the gene construct disrupts only development of the tapetal tissue and its end product, pollen. Plants transformed with this construct were male-sterile but otherwise normal.

Although male-sterile plants are valuable for hybrid seed production, they have limited value when it comes to crop production. Fertility must be restored to crops such as wheat, rice, and tomato, in which the seed or fruit is the harvested product. Fortunately, the ribonuclease is inhibited very much by a simple protein, named barstar. One can thus cross the male-sterile plant with a male-fertile variety in which the gene for barstar has been introduced, and the result is progeny with viable pollen and restored fertility.
A closely related approach has been criticized as "terminator technology" as it is seen by its critics as a way for companies to protect and enforce their patents. In any case, several genetically modified crops with barstar and barnase are available. A very useful database of genetically modified crops is available at http://www.agbios.com/dbase.php.

Antisense RNA
Antisense RNA refers to nucleotide strands that are produced in a cell and that are complementary to a particular mRNA. Antisense RNA can be produced, for example, by inverting the coding region of a gene with respect to its promoter. The antisense RNA can hybridize with its corresponding mRNA, making it double-stranded. The double-stranded mRNA no longer can be recognized by the protein-synthesizing machinery (the ribosomes), and thus expression of this mRNA is suppressed. Also, in many systems double-stranded mRNA is very unstable and is broken down quickly. Thus, one can inactivate specific genes while not interfering with others.
Antisense approaches already are used to protect plants from damage by plant viruses. For example, reversal of a gene from bean yellow mosaic virus (BYMV), and putting it into tobacco under a reasonably strong promoter, has led to a tobacco variety that is quite resistant to BYMV (http://www.actahort.org/books/377/377_28.htm). A similar approach is used to transfer viral resistance to other plants. This finding is of significance, in that currently no effective, environmentally friendly methods exist to control many plant viruses.

Very related to this approach is the RNAi (RNA interference) approach (for example, see http://www.ambion.com/techlib/hottopics/rnai/). This is very useful for both agriculture and medicine, and the first examples of practical applications of this RNAi technology are appearing. As with any new technology, the initial pilot projects are sort of pedantic (including manipulation of flower color (http://www.biologynews.

net/archives/2005/04/06/_roses_are_red_and_now_blue_with_the_help_of_csiro_technology.html) and of the speed of fruit ripening). However, more exciting application possibilities abound. Obviously, RNAi technology provides an excellent approach for reverse genetics in eukaryotes. With this method one can turn off genes, and see what the consequences are.

Agricultural applications in developing countries

Perhaps indicative of the large potential and relative ease of genetic engineering, developing countries (particularly China) are progressing rapidly in development and application of genetically engineered crops. Some have gone into commercial production well ahead of similar crops in the US. In China, for example, tomatoes that have been engineered for improved virus resistance have been on the market since late 1992. There are two main reasons for the more rapid commercialization of bioengineered crops in the developing world: (1) less tight governmental approval mechanisms, and (2) hungrier populations. While in developed countries the main value of biotechnological applications may be to reduce production costs, in the developing world a main factor is the production of more food. Indeed, genetic engineering applications seem to be pretty successful to cut down on pathogen-induced losses. For example, genetic modification of papaya plants (expression of the ringspot virus coat protein in the plant) protects very well against the very destructive ringspot virus.

A website that is critical of plant genetic engineering and genetically engineered foods is http://www.bio-integrity.org/. One area of particular concern for some people is the lack of labeling of genetically engineered foods, and legislation may be introduced to address this issue (for example, see http://www.thecampaign.org/ and http://www.safe-food.org/). On the other hand, as so many plants (soybean, corn, etc.) are genetically modified and the nature of the genetic modification is not necessarily easy to explain, it may be simpler to label those foods that are guaranteed free of "genetically modified organisms" or their products. However, keep in mind that essentially all agricultural products have been genetically modified by traditional breeding, so it may be difficult to define what is actually free of genetically modified organisms.

Several plant biotechnology companies have increased their efforts to provide information regarding the full, global scope of impacts of plant biotechnology.

For example,Monsanto(http:/www.biotechknowledge.monsanto.com/) and Du Pont (http://www.dupont.com/biotech/) have useful web sites. These company sites probably are just as subjective as some of the sites listed above that are very critical of genetically modified organisms, and the best solution is to read information provided by both sides and to see what is reasonable.

4.5.4.7 Summary

The production of transgenic animals and plants became easy due to effective gene transfer mechanisms like microinjection and agro bacterium mediated transfer . The technology has already produced transgenic animals such as mice, rats, rabbits, pigs, sheep, and cows.Sreveral trans genic plants are already in use.Trans genic animals have wide applications in various fields like agriculture medicine and other allied fields.

Trans genic plants also proven to be good for medicine as they are being used for the production of antibodies ,called plantibodies.

4.5.4.8 Model questions

1) Give a detailed account on applications of transgenic animals and plants in various fields of biology?

4.5.4.9 Reference books

1) Principles of gene manipulations by OLD and PRIMROSE
2) Recombinant DNA technology by Watson

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