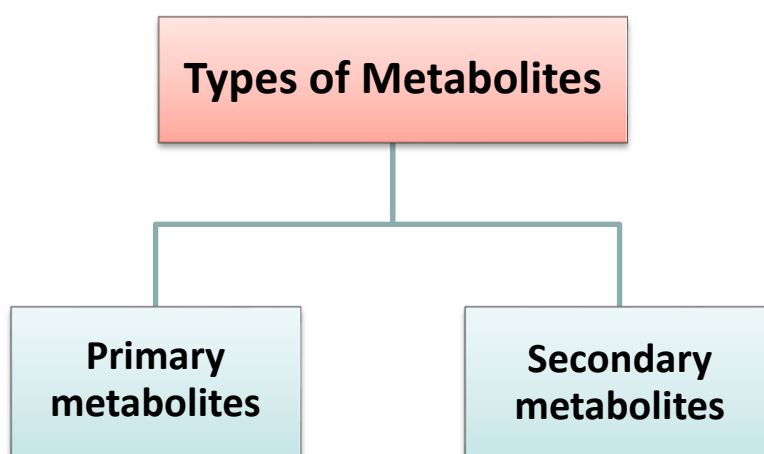


PRIMARY AND SECONDARY METABOLITES

INTRODUCTION

Metabolism-Metabolism constitutes all the chemical transformations occurring in the cells of living organisms and these transformations are essential for life of an organism.

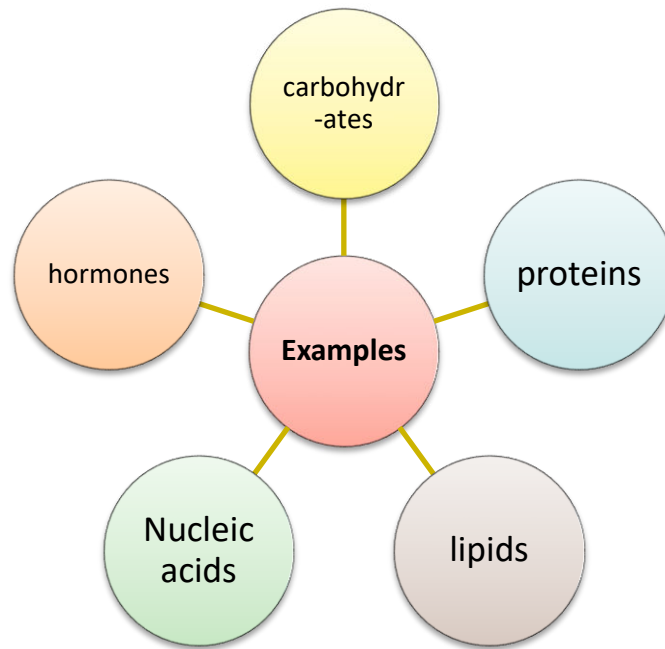
Metabolites-End product of metabolic processes and intermediates formed during metabolic processes is called metabolites.



Primary metabolites

A **primary metabolite** is a kind of [metabolite](#) that is directly involved in normal growth, development, and reproduction. It usually performs a physiological function in the organism (i.e. an intrinsic function). A primary metabolite is typically present in many organisms or cells. It is also referred to as a central metabolite, which has an even more restricted meaning (present in any autonomously growing cell or organism). Some common examples of primary metabolites include: [ethanol](#), [lactic acid](#), and certain [amino acids](#).

In higher plants such compounds are often concentrated in seeds and vegetative storage organs and are needed for physiological development because of their role in basic cell metabolism. As a general rule, primary metabolites obtained from higher plants for commercial use are high volume-low value bulk chemicals. They are mainly used as industrial raw materials, foods, or food additives and include products such as vegetable oils, fatty acids (used for making soaps and detergents), and carbohydrates (for example, sucrose, starch, pectin, and cellulose). However, there are exceptions to this rule. For example, myoinositol and β -carotene are expensive primary metabolites because their extraction, isolation, and purification are difficult.



A plant produces primary metabolites that are involved in growth and metabolism. A primary metabolite plays a very important role in plant metabolism and is essential for the plant existence. Some primary metabolites are precursors of secondary metabolites.

Conversely, a [secondary metabolite](#) is not directly involved in those processes, but usually has an important [ecological](#) function (i.e. a relational function). A secondary metabolite is typically present in a taxonomically restricted set of organisms or cells (Plants, Fungi, Bacteria...).

Some common examples of secondary metabolites include: [ergot alkaloids](#), [antibiotics](#), [naphthalenes](#), [nucleosides](#), [phenazines](#), [quinolines](#), [terpenoids](#), [peptides](#) and [growth factors](#).

Plant growth regulators may be classified as both primary and secondary metabolites due to their role in plant growth and development. Some of them are intermediates between primary and secondary metabolism.

Example:-Alcohol

Ethanol

Aminoacid

Gluromic & Aseptic acid

Organic acid

Lactic acid

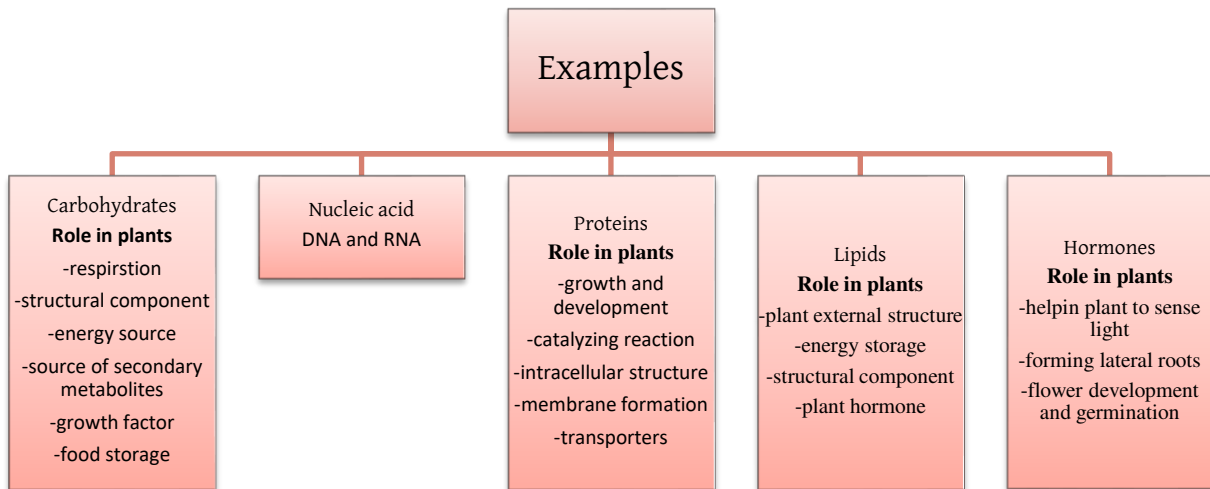
Antioxidant

Isoarobic

Vitamins

B₁₂

Examples of plant primary metabolites include:



Secondary metabolites

Secondary metabolites are not essential as primary metabolites as these are not directly involved in growth, development and reproduction of organisms. They are organic compounds which are not directly involved in survival of plants but they produce some products which aid them in their normal growth and development. Secondary metabolites are compounds biosynthetically derived from primary metabolites but more limited in distribution in the plant kingdom, being restricted to a particular taxonomic group (species, genus, family, or closely related group of families).

Secondary compounds have no apparent function in a plant's primary metabolism but often have an ecological role; they are pollinator attractants, represent chemical adaptations to environmental stresses, or serve as chemical defenses against microorganisms, insects and higher predators, and even other plants (allelochemicals). Secondary metabolites are frequently accumulated by plants in smaller quantities than are primary metabolites. In addition, secondary metabolites, in difficult. As a result, secondary metabolites that are used commercially as biologically active compounds (pharmaceuticals, flavors, fragrances, and pesticides) are generally higher value-lower volume products than the primary contrast to primary metabolites, tend to be synthesized in specialized cell types and at distinct developmental stages, making their extraction and purification metabolites. Thus, compared to primary metabolites (bulk chemicals), many secondary metabolites can be considered as specialty materials or fine chemicals. Secondary metabolites are often large organic molecules that require a large number of specific enzymatic steps for production

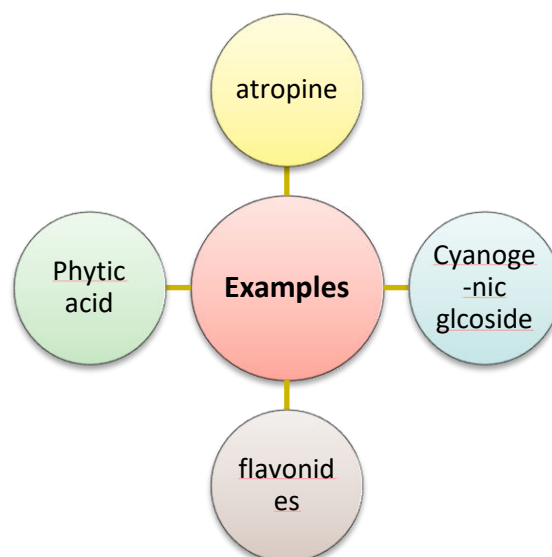
- Synthesis of tetracycline requires at least 72 separate enzymatic steps

- Starting materials arise from major biosynthetic pathways

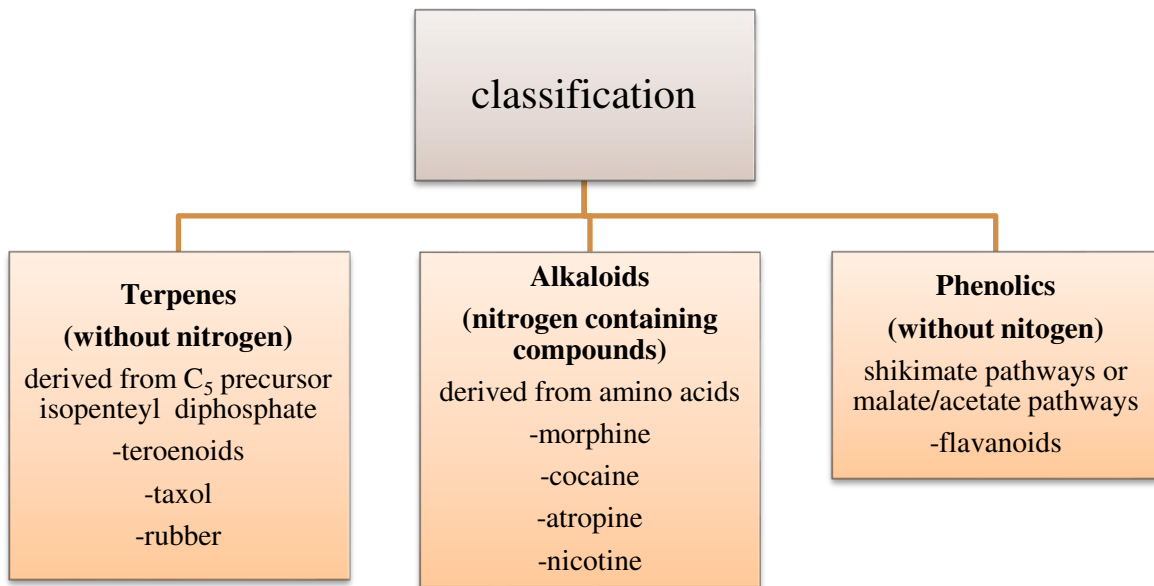
Examples of commercially useful plant secondary metabolites are nicotine, the pyrethrins, and rotenone, which are used in limited quantities as pesticides, and certain steroids and alkaloids, which are used in drug manufacturing by the pharmaceutical industry.

The steroids and alkaloids include steroidal sapogenins, *Digitalis* glycosides, the anticancer *Catharanthus* (formerly *Vinca*) alkaloids, belladonna alkaloids (for example, atropine, hyoscyamine, and scopolamine), cocaine, colchicine, opium alkaloids (codeine, morphine, and papaverine), physostigmine, pilocarpine, quinine, quinidine, reserpine, and *d*-tubocurarine. Other secondary plant metabolites are used in limited quantities as pharmacological tools to study various biochemical processes. For example, diterpene esters (among which are phorbol derivatives) from the latices of various species of *Euphorbia* (members of the spurge family) are potent irritants and cocarcinogens and are useful in studies of chemical carcinogenesis. Secondary metabolites are often large organic molecules that require a large number of specific enzymatic steps for production

- Synthesis of tetracycline requires at least 72 separate enzymatic steps
- Starting materials arise from major biosynthetic pathways



Classification of secondary metabolites:



Compartmentation of secondary metabolites:

Cytosol	Hydrophilic compounds
Chloroplast	Alkaloids and Terpenoids
Mitochondria	Some amines, alkaloids
Vesicles	Alkaloids
Endoplasmic Reticulum	Lipophilic

The primary metabolites consist of the vitamins, [ethanol](#), nucleosides, [organic acid](#), and certain [amino acids](#). Which are necessary at the time of logarithmic phase of microbial growth. But the products like alkaloids, steroids, antibiotics, gibberellins, toxins are the secondary metabolite compound produced during the stationary phase of the cell growth.

Ethyl Alcohol or Ethanol Production from Molasses by Fermentation

Sugar industries produce molasses from the sugar cane processing. Molasses have 50 -55% concentration of sugar in the form of sucrose, with chemical formula $C_{12}H_{22}O_{11}$. This source of compound is used for preparing ethyl alcohol. Ethanol in the form of absolute and rectified spirit can be made from molasses. Basis raw materials for an industry to produce 1 ton of ethyl alcohol requires, molasses up to 5.6 tons, sulfuric acid 27 kg and ammonium sulphate 2.5 kg.

The chemical reactions involved for ethanol production from molasses:

Main reaction:

$C_{12}H_{22}O_{11} + H_2O \rightarrow 2C_6H_{12}O_6$,with enzyme invertase.

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$ -31.2 KCal ,with enzyme zymase.

Side reaction:

$2C_6H_{12}O_6 + H_2O \rightarrow ROH + RCHO$ (high molecular weight alcohols C_2H_5OH)

Description of the ethanol production process:

Large volume storage tanks of molasses provide continuous supply of molasses and store the fresh molasses from sugar processing section during the fermentation process. The molasses from the tanks are diluted with water to obtain the sugar concentration around 10-15%. The acidic nature of molasses supports the growth of yeast during breaks up of sucrose, for that purpose acids are added to maintain the pH between 4 and 5.

Continuous diluter equipment takes up this task. A yeast culture tank, which is provided with nutrition supply of ammonium and magnesium phosphate or sulphate, is used as nutrient to the yeast. The acidic condition favours the yeast to produce catalytic enzymes, invertase and zymase.

Diluted and treated molasses and the yeast from storage are fed to the fermentation chamber. Modern fermentation tanks are made with stainless steel material provided with heating coils or jacket provision. The temperature 20-30 °C is maintained in the tanks by the heating and cooling system.

The process of fermentation takes place around 30-70 hours based on the temperature and sugar concentration to yeast count. Final temperature 35 °C is attained at the end of the process. During the fermentation process, microorganism yeast produces carbon dioxide as by-product. After the process cycle, the product liquid mixture is fed to beer still to perform distillation. Solid and slurry mass is separated leaving the solution of alcohol and water. The concentration of alcohol in the liquid mixture would around 8-10%.

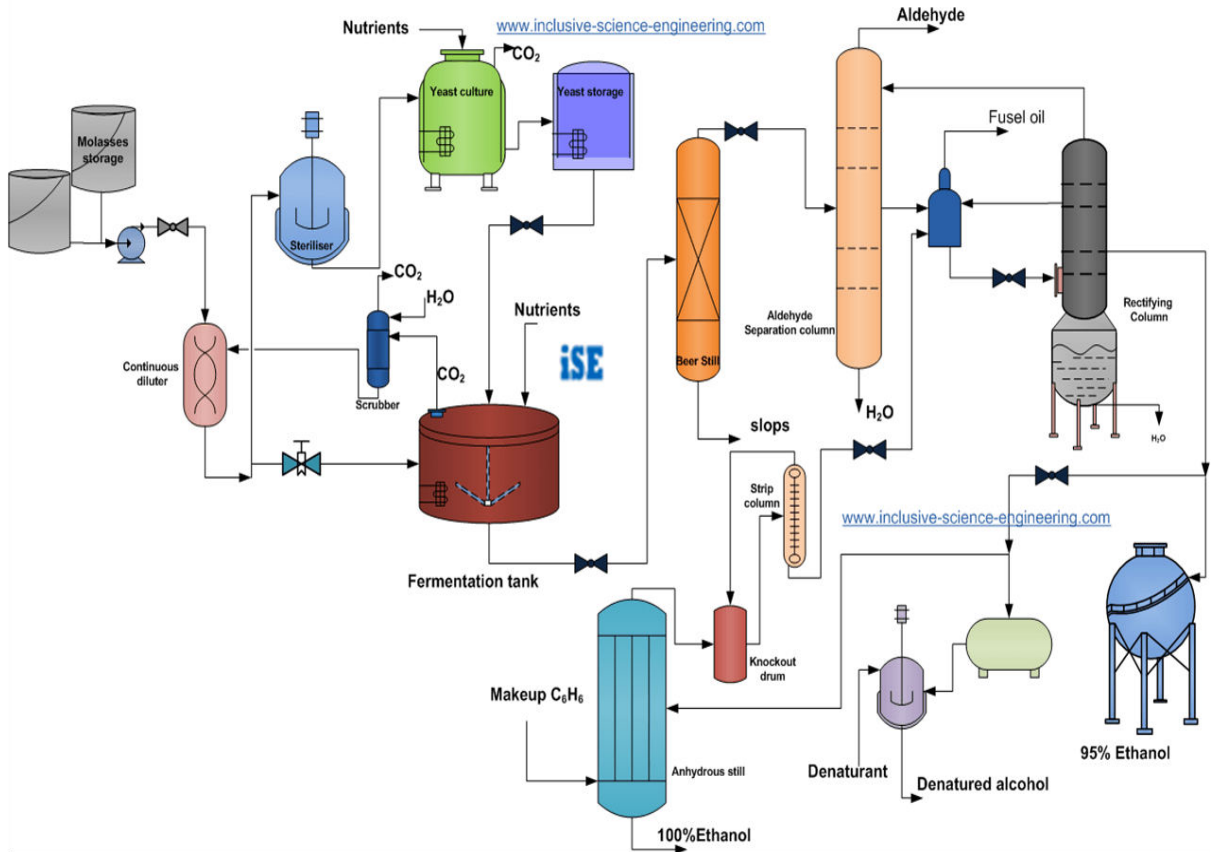
A series of beer still work out to produce different quality of beer products. The slurry form of material obtained from bottom of beer still is called as slops. It is used for cattle feed and fertilizer after some waste treatment operations.

However, the aldehydes are not allowed in consumable beer so aldehydes present in the solution are removed by aldehyde column. The streams coming out at different section of the column are aldehydes from top, fusel oil and ethanol mixture from middle and bottom stream with water.

The middle stream is fed to rectification column to produce a product called rectified spirit having 95% ethanol. [Rectified spirit further made to absolute alcohol](#) by anhydrous still using benzene as third component.

Absolute alcohol with 100% ethanol concentration is a standard product used as intermediate for producing other chemical products and blending agent in power fuels. The end use of the ethanol would be largely in solvent, acetaldehyde, [acetic acid](#), polyethylene and synthetic rubber production

Ethyl alcohol manufacturing by molasses fermentation



Industrial production of ethanol from molasses

Ethanol production can be increased by utilizing sugarcane juice also. Fermentation molasses is not disturbed if treated sugarcane juice is added at frequent intervals.

Other methods for ethanol production:

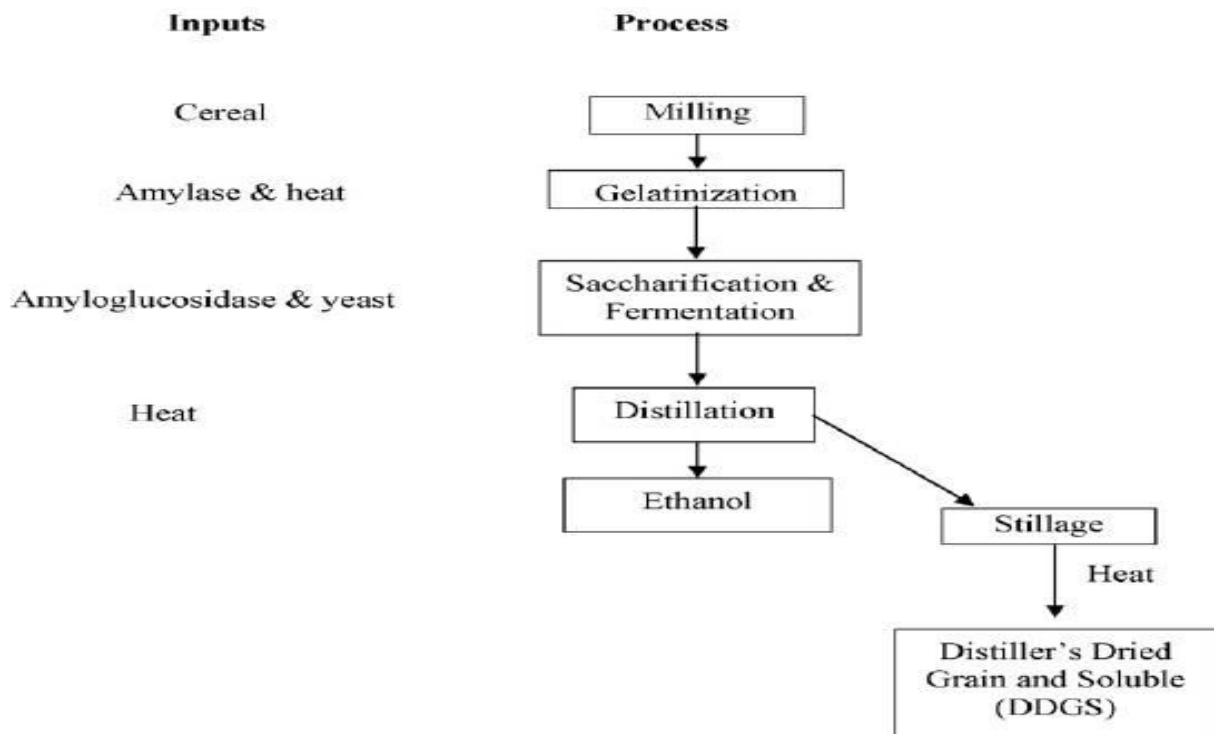
- Ethanol manufacturing by catalytic hydration of ethylene: Direct method
- Indirect method using sulfuric acid

Application of ethanol as fuel in IC engines:
Gasoline is blend with methanol or ethanol that was produced biologically. This type of fuel is used for cars and petrol engine vehicles that already run on roads. Scientists claim that using such fuels will reduce the greenhouse gas emission. Petrol engines used in automobiles used spark plug ignition technology and the fuel vapours are combusted in the cylinder.

Gasoline itself is not the outstanding fuel in terms of octane number. The octane value of the fuel has increased by adding additives in recent times. The additives that were used in gasoline has harmful effect on environment for example tetraethyl lead is toxic compound, MTBE cause water pollution and when coming toluene and benzene they are best cancer causing compounds.

Ethanol now used as additive that replaces all other harmful compounds. Due to oxygen content in its structure it improve combustion and decrease the emission of harmful unburnt hydrocarbons and carbon monoxide.

Flow chart of ethanol production from cereal grains



Strain improvement of industrially important microorganisms



Strain Improvement

The Science and technology of manipulating and improving microbial strains, in order to enhance their metabolic capacities for biotechnological applications, are referred to as strain improvement.

Targets of strain improvement

- Rapid growth
- Genetic stability
- Non-toxicity to humans
- Large cell size, for easy removal from the culture fluid
- Ability to use cheaper substrates
- Elimination of the production of compounds that may interfere with downstream processing
- Increase productivity.
- To improve the use of carbon and nitrogen sources.
- Reduction of cultivation cost
 - lower price in nutrition.
 - lower requirement for oxygen.
- Production of
 - additional enzymes.
 - compounds to inhibit contaminant microorganisms.

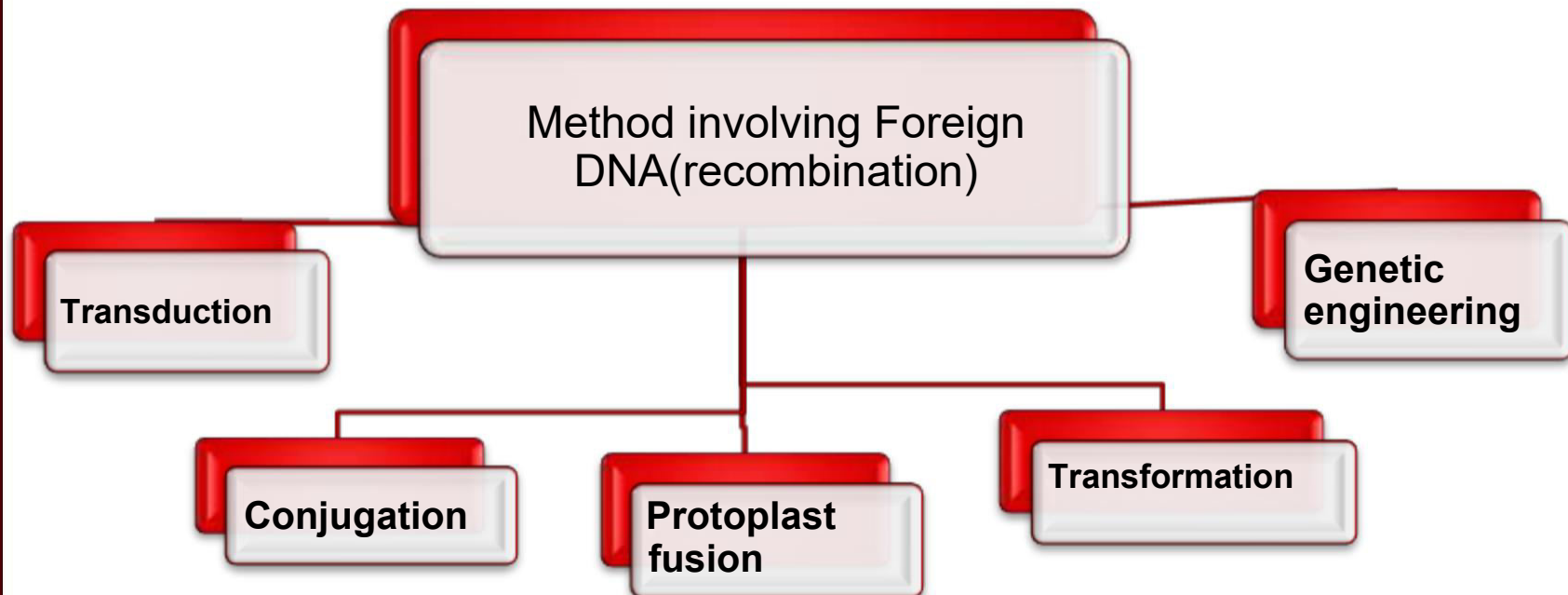
Optimization of microbial activity

It can be done by

- ❑ Optimizing environmental conditions
- ❑ Optimizing nutrition of microorganisms
- ❑ Other includes

1. Method not involving foreign DNA—**Mutagenesis**

2.



Optimizing environment conditions

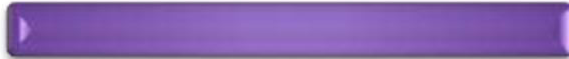
- ❖ Modification of physical parameter (temperature, agitation, etc)
- ❖ Modification of chemical parameter (pH, O₂ concentration)
- ❖ Modification of biological parameter (enzymes)

Optimization of nutrition of microorganisms

- Carbon sources
- Nitrogen sources
- Mineral sources and other sources
- Precursor
- Enzymes

Proper strain used in Industry
Genetically regarded as safe
GRAS

Bacteria



– *Bacillus subtilis*

– *Lactobacillus bulgaricus*

– *Lactococcus lactis*

– *Leuconostoc oenos*

Yeasts

– *Candida utilis*

– *Kluyveromyces marxianus*

– *Kluyveromyces lactis*

– *Saccharomyces cerevisiae*

Filamentous fungi

– *Aspergillus niger*

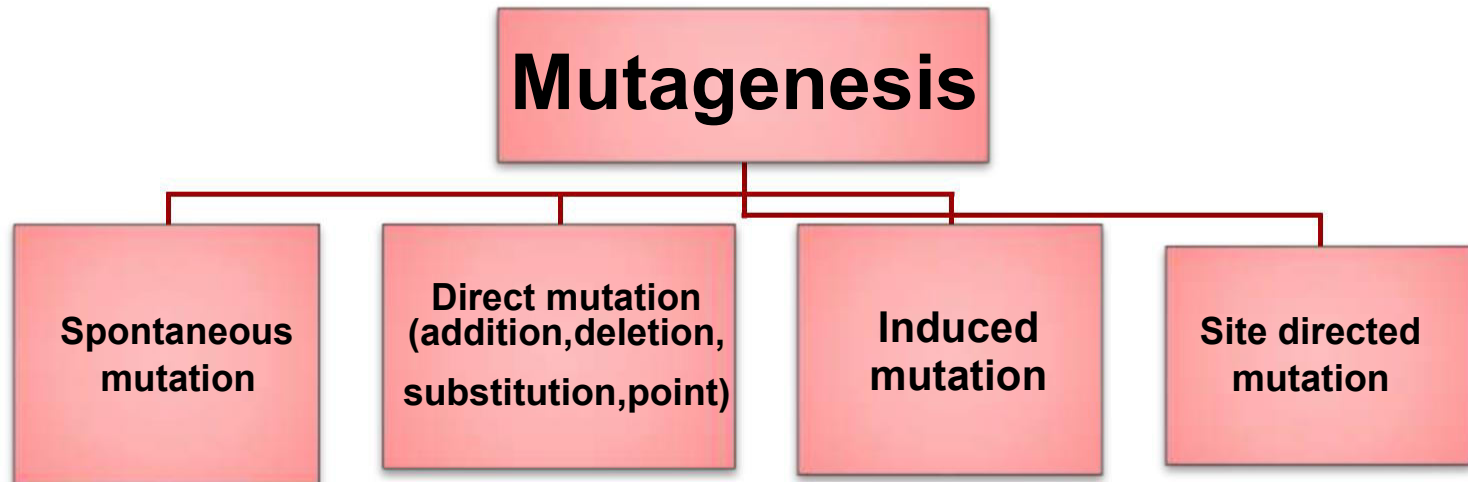
– *Aspergillus oryzae*

– *Mucor javanicus*

– *Penicillium roqueforti*

1. MUTAGENESIS

- Mutagenesis is a process of treatment given to microorganism which will cause an improvement in their genotypic and phenotypic performances



Types of mutation

1. Spontaneous mutation:

- Occur spontaneously at the rate of 10^{-10} and 10^{-15} per generation.


2. Induced mutation:

- The rate of mutation can be increased by various factors and agents called mutagens.
- ionizing radiations (*e.g.* X-rays, gamma rays)
- non-ionizing radiations (*e.g.* ultraviolet radiations)
- various chemicals (*e.g.* mustard gas, benzene, ethidium bromide, Nitroso guanidine-NTG)

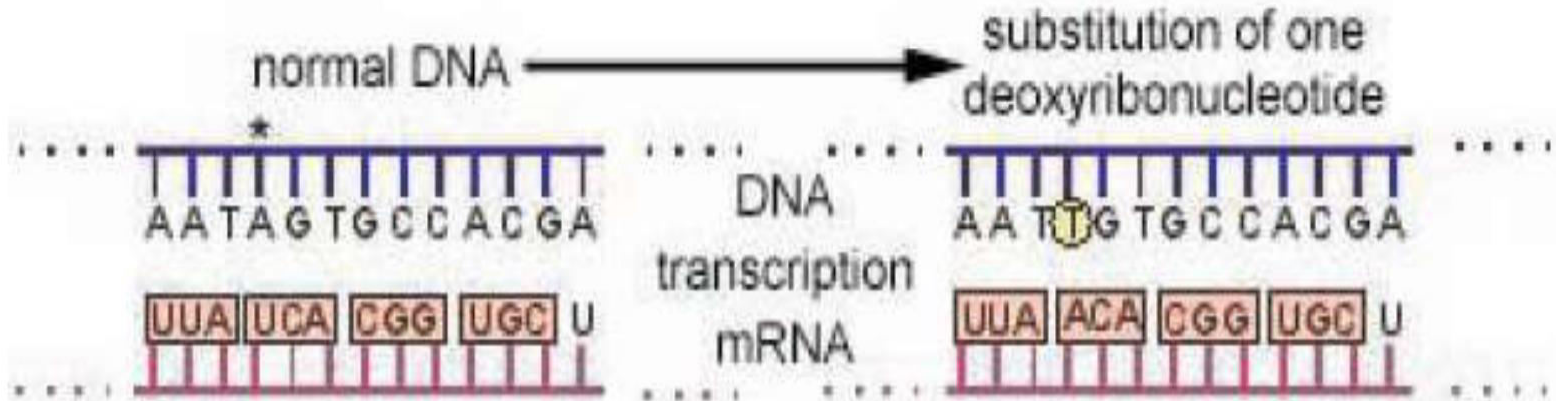
MUTAGEN	MUTATION INDUCED	IMPACT ON DNA	RELATIVE EFFECT
Ionizing Radiations-X Rays,gamma rays	Single or double strand bearkage of DNA	Deletion/structura l changes	high
UV rays,chemicals	Pyrimidine dimerisation	Trnsversion,deleti on,frameshift transitions from GC AT	Medium
Hydroxylamine(NH ₂ OH)	Deamination of cytosine	GC A T transitions	low
N-Methyl –N'- Nitro N-Nitrosoguanidine	Methylation of bases and high pH	GC AT transitions	high
Nitrous acid(HNO ₂)	Deamination of A,C&G	Bidirectional transitions,deletio n,AT GC/GC AT	Medium
Phage,plasmid,D NA transposing	Base substitution,break age.	Deletion,duplicati on,insertion.	high

CHOICE OF MUTAGEN

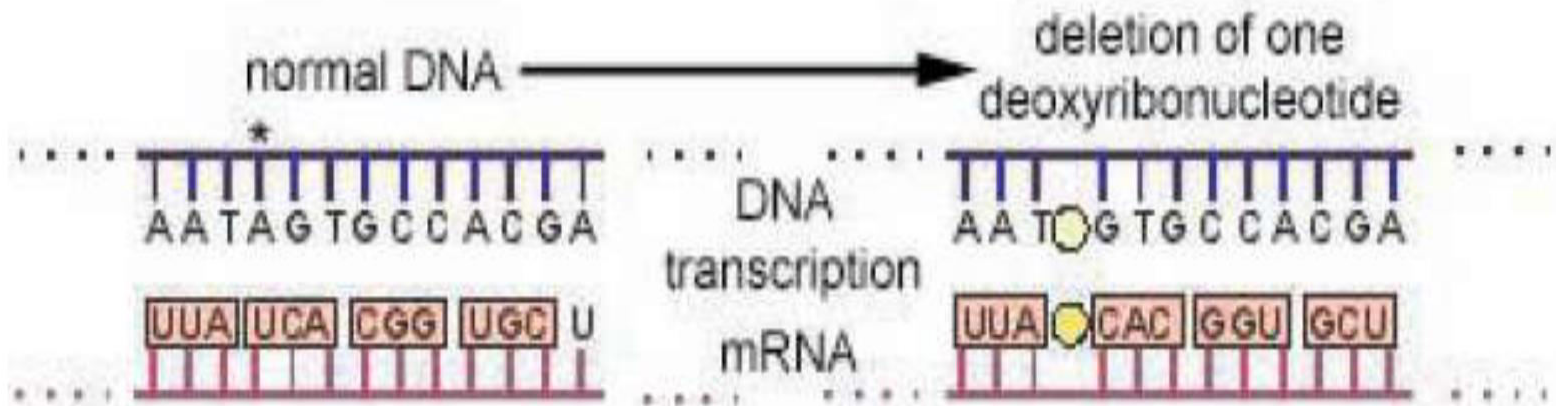
- Mutagenic agents are numerous but not necessarily equally effective in all organisms.
- Other factors--
 - (a) the safety of the mutagen.
 - (b) simplicity of technique.
 - (c) ready availability of the necessary equipment and chemicals.

- 
- Among physical agents, UV is to be preferred since it does not require much equipment, and is relatively effective and has been widely used in industry.
 - Chemical methods other than NTG (nitrosoguanidine) are probably best used in combination with UV.
 - The **disadvantage** of UV is that it is absorbed by glass; it is also not effective in opaque or colored organisms.

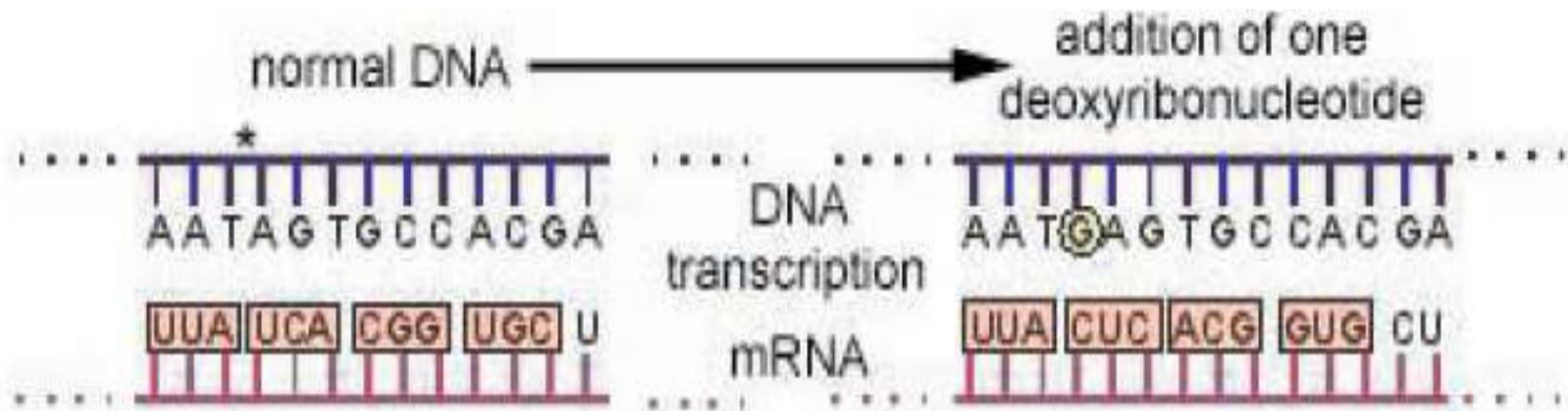
3. Direct mutations



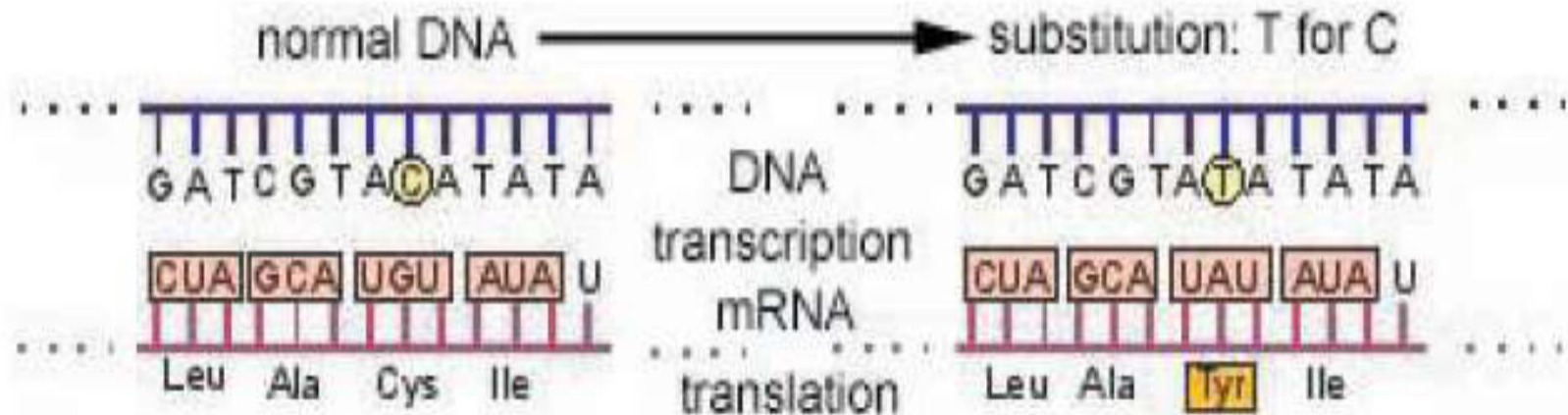
i) Point Mutation or Substitution of a Nucleotide



ii) Deletion of a nucleotide



iii) Addition of a Nucleotide



iv) Substitution of a nucleotide: Results in one wrong codon and one wrong amino acid

3. Site directed mutations(SDM)--

- ✓ Change in the base sequence of DNA
- ✓ changing the codon in the gene coding for that amino acid.
- ✓ It has helped to raise the industrial production of enzymes, as well as to produce specific enzymes

STEPS INVOLVED IN SITE DIRECTED MUTAGENESIS

Isolate required enzyme gene, *e.g.* via mRNA and its conversion into cDNA.



Sequence the DNA of the gene (in order to decide on change required for primer in stage 5).



Splice gene into M13 vector dsDNA and transduce *E. coli* host cells.



Isolate ssDNA in phage particles released from host cells.



Synthesize an oligonucleotide primer with the same sequence as part of the gene but with altered codon (mismatch/mispair) at desired point(s).

For example, one of the codons in DNA coding for the amino acid Alanine is CGG. If the middle base is changed by SDM from G to C the codon sequence becomes CCG which codes for a different amino acid (Glycine).

Cont..

Mix oligonucleotide with recombinant vector ssDNA.

Carried out at low temperature (0-10⁰ C) and in high salt concentration to allow hybridization between oligonucleotide and part of gene.

Use DNA polymerase to synthesize remainder of strand. (Oligonucleotide acts as a primer for the DNA synthesis). Then add ligase to join primer and new strand

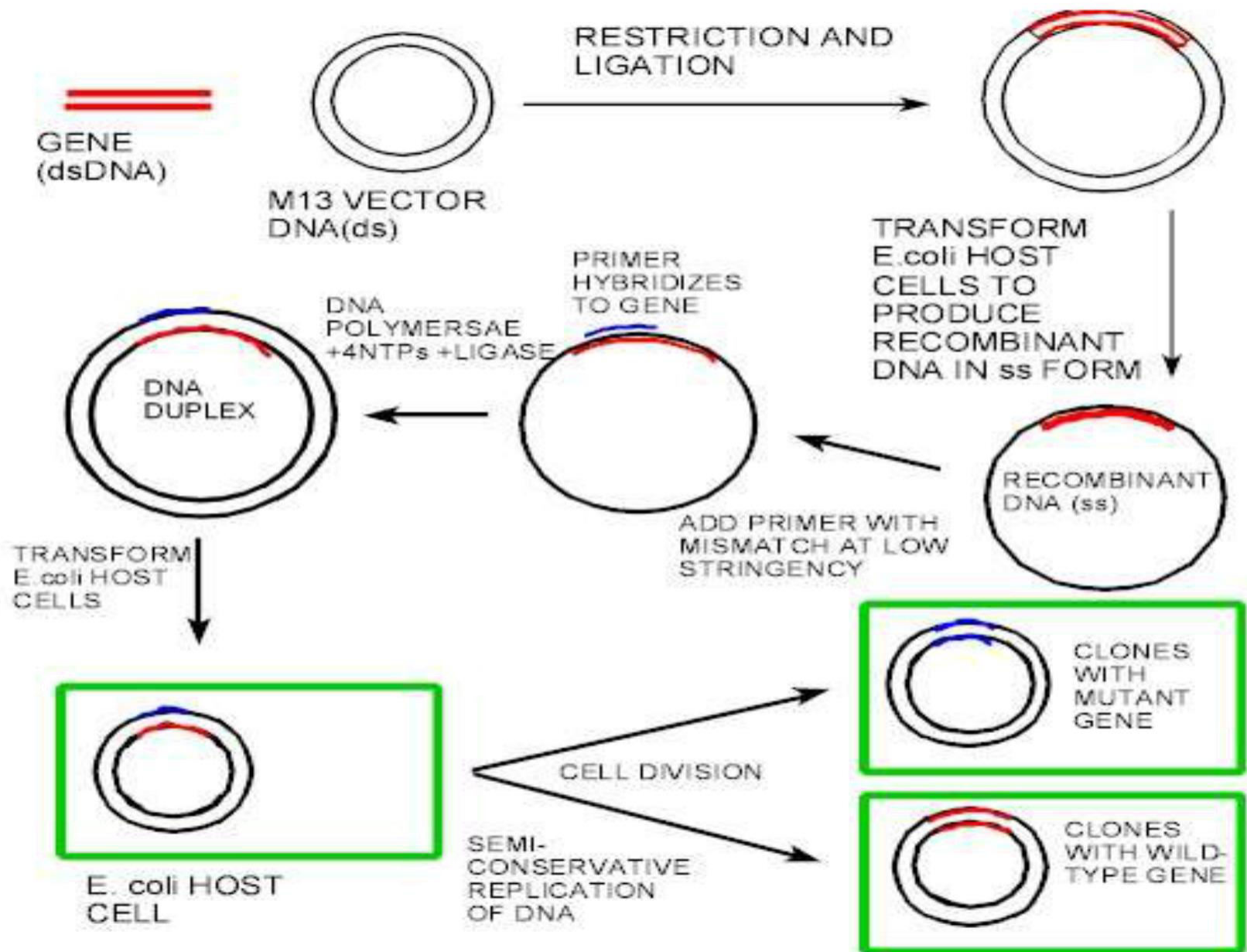
dsDNA molecule.

Transform *E. coli* cells and allow them to replicate recombinant vector molecule.

DNA replication is semi -conservative, therefore two types of clone are produced each of which excretes phage particles containing ssDNA:

- Type 1: contain the wild-type gene (*i.e.* unaltered)
- Type 2: contain the mutated gene!!!

SDM USING M13 PHAGE VECTOR



Reports on strain improvement by mutation-

- Karana and Medicherla (2006)- lipase from *Aspergillus japonicus* MTCC 1975- mutation using UV, HNO₂, NTG showed 127%, 177%, 276% higher lipase yield than parent strain respectively.
- Sandana Mala *et al.*, 2001- lipase from *A. niger* - Nitrous acid induced mutation – showed 2.53 times higher activity.
- Medically useful products Demethyltetracycline and doxorubicin were discovered by mutations from tetracycline and daunorubicin(Shir *et al.*, 1969).Hybramycines were also made by this way.
- First superior penicillin producing mutant, *Penicillium chrysogenum* X-1612, was isolated after X ray

Random Screening

- After inducing the mutations ,survivors from the population are randomly picked and tested for their ability to produce the metabolite of interest.
- **A very large number of colonies must be tested**
- **Advantage-**
over genetic engineering ,with minimal startup time and sustaining for years.
- **Disadvantage-**
Non-targeted and non-specific.

Rational Screening

Rational screening requires some basic understanding of product metabolism and pathway regulation which gives information about metabolic check points and suggest ways to isolate mutants with specific traits.

- Environmental conditions i.e. pH, temp, aeration can be manipulated or chemicals can be incorporated in the culture media to select mutants with desired traits.

Applications -

- **Selection of mutants resistant to the antibiotic produced**
- **Selection of morphological variants**
- **Reversion of nonproducing mutants**
- **Selective detoxification**
- **Selection of overproducers of a biosynthetic precursor**




Three stages before
a mutant can come
into use:


(i) *Exposing organisms to the mutagen:*

- The organism undergoing mutation should be in **the haploid stage** during the exposure.
- **Bacterial** cells are haploid; in **fungi** and **actinomycetes** the haploid stage is found in the spores.
- The use of haploid is essential because many mutant genes are recessive in comparison to the parent or wild-type gene.

Selection for mutants:

- ❑ Following exposure to the mutagen the cells should be suitably diluted and plated out to yield 50 – 100 colonies per plate.
- ❑ The selection of mutants is greatly facilitated by relying on the morphology of the mutants or on some selectivity in the medium.
- ❑ When morphological mutants are selected, it is in the hope that the desired mutation is pleotropic (i.e., a mutation in which change in one property is linked with a mutation in another character).
- . The classic example of a pleotropic mutation is to be seen in the development of penicillin-yielding strains of *Penicillium chrysogenum*.
- After irradiation, strains of *Penicillium chrysogenum* with smaller colonies and which also sporulated poorly, were better producers of penicillin.

- 
- Similar increases of metabolite production associated with a morphological change have been observed in organisms producing other **antibiotics: cycloheximide, nystatin and tetracyclines.**
 - It is desired to select for mutants able to stand a higher concentration of alcohol, an antibiotic, or some other chemical substance, then the desired level of the material is added to the medium on which the organisms are plated. Only mutants able to survive the higher concentration will develop
 - Most efficient method is to grow them on selective media, which contain increasing concentrations of pollutant.
 - Most of bacteria might well grow on 1-2% concentration of this substance. However, as the

- 
- The concentration of the toxic pollutant could be gradually increased in the growth medium thus selecting the most resistant ones. This method is called acclimatization.

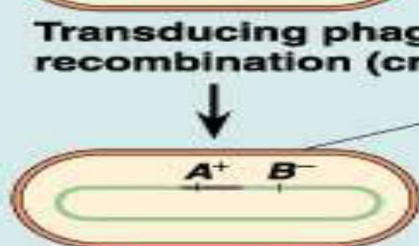
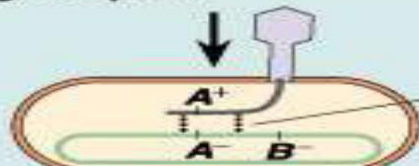
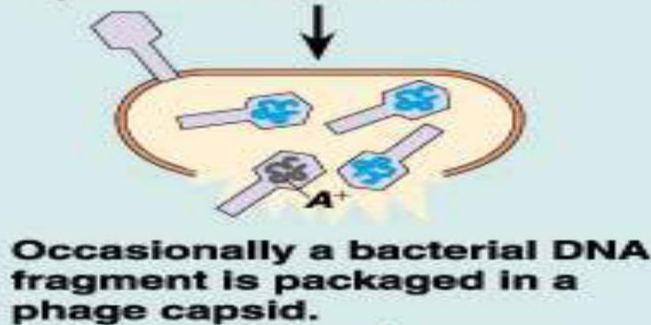
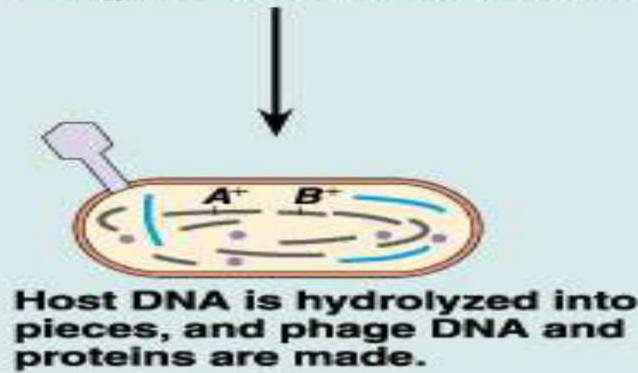
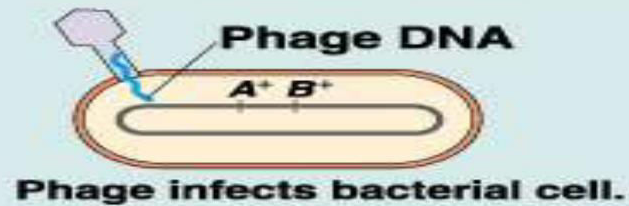
Screening of mutant:

- Screening must be carefully carried out with statistically organized experimentation to enable one to accept with confidence any apparent improvement in a producing organism.
- better Use in industrial practice where time is important to carry out as soon as possible a series of mutations using ultraviolet, and a combination of ultraviolet and chemicals and then

2. Transduction-

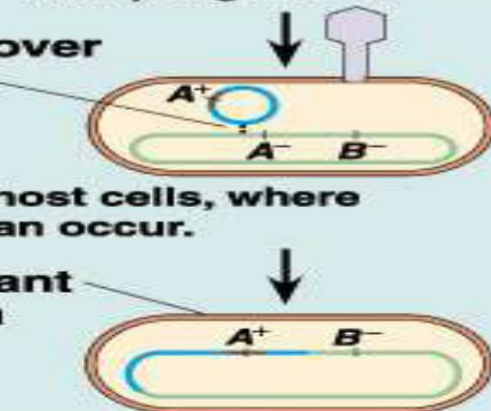
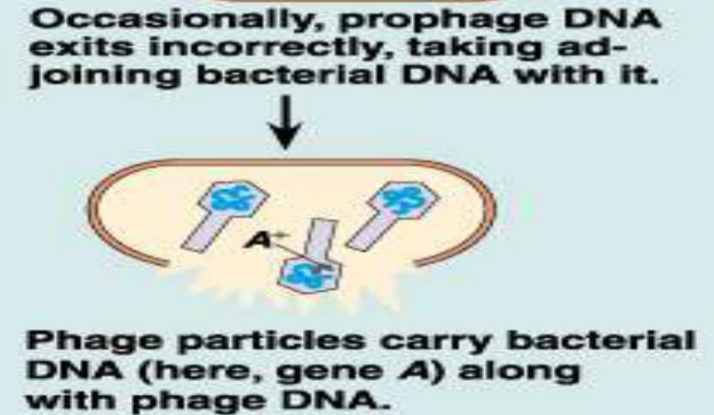
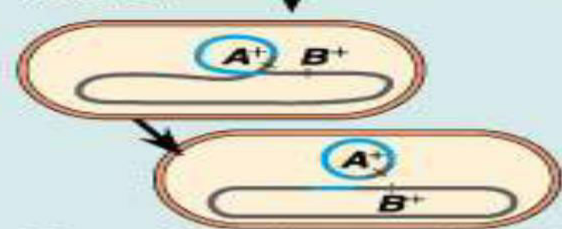
- Transduction is the transfer of bacterial DNA from one bacterial cell to another by means of a bacteriophage.
- Two types:
 - *general transduction* and
 - *specialized transduction*.
- In general transduction, host DNA from any part of the host's genetic apparatus is integrated into the virus DNA.
- In specialized transduction, which occurs only in some temperate phages, DNA from a specific region of the host DNA is integrated into the viral DNA and replaces some of the virus' genes.
- The method is a well-established research tool in **bacteria** including **actinomycetes** but prospects for its use in **fungi appear limited**.

(a) Generalized transduction



The recombinants have genotypes (A⁺ B⁻) different from either the donor (A⁺ B⁺) or recipient (A⁻ B⁻).

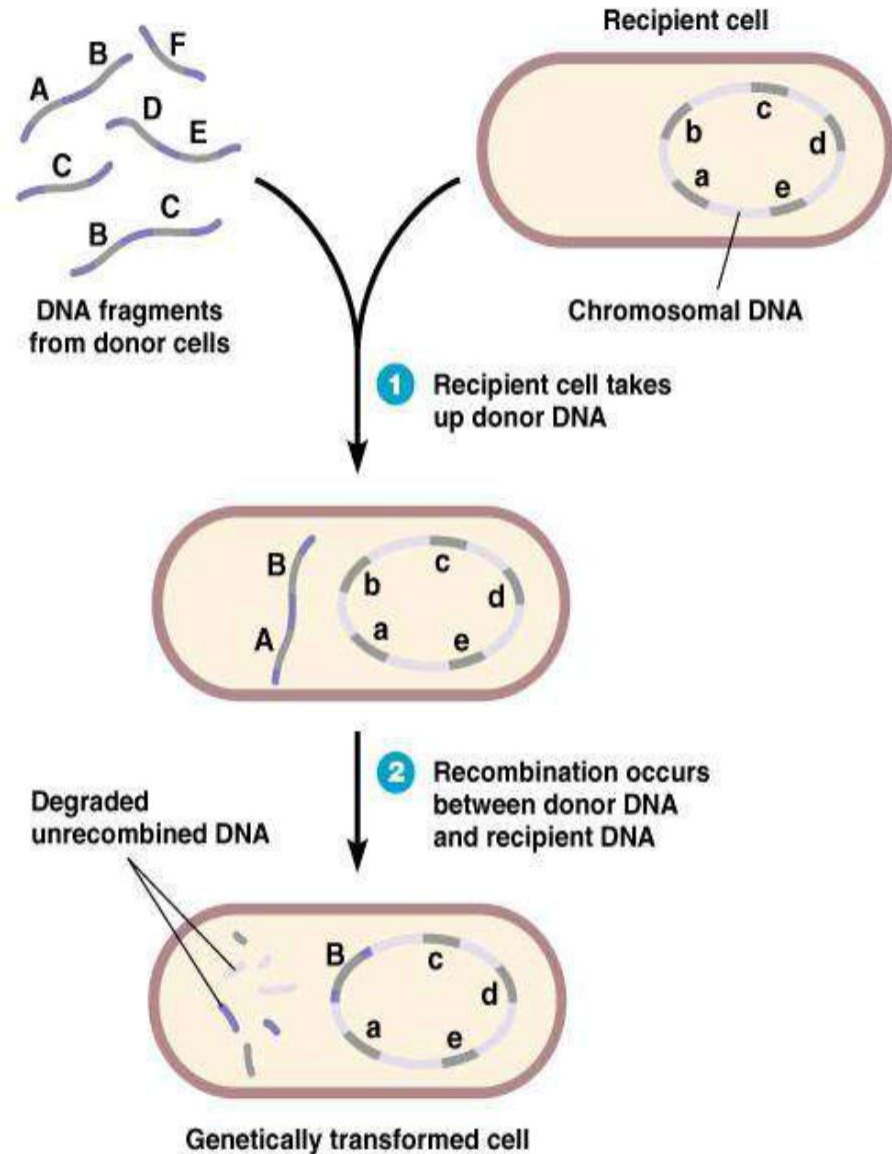
(b) Specialized transduction




The recombinants have genotypes (A⁺ B⁻) different from either the donor (A⁺ B⁺) or recipient (A⁻ B⁻).

3. Transformation

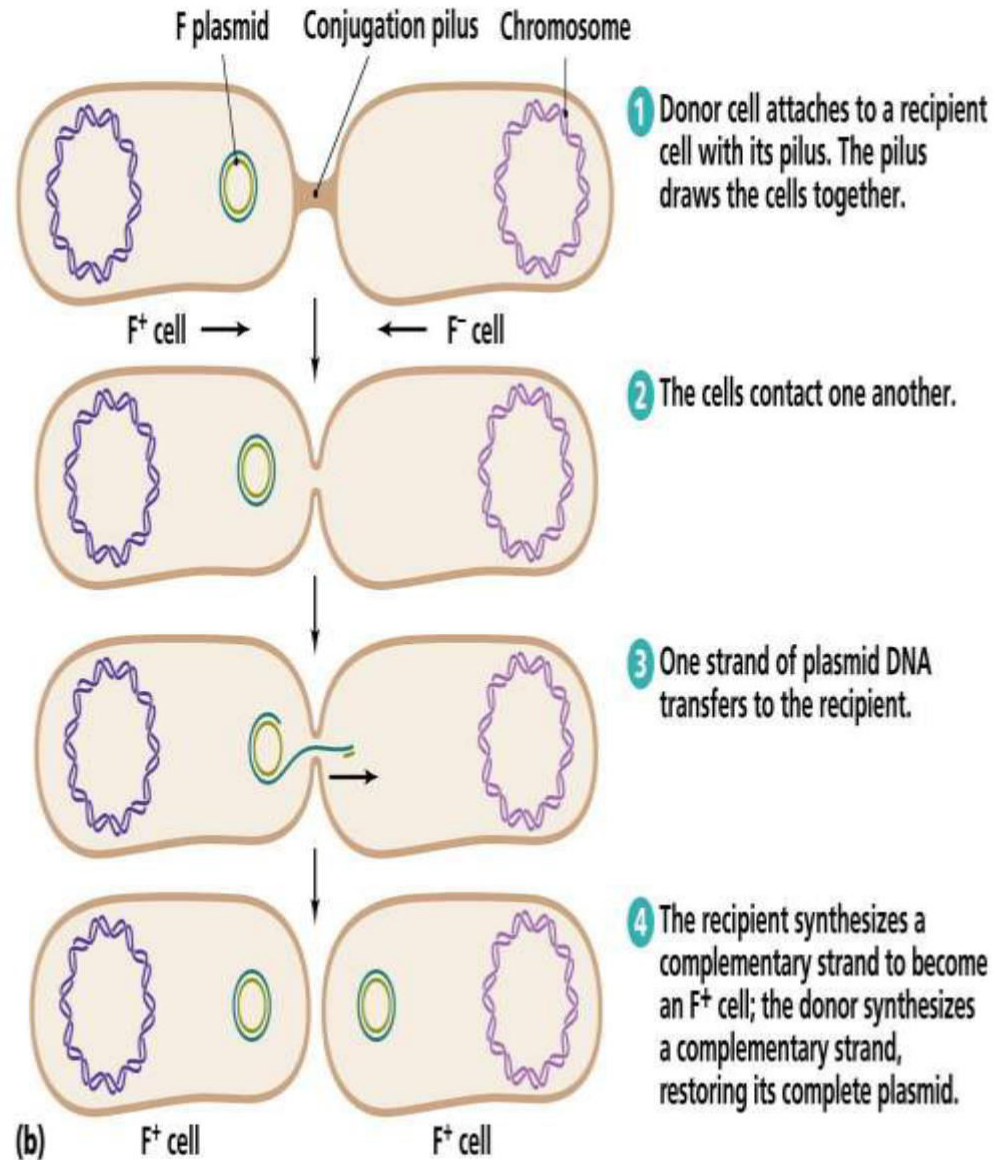
- When foreign DNA is absorbed by, and integrates with the genome of, the donor cell.
- Cells in which transformation can occur are 'competent' cells.



- 
- In some cases competence is artificially induced by treatment with a calcium salt.
 - The method has also been used to increase the level of protease and amylase production in *Bacillus* spp.
 - The method therefore has good industrial potential.

4. Conjugation--

- ❖ Conjugation involves cell to cell contact or through sex pili (*singular*, pilus) and the transfer of plasmids.
- ❖ The donor strain's plasmid must possess a sex factor as a prerequisite for conjugation; only donor cells produce pili.
- ❖ The sex factor may on occasion transfer part of the hosts' DNA.
- ❖ Plasmids play an



5. PROTOPLAST FUSION

- Protoplasts are formed from bacteria, fungi, yeasts and actinomycetes when dividing cells are caused to lose their cell walls.
- Fusion from mixed populations of protoplasts is greatly enhanced by the use of polyethylene glycol (PEG).
- The method has great industrial potential and experimentally has been used to achieve higher yields of antibiotics through fusion with protoplasts from different fungi.
- Protoplast fusion has been demonstrated as an efficient way to induce hetero-karyon formation and recombination with high frequency (Anne and

- Protoplast fusion has been successfully done with

Bacillus subtilis and *B. megaterium* (Fodor and Alföldi, 1976) among several species of *Streptomyces* spp. Like *S. coelicolor*, *S. acrimycini*, *S. olividans*, *S. pravulies* (Hopwood *et al.*, 1977) between the fungi *Geotrichum* and *Aspergillus* (Ferenczy *et al.*, 1974) and Yeasts (Sipiczki and Ferenczy, 1977)


Reports on strain improvement by protoplast fusion

- Kim *et al.*, 1998 did a comparative study on strain improvement of ***Aspergillus oryzae*** for protease production by both mutation and protoplast fusion.
 - UV radiation – 14 times higher yield.
 - Ethyl methane sulphonate – 39 times higher yield.
 - Protoplast fusion – using PEG and CaCl₂ – 82 times higher yield.
- An intergeneric hybrid was obtained from ***Aspergillus niger*** and ***Penicillium digitatum***

for enhancing the production of verbenol, a highly valued food flavorant (Rao *et al*, 2003)

6. Genetic Engineering

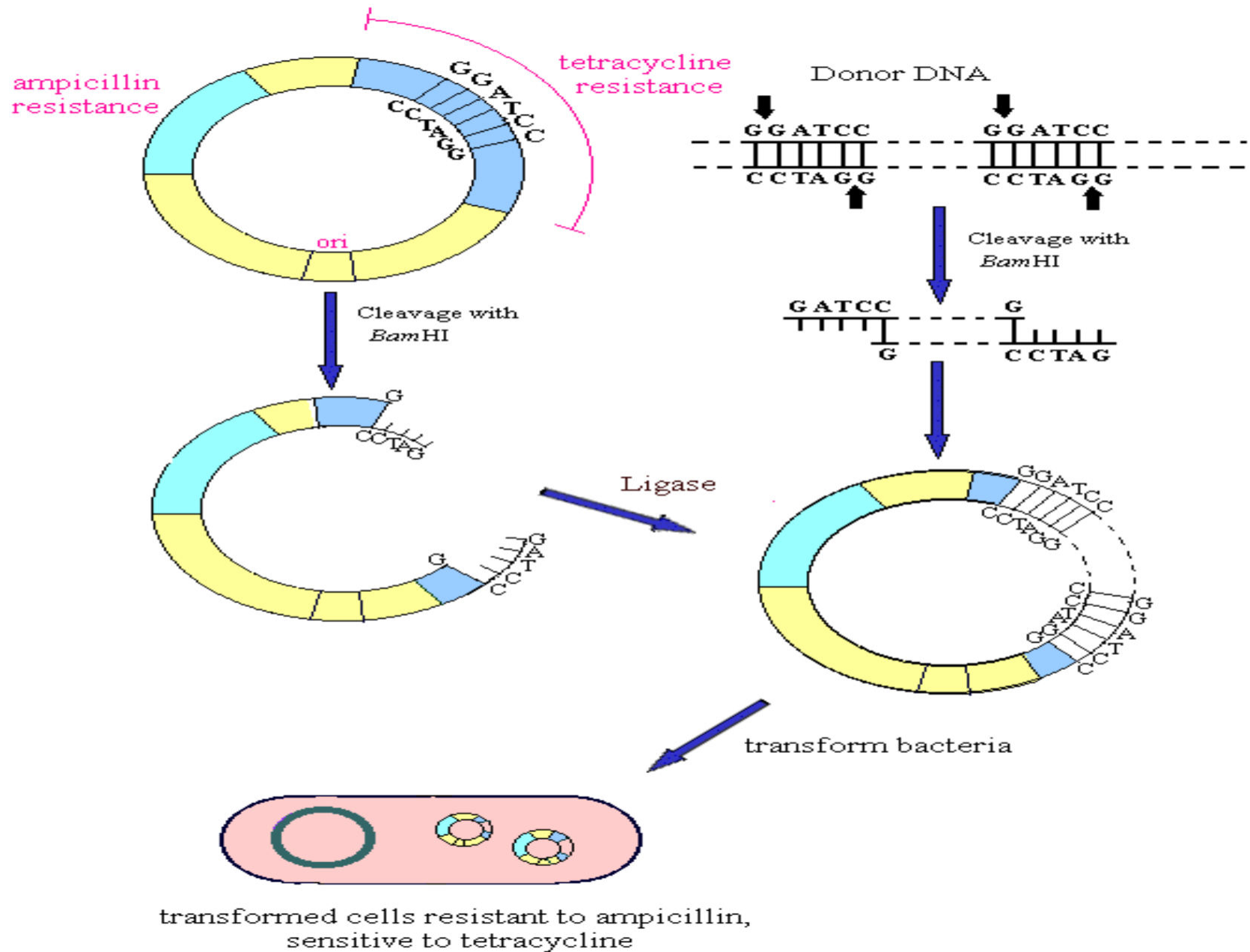
- ❖ Genetic engineering, also known as recombinant DNA technology, molecular cloning or gene cloning.
- ❖ Recombinant DNA Technology enables isolation of genes from an organism, this gene can be amplified, studied, altered & put into another organism
- ❖ Recombinant DNA procedure:
 - i. Cutting of donor DNA : Restriction endonucleases cut DNA molecule at specific sites and desired fragment is isolated by gel electrophoresis.
 - ii. Cloning of a gene : DNA fragment, which wanted to be cloned, is joined to one of vectors (plasmid, phage, cosmid). For this purpose, vector and donor DNA are first cleaved with the same restriction endonuclease, or with the ones producing the same ends

- 
- Then by using DNA ligase, DNA fragment and vector DNA is joined. If fragment has no sticky ends, homopolymer tailing or linker DNA segments can be applied for this step.

iii. **Transformation** : Recombinant vector is put into suitable host organism, like; bacteria, yeast, plant or animal cells, by several physical or chemical methods. Transformed cells are identified by several ways:

- a. Insertional inactivation (of antibiotic resistant genes on plasmids),
- b. nucleic acid hybridization
- c. labeled Ab's for specific proteins (immunological test) are helpful for screening recombinant colonies.

Insertional inactivation-



b. Nucleic acid hybridization

- ***Probe is nucleic acid sequence of the gene of interest, can be whole or partial sequence, can be RNA or DNA***
- If nucleic acid sequence of interested gene is known, synthetic probes can be designed easily, also amino acid sequence is used for probe preparation.

VECTORS

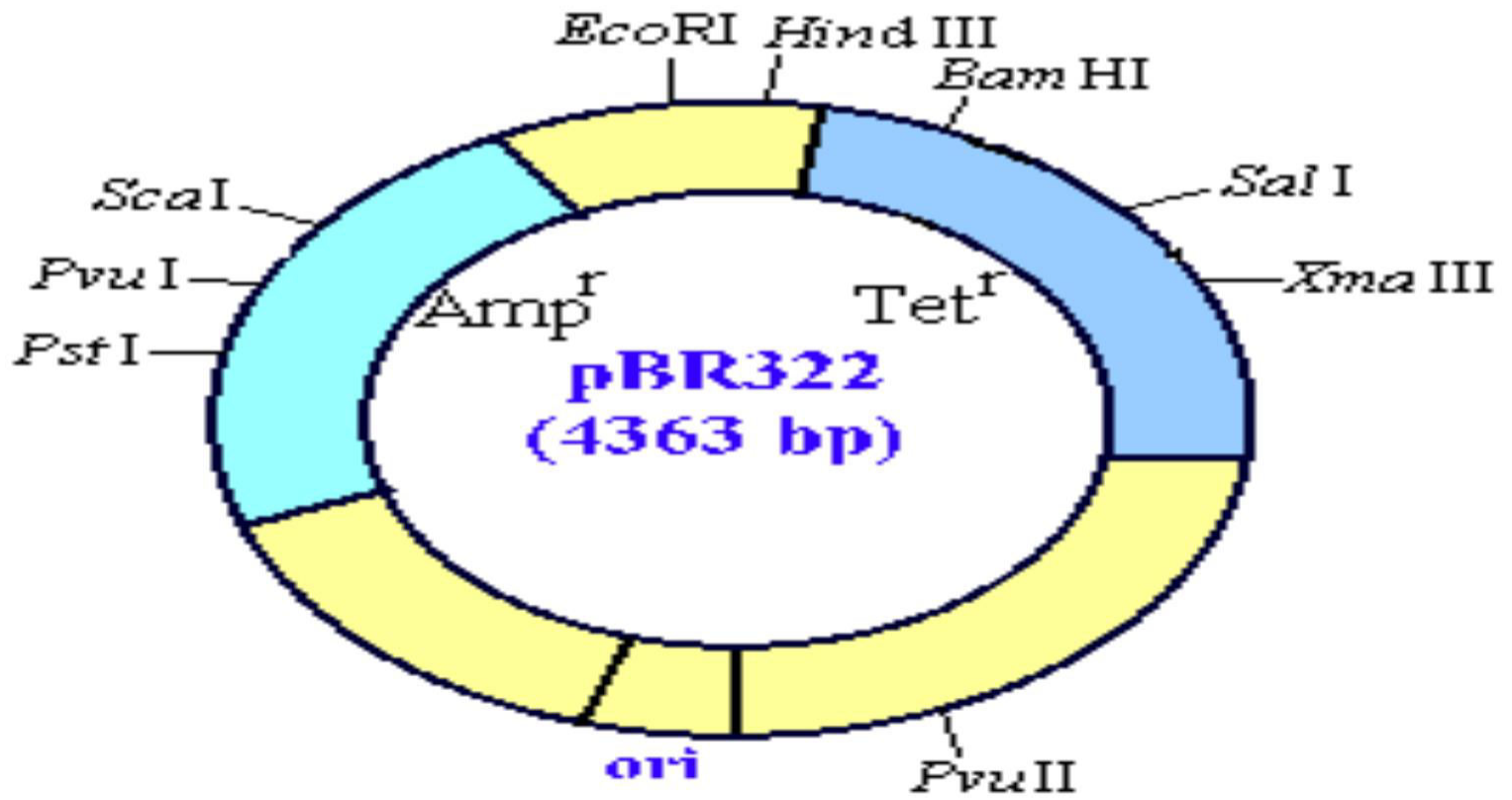
a.) Phages

- small, circular, dispensable genetic elements, found in most prokaryotic and some eukaryotic species.
- have replication origin and can replicate autonomously in the host cell.
- can be beneficial to host cell, since it can provide drug or heavy metal resistance or produce some toxic proteins.
- artificial plasmids can be constructed with useful characteristics of natural plasmids for the purpose of cloning

Characteristics of artificial plasmids


- high copy number,
- non-conjugative,
- carry at least two selection markers (one of them carry restriction site for enzyme),
- have more than one unique restriction site,
- accommodate large DNA fragment

pBR322 is one of the most widely used vector . It carries two antibiotic resistance genes: ampicillin and tetracycline. If foreign DNA is inserted into one of the restriction sites in the resistance genes, it inactivates one of the markers. This can be used for selection of recombinants.



b. Phages

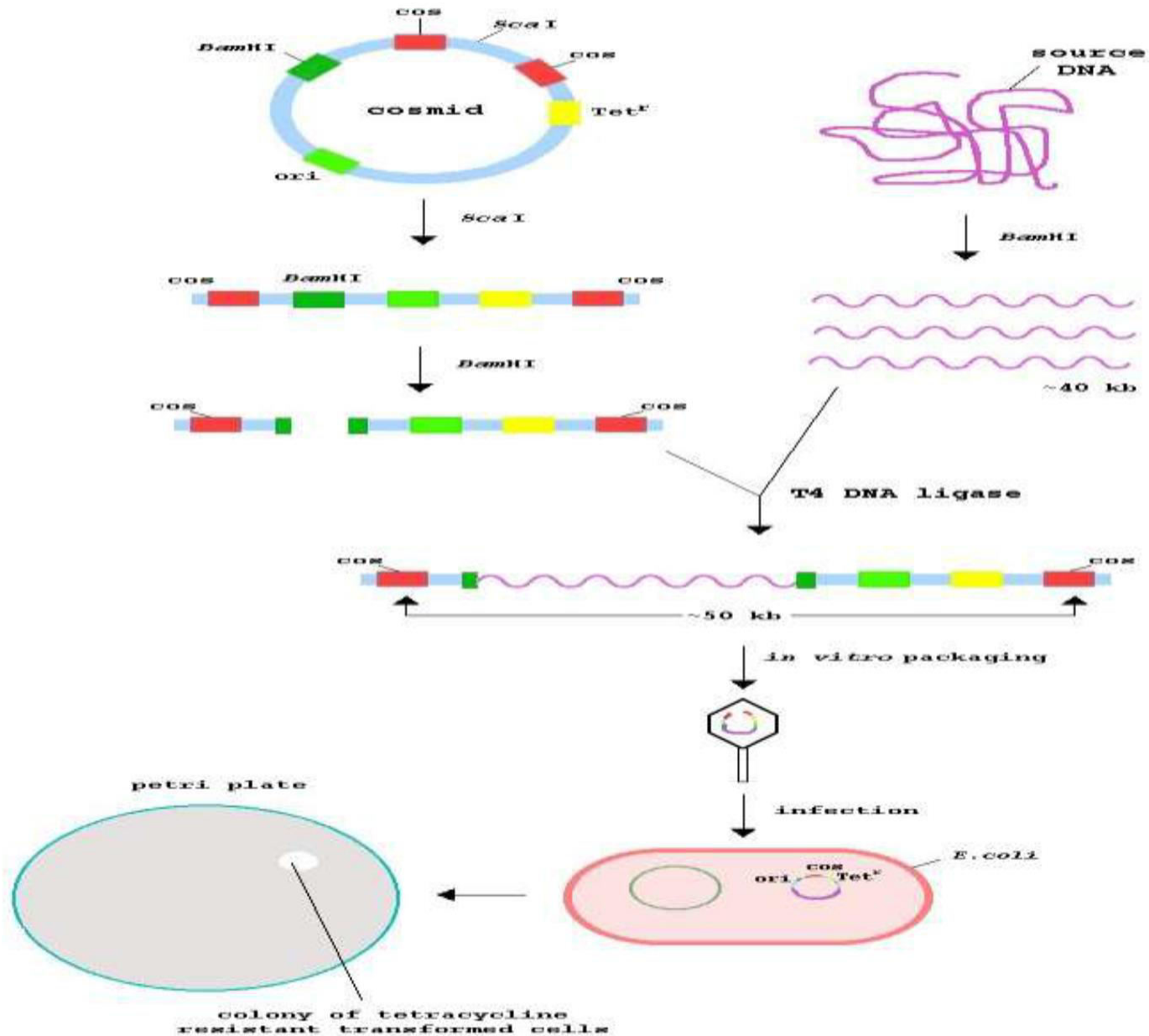
- viruses of bacteria
- consist of a molecule of DNA or RNA and protein coat.
- bind to receptors on bacteria and transfer genetic material into the cell for reproduction.
- can enter a lytic cycle which leads to lysis of host cell and release of mature phage particles or they can be integrated into host chromosome as prophage and maintained (lysogeny).

- 
- **Phage lambda** has double stranded DNA, around 48.5 kbp,. There contain single stranded, 5' projections at each end, called as **cos** sites. These are complementary in sequence. When it is injected into host cell, phage DNA circularize by means of these sequences.
 - By mixing purified phage heads, tails and bacteriophage lambda DNA, infective particles can be produced in reaction tube, this is called as in vitro packaging. During packaging, DNA sequences between two cos sites are packed into phage heads.

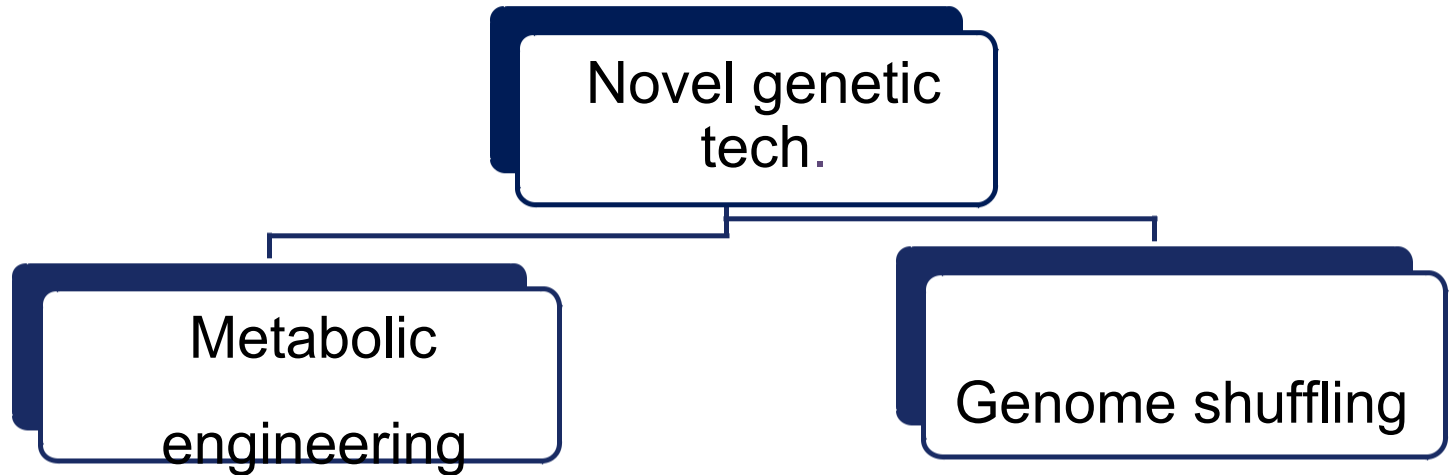
c. Cosmids--

- are artificial vectors prepared by DNA segments from plasmids and phages.
- replicate in the host cell like plasmids at a high copy number.
- like phage vectors, contain **cos sequences**, *in vitro* packaging is possible.
- **transformation efficiency is higher than plasmid vectors since transformation occurs by infection.**
- carry a selectable genetic marker and cloning sites.
- ~40 kb fragments can be inserted between cos sites

A cosmid cloning system



Novel genetic technologies



Metabolic engineering-

- The existing pathways are modified, or entirely new ones introduced through the manipulation of the genes so as to improve the yields of the microbial product, eliminate or reduce undesirable side products or shift to the production of an entirely new product.
- It has been used to overproduce the amino acid isoleucine in *Corynebacterium glutamicum*, &
- ethanol by *E. coli* and has been employed to introduce the gene for utilizing lactose into *Corynebacterium glutamicum* thus making it possible for the organism to utilize whey which is plentiful and cheap.

□ Product Modification by Metabolic Engineering include the new enzymes which modifies the product of existing biosynthetic pathway
e.g.conversion of Cephalosporin C into 7-aminocephalosporanic acid by D amino acid oxidase(in *A. chrysogenum*)

Completely new metabolite formation include in which all the genes of a new pathway are transferred
e.g *E.coli* ,transfer of two genes for Polyhydroxybutyrate synthesis from *Alcaligenes eutrophus*.

Enhance growth include enhanced substrate utilization
e.g *E.coli* ;glutamate dehydrogenase into *M.methylotrophus*
;carbon conversion increased from 4% to 7%

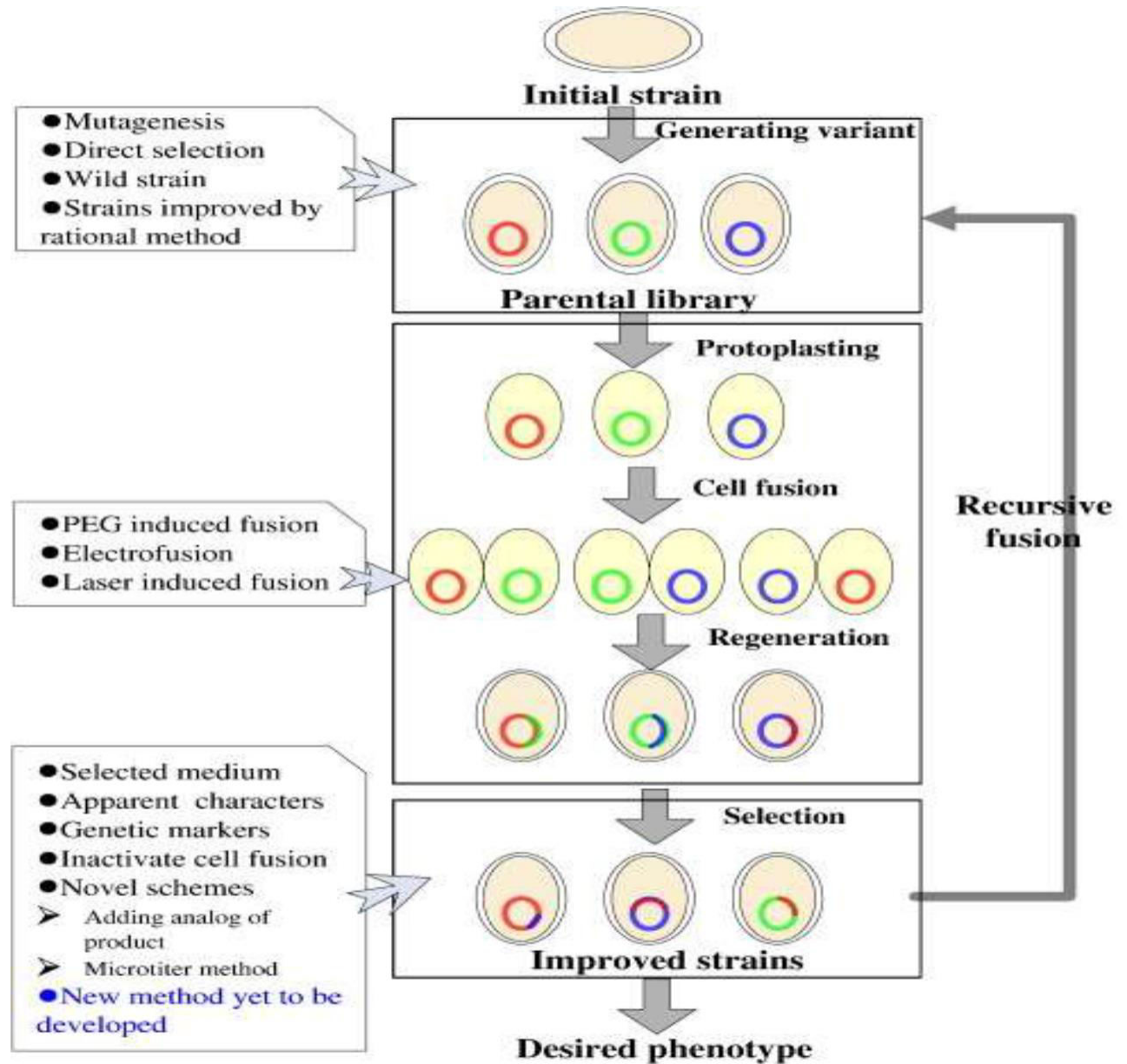



The Strain of *E.coli* has been engineered for the production of **lycopene**(Farmer and Liao,2000;Alper *et al*,2005) **amino acids** (Lee *et al*,2007;Park *et al*,2007) and **alcohols**(Atsumi *et al*,2008)through metabolic engineering methods.

The improvement of *Saccharomyces cerevisiae* for the production of **ethanol** by metabolic engineering method (Nissen *et al*,2000;Alper *et al*,2006;Bro *et al*,2006)

Genome Shuffling– is a novel tech for strain improvement allow for recombination between multiple parents at each generation and several rounds of recursive genome fusion were carried out resulting in the final improved strain involving genetic trait from multiple initial strains.

Genome shuffling--



- 
- Biochemically products such as **astaxanthin** (Zheng and Zhao,2008), **ethanol**(Gong *et al*,2008;Shi *et al*,2009) and **bioinsectisides** (Jin *et al*,2009) were successful in improving the yield by genomic shuffling.
 - Used to increase acid and glucose tolerance in *Lactobacillus*(Patnaik *et al* ,2002;Wang *et al*, 2007;John *et al*,2008), improve acetic acid tolerance in *Candida krusei*(Wei *et al*,2008), enhance Pristinamycin tolerance in *Streptomyces pristinaespiralis*(Xu *et al*,2008),improve thermotolerance and ethanol tolerance in *S.cerevisiae* (Shi *et al*,2009)

CONCLUSION-

- These steps have been taken by firms in order to gap the bridge between basic knowledge and industrial application.
- The task of both discovering new microbial compounds and improving the synthesis of known ones have become more and more challenging.
- The tremendous increase in fermentation productivity and resulting decreases in costs have come about mainly by using mutagenesis. In recent years, recombinant DNA technology has also been applied.
- The promise of the future is via extensive use of new genetic techniques-
 - Metabolic engineering
 - Genomic shuffling
- The choice of approaches which should be taken will be driven by the economics of the biotechnological process and the genetic tools available for the strain of interest.

STRATEGIES FOR DEVELOPMENT OF INDUSTRIAL MICROBIAL STRAINS WITH SCALE UP PRODUCTION

Characteristics of industrial strains
<ul style="list-style-type: none">▪ Genetically stable and rapid cell growth▪ High rate of production of the desired product▪ Ability to utilize wide variety of inexpensive substrates▪ Shorter time of fermentation▪ Fewer byproducts which makes simpler downstream processing▪ Ability to undergo genetic manipulation▪ Ready harvesting ability of the product

So development of industrial strain, the industrial strain appears to be the one of the important factor that we have with the particularly in the bio chemical industries and this industrial strain should have different characteristics and first characteristic is to be the genetically stable and genetically stable means the bio chemical characteristics of the organism should be same, because you know that organism microorganism has stable generation, as they have change the generation, the characteristics of the organism should not be changed, genetically is stable organism is very important.

Now if you compare these with the wild strain, wild strain this is most unstable genetically unstable. So suppose we work with the wild strain and get some good product formation, but next time we can have the repeatability of these reactions. Now most of the industry, they when they involved they never they think about that you know that they cannot compromised with the productivity. Productivity of the industry should remain same, because at the end of day the (())(1:39) management will see that how much product is produced in the industry if the product formation is less naturally that is not desirable for any industry.

So they are come up with lot of (())(1:51) question to the to the operation people. So genetical stability of the organism is very important and it should have rapid growth, when (())(2:00) would have rapid growth characteristics and the high production rate, because here I **to** want to (())(2:08) tell you that the product concentration plays very important role in any kind of chemical and bio chemical industries. Now (if we) if the product concentration is less than our recovery cost, because I told you that whenever we market any kind of product, it should in a purified form if the concentration is very less your purification cost will be very high

which is undesirable. So we have the so this industrial strain has high productivity than the high rate production high concentration of product that formation takes place.

The another very important factor is that you know, to utilize the wide variety of the inexpensive substances, because you know that organism should have the capability of wide type of different type of substrate. If they use one type suppose, they are using one type of substrate for the timing it may be cheaper, but as the time passes or (3:08) then since that showed with the peoples (3:11) the source to form where he (3:13) is collecting the raw materials thereby finding that this has lot of demand they keep on rising increasing the price of that particular material.

So if you have multiple choice of the raw materials naturally that you know you can you can control the cost of your production to a great extent. Their short time of the fermentation, because it is because we know in the industry every time is very costly, because they count money for that. So the time of fermentation plays very important role in the fermentation industry that should be as lowest possible, because by using the industrial strain that time of fermentation can be reduced, the fewer than in a byproducts formation that is also very important aspect that byproduct formation should be as low as possible, more byproduct formation that means your substrate will go for some not to produce your desire product, but the other than the desire product which is not to acceptable.

So industrial strain give you fewer byproduct that is most desirable thing, ability to undergo genetic manipulation, because the why it is required, because to get desired change in the organism so that we can increase our productivity increase our product concentration we can get our desire product and ready harvesting that is also very important, because cell particularly we have if I told you that different type of cells we have, we have bacteria, we have algae, we have fungi, so you know in case of bigger cells like fungi, we do not have any separation problem, but when you go for the when you go for bacteria, the bacteria is very tiny particle.

So bacterial size varies from 0.5 to 2 micron, so separation problem is there, so harvesting is a very important aspects of that, so how quickly you separate that organism that is also very important that plays very important role. So what we have covering in this course that not only on the development of this how the industrial strain and how to preserve this strain that also we are going to discuss.

Difference between wild strain and industrial strain	
Conventional wild strains are subjected to various strategies until a potent industrial strain is developed	
Wild strains	Industrial strains
✓ Poor reproducibility (Growth rate, Product formation rate and titre)	✓ High reproducibility in product formation rate and titre
✓ Are susceptible to product inhibition	✓ Highly tolerant to product inhibition
✓ Poor genetic stability	✓ High genetic stability
✓ Ability to use various substrates	✓ Acclimatized to use cheaper and wide variety of substrates
✓ Poor substrate	✓ Very high substrate

Now let initially let us discuss about the what is the difference between the wild strain and the industrial strain, as we know wild strain, basically by the in the environment, because you look at this soil we have different type of bacteria that is available different type of organism whatever organisms we have all organism most of the organism is available in the soil. So that that if we bring that an isolate in the lab and this organism we consider is a wild strain.

So wild strain is the reproducibility that is growth rate and product formation rate and titer is very poor as compared to the industrial strain. Industrial strain that the high predictability in product formation rate and titer and I told you that concentration of the products has great influence on the cost of production of because recovery cost will be reduce to a great extent if the concentration product is very high. Then another important that drawbacks is that that wild strain, they are susceptible to the product inhibition, because as the product concentration increases it inhibit the growth of the organism, so in case of industrial strain high tolerance on the product inhibition, I can give the example of the yeast that you know approximately one gram one gram of glucose produces 0.51 gram of the ethanol.

Now if I want to increase the alcohol concentration in the fermentation, but now if we look at in the early days that most of the fermentation industry, they produce the ethanol about 7 to 8 percent and now it is the industry they are producing about for 14 to 15 percent. Now for producing the 14 to 15 percent there so the glucose concentration should be as high as 30 percent which is very high and as we increase the glucose concentration than the osmotic pressure on the fermentation block will be high. So in that cusses the shrinkage of the organism.

So organism cannot grow properly, so that is the that means the organism should have the osmo tolerant. So industrial strain they have that capability they can withstand that high

osmotic shock this is another advantage we have, then poor genetical stability I told you before also this is wild strain, they have poor genetical stability whereas the industrial strain, they have high genetical stability. Ability to use the various substrate here also we ability use the various substrate, but we use the cheaper substrate and wide variety of substrate can be used by the industrial strain and in case of wild strain we have the poor substrate conversion rate and here very high substrate conversion.

So this is the different advantages we have when we use the industrial strain, so that is why the industrial strain is preferred by the industry and naturally the cost of the industrial strain is very high, because this is the specially prepared by the different lab for the industrial purpose.

Now if you look at the how the industrial strain development is taking place that we have 3 things we have we can see, the strain development that is in the upstream processing then midstream process and downstream process that that is like this, in the strain development, first we select the microbial host so that where we want to have the changes that you know, then construction of the biosynthetic pathway for the non-native bio products. Improvement of self-tolerance against the bio products, removal of the negative regulation, flux (())(9:15) for cofactor and precursor the optimization, optimization of metabolic fluxes through the relevant biosynthetic pathway, use of high throughput tools for system level metabolic analysis .

So we know that this is the this is then that we want to work with the for the strain development, because I shall discuss that how we developed it, but this is the main purpose of the in this way we want to guide the organism, in such a way we can get the higher amount of product formations, then if you come to the fermentation process, use of effective and cheap and easily obtainable carbon source and chemically define media, because this the this should influence that one then optimization of culture condition and feeding strategy, performance of batch and fed-batch culture, evolution of product performance and scale up of bio reactors that is very important.

And in the separation use of effective and cheap keep and easily obtainable carbon source and chemically defined minimal media and minimization of byproducts I told you before and optimization of culture condition that is are at a different pH we to want to carry out. So this are our interactive things that we have that is how it influencing the fermentation process.

(Refer Slide Time: 10:40)

Industrial microbial strain development

The following points highlight the five main steps involved in developing producer strains.

- 1. Isolation of Industrial Microorganisms**
- 2. Screening for new Products**
- 3. Identification of Metabolites**
- 4. Maintenance of Microbial Isolates**
- 5. Strain Improvement.**

Now industrial that that is strain that is develop in that is five main steps involved, one is the isolation of the industrial microorganism, which organism we are looking for which organism we as for example *saccharomyces cerevisiae* that is used for alcohol production, now the suppose the *saccharomyces cerevisiae* is mainly use the hexo sugar.

Now we want to use the also the pentose sugar, because the suppose we want to use some kind of pentose sugar for the formation of ethanol, naturally that we shall have to improve upon this organism we shall have to do the genetic modification of the organism to have the capability of the organism so that you know we can covert the pentose into the ethanol. The screening (of) for new products that we that we that is very important then identification of metabolites that is also important, maintenance of microbial isolates and strain improvement.

These are the several things that is involve with the industrial strain development.

Methods of industrial strain development

Screening of new metabolites

There are five distant approaches for obtaining new microbial metabolites from microbes

- **Screening**
- **Chemical modification of known microbial substances**
- **Biotransformation**
- **Interspecific protoplast fusion**
- **Gene cloning**

Now another that the other five distant approaches for obtaining the new microbial metabolites from the microbes that is screening we do the screening we do the microbial

screening and try to find out which microbes is good for the product formation, then chemical modification of known microbes have microbial substances, bio transformation that also by we can easily carried out with the help of microbes and interspecific protoplast fusion, because we know the different organism has different characteristics.

So if you do the protoplast fusion, it is possible that characteristics the another organism can switch over to other organism and you get the properties of that organism in the system and genetic cloning I told you that insulin, it kind of disease this is used for the diabetic patient and insulin is kind of protein that is available in the human system. So we can we can produce microbially how we can produce we isolate the gene, which is produce that insulin and we with that gene we over express in the in out host that is E. Colli (13:05), so that we can pipe through the fermentation process we can produce the insulin and we can use for our day to day life.

Then question comes how to do the preservation of the industrial strain industrial culture must be preserved by maintaining such a way to eliminate the genetic changes protect against the contamination and retain viability. This is very important. All these 3 factors is to be considered that one is we should keep in a manner so that you know genetic change should not be there. So you should not keep exposure to the UV rays and other chemicals other the adverse conditions so that some kind of changes can take place and then protect against the contamination, because our air atmosphere contains lot of microorganism, because particularly contamination problem is very problematic in the industry I work with citric acid industry I can we use the cane molasses (14:10) for the production of citric acid and we observe that our main problem this fermentation process with the contamination by the yeast cells, because cane molasses can be easily used by yeast cells for the production of ethanol.

So and if you look at the doubling time of yeast is much less as compared to fungi. So yeast can grow very rapidly as compared to fungi. So that is why that our the citric industry (14:40) well scaled of these yeast cells and we try to remove this organism so that we do not have any (14:47) problem with the citric acid productivity and retain the viability, because viability as you know that the organism should be viable, so that we can it can grow properly and give the desired product and mostly that industrial fermentation process the when we when you do the inoculation of the organism with the low, it should be either mid lock phase to lead lock so that that organism should grow in a proper manner.

Now about the preservation of the industrial strain is concerned, the methods to keep the organism metabolically active, the periodically transfer to the fresh media, this is one important aspects that we have, overlaying the culture with the mineral oil that is another thing that we have, storage of sterile soil then saline suspension. These are the different methods through which we can keep this when the organism metabolically active, the methods where the organisms are submerged in metabolic state, like drying in vacuum then lyophilization.

Lyophilization consider as a phis drying, phis (16:00) drying phis drying means that organism that with the frozen then they do the then that water molecule that presents in the solids phase (16:11) that will go to the vapour phase. So the organism can be preserve for a longer period of time around 30 years we can preserve that organism in the viable conditions, then use of liquid nitrogen a that is another way that we can do that, then storage of silica gel, storage in silica gel that is a another way we can preserve this microorganisms.

Preservation of industrial strains – Comparison of various methods	
Method	Merits and de-merits
Periodic transfer	Variables of periodic transfer to new media include frequency, medium used and holding temperature. This can lead to increased mutation rates and production of variants.
Mineral oil slant	A stock culture is grown on a slant and covered with sterilized mineral oil; the slant can be stored at refrigerator temperature.
Minimal medium, distilled water, or water agar	Washed cultures are stored under refrigeration; these cultures can be viable for 3-5 months or longer.
Freezing in growth medium	Not reliable, can result in damage to microbial structures, with some microorganisms, however this can be a useful means of culture maintenance.
Drying	Cultures are dried on sterile soil (solid stocks), on sterile filter paper disks, or in gelatine drops; these can be stored in a desiccator at refrigeration temperature or frozen to improve viability.
Freeze-drying	Water is removed by sublimation, in the presence of a cryoprotective agent; sealing in an ampoule can lead to long term viability, with 30 years having been reported.
Ultrafreezing	Liquid nitrogen at -196°C is used, and cultures of fastidious microorganisms have been preserved for more than 15 years.

Now the preservation of the industrial strain and comparison with the different methods is given here, one is periodical transfer the variable of periodical transfer to new media includes the frequency, media used and holding temperature. This can lead to increase the mutation rates and production of variant. Then mineral oil slant, this is another important aspect that we have, a stock culture is grown on in a on a slant and covered with sterilized mineral oil and slant can be stored at refrigerator temperature.

The then minimal medium and distilled water or water agar that is the use the washed culture was stored under refrigeration and this culture can be viable only for 3 to 5 month or longer

not for very long period of time. The freezing of the growth media, a not real reliable, because can result in damage of microbial structure, with some microbes and however this can be useful mean of the culture maintenance. Drying this is another important thing that is the culture are dried on sterile sterilized oil soil and on sterile the filter paper disk in gelatin drops and these can be stored in a dessicator at refrigerator temperature or frozen to improve the viability.

The most the technique that I was telling you that which is used by the industry that is the freeze drying technique. Freeze drying technique, water is removed by sublimation process, in presence of cryoprotective agent and sealing it in a ampoule and can lead to the long term viability with 30 years having been reported, because 30 years we can preserve this organism in the viable condition. Then ultra-freezing, this is another aspect that we have liquid nitrogen at minus 196 degree centigrade is used, culture or fastidious microorganism have been preserve for more than 15 years. So these are the different way we can preserve the microorganism.

Methods of industrial strain development

Strain improvement

Optimization of microbial activity

- Optimization of environmental parameters
- Nutrition etc.

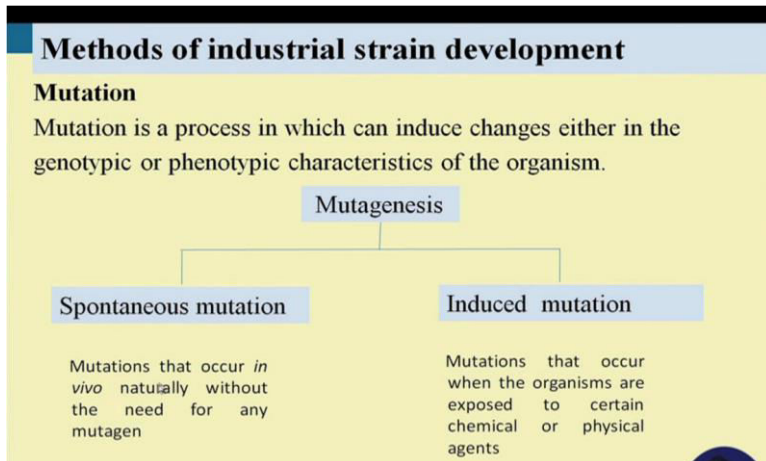
One of the most fundamental approaches to strain improvement is done with the help of recombinant DNA techniques. Other examples include **site-directed mutagenesis**, **protoplast fusion**, **metabolic engineering** etc.

Most of the strain improvement procedures aim to increase the yield of the product.

Now let me discuss about the method of industrial strain development. The strain improvement here, optimization of microbial activity we can do by optimizing the environmental parameters, like the in a temperature, pH, substrate concentration I told you that osmo tolerant culture that can we increase the microbial activity. Then nutrition this is very important ligand, recently we work on that and we find that that you know particularly if that, in case of when we use some kind of waste material for the production of some useful product, waste material usually contain does not contain much of nutrient has less (19:45) nutrients I am also if with the micro (19:48) we add to the waste material then your microorganism can grow properly and give the desired product that that is there.

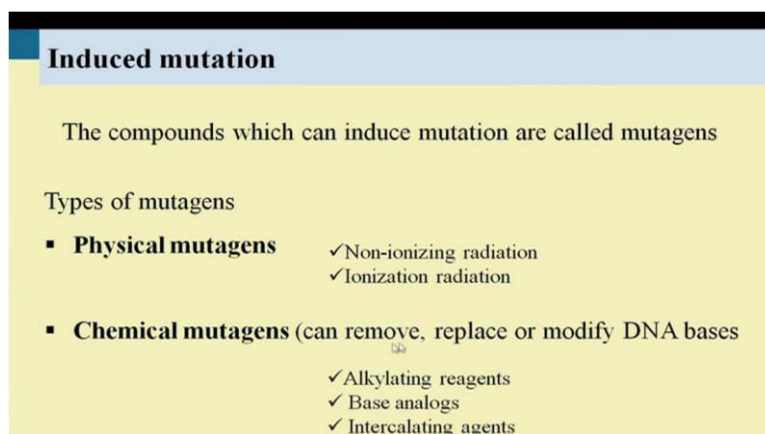
One of the most fundamental approach is of the strain development is done with the help of recombinant DNA techniques, because I told you the example is the insulin production and

that the other examples as site-directed mutagenesis, protoplast fusion and metabolic engineering, metabolic engineering plays very important role, because you cannot (20:18) the metabolic pathway to get the desire amount of product, because this is very important. Most of the strain improvement procedure aim to increase the yield of the product. This is the main purpose that.



So mutation is very known technique that is used in the industry for the improvement of the productivity of the strain. Mutation is a process, which can induce changes either in the genotype or phenotype characteristics of the organism. The two type of changes that occurred in the organism, one is genetically another phenotype change that can be done with the help of mutation and mutation may be of two type, one is call spontaneous mutation another is induced mutation.

The spontaneous mutation, which is occurred in vivo, (21:05) in vivo means they are itself organism itself the mutation that occurs naturally without the need of any mutagens. The induce mutation the mutation that occurs when the organisms are exposed to certain chemical or physical agents.



So now we have different type of mutation we have **we have** physical mutation, we have chemical mutations. Physical mutation we have non-ionizing the radiation, ionization radiation then chemical mutagens we have alkylating agent, base analogs and intercalating agents.

Non-ionizing radiation

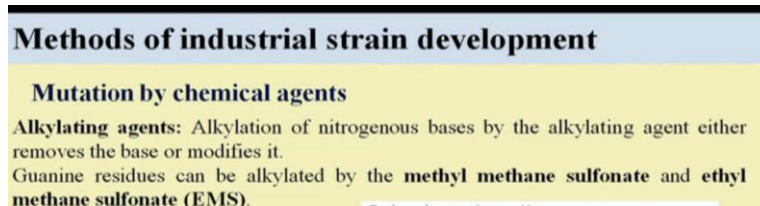
- Long ultraviolet radiation
- They have low penetration power
- The major effect of UV radiation is formation of pyrimidine dimers, particularly between two thymines.
- Cytosine-cytosine and cytosine-thymine dimers are less prevalent.

So different type of mutagens we use in the industry for the development of the strain. Let me take the example of the non-ionizing the radiation examples is the ultraviolet radiation and they have long penetration power you know that ultraviolet radiation as very shorter wavelength and major effect of ultraviolet radiation is the formation of pyrimidine dimer, because you can see that here, 2 nitrogen bases is there, this is this is thymines and there how the dimer formation is there that you can see that here dimer formation takes place, but cytosine-cytosine and cytosine-thymine dimers are less prevalent.

Ionizing radiation

- They include β -rays, γ -rays and α -rays which cause the ionization of the medium
- They are used for mutagenesis only if other mutagens cannot be used (e.g. when the cells are impermeable to UV rays)
- They have more penetrating power as compared to UV rays
- Cells when exposed to these radiations result in the formation of free radicals which further cause genetic mutations.

Now ionization radiation this is actually apply where that non-ionization that radiation is is not effective, they include the gamma the beta rays, gamma rays, alpha rays which causes the ionization in the media. They are used for mutagenesis only if other mutagens cannot be used when the cells are impermeable to the UV rays. They have more penetrating power as compared to UV rays. Cells when exposed to these radiations results in the formation of free radicals which further cause the genetic mutation. So this is the (())(23:10) effective more effective as compared to non-ionizing radiation.

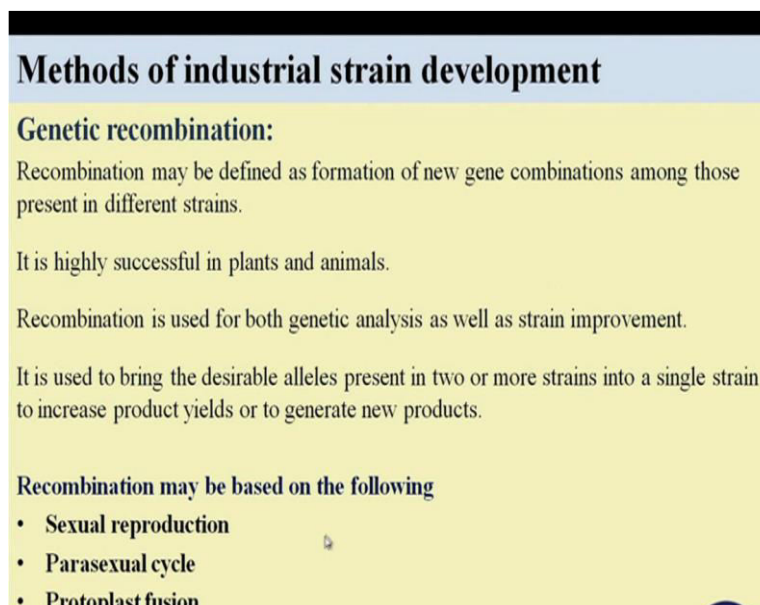


Methods of industrial strain development

Mutation by chemical agents

Alkylating agents: Alkylation of nitrogenous bases by the alkylating agent either removes the base or modifies it.
Guanine residues can be alkylated by the **methyl methane sulfonate** and **ethyl methane sulfonate (EMS)**.

Now we have chemical mutagens that we use mainly, this chemical mutagens we use to change the nitrogenous bases that you know alkylation of nitrogen bases by alkylating agent either remove or the base is or base or modifies it you can see this is the thymine, adenine cytosine and guanine that here how by using EMS, EMS means the ethyl methane sulphonate, methyl methane sulphonate, there are two alkylating agent that can be used for mutation purpose. This is how we use to change the characteristics of this nitrogen base that is call there is part of the gene.



Methods of industrial strain development

Genetic recombination:

Recombination may be defined as formation of new gene combinations among those present in different strains.

It is highly successful in plants and animals.

Recombination is used for both genetic analysis as well as strain improvement.

It is used to bring the desirable alleles present in two or more strains into a single strain to increase product yields or to generate new products.

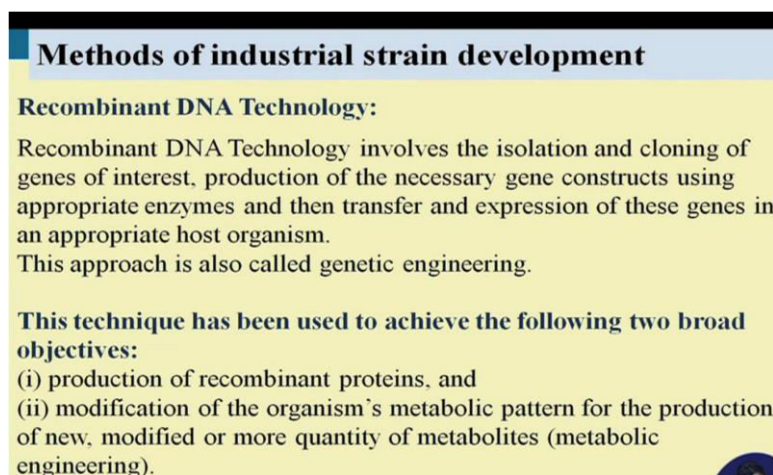
Recombination may be based on the following

- Sexual reproduction
- Parasexual cycle
- Protonlast fusion

Then methods of industrial strain improvement that is also very important. The one is the genetic recombination the recombination may be defined as the formation of new gene combination among those present in the different stain as I told you suppose one organism does not have a particular gene we want that particular gene in that particular organism. So

we try to take the gene from other organism and we clone in a kind of plasmid and then ((
(24:38) when these organism to get the desired property on the in that particular organism
atht is call recombination techniques and it is highly successful in plants and animals.

Recombination is used for both genetic analysis as well as strain improvement. it is used to bring the desirable the present 2 or more strain into single strain to increase the product yield and to generate the new products. The recombination may be based on the following sexual reproduction, Para sexual cycle and protoplast fusion. The different base that we can do the recombination.



Methods of industrial strain development

Recombinant DNA Technology:
Recombinant DNA Technology involves the isolation and cloning of genes of interest, production of the necessary gene constructs using appropriate enzymes and then transfer and expression of these genes in an appropriate host organism.
This approach is also called genetic engineering.

This technique has been used to achieve the following two broad objectives:
(i) production of recombinant proteins, and
(ii) modification of the organism's metabolic pattern for the production of new, modified or more quantity of metabolites (metabolic engineering).

Now finally I want to tell you that recombinant what is the recombinant DNA technology you might be knowing that see, let me tell you the recombinant DNA technology involve the isolation and cloning of the gene of interest, because gene of interest me the what particular gene you know that the why if the gene is coding a particular protein and the production necessary gene construct using the appropriate enzymes and then transfer and express this is into the appropriate host organism.

So this is the technique this is the kind of innovative technology that I recently has develop to get the desire characteristics of the microorganism that we call it genetically the improvement of the microbial strain and this technique has been used to achieve the following two broad objectives: one is the production of recombinant proteins as I mention that this is very important and modification of the organisms, metabolic pattern for the production of new modified and more quantity of metabolites that is a metabolic engineering when we when we study the different bio chemical pathway so we easily find out that you know which pathway can give us the maximum amount of product formation.

So through this metabolic pathway is we can through this recombinant DNA technology we can develop that particular desire enzyme. So that the activity of that enzyme can be increased so the we can be increase the productivity of the particular product metabolize to a great extent. So this is the whole things that I want to emphasize here that is there how the industrial strain development are how preservation takes place and this is largely used by the industry for getting desired products, thank you very much.