STRAIN IMPROVEMENT OF INDUSTRIALLY IMPORTANT MICROORGANISMS

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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.

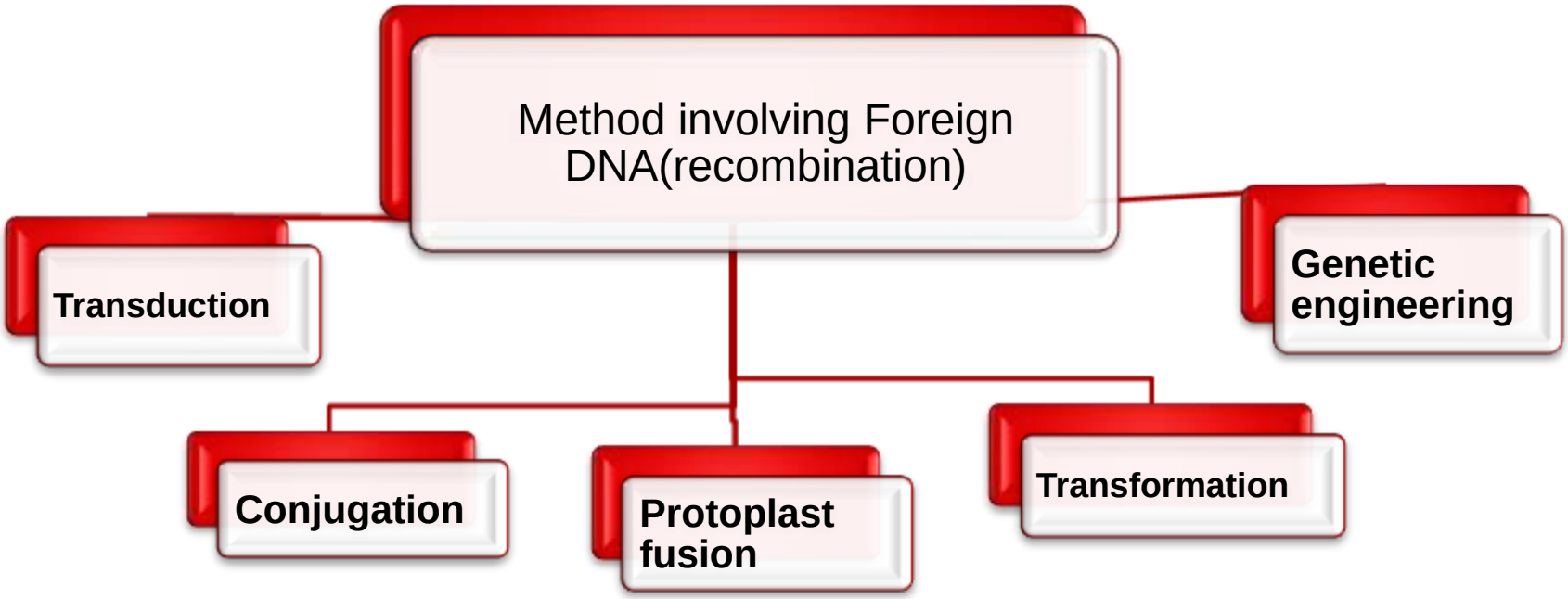
Optimization of microbial activity

It can be done by

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

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

2.



Optimizing environment conditions

- Modification of physical parameter (temperature,agitation,etc)
- Modification of chemical parameter (pH,O_2 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

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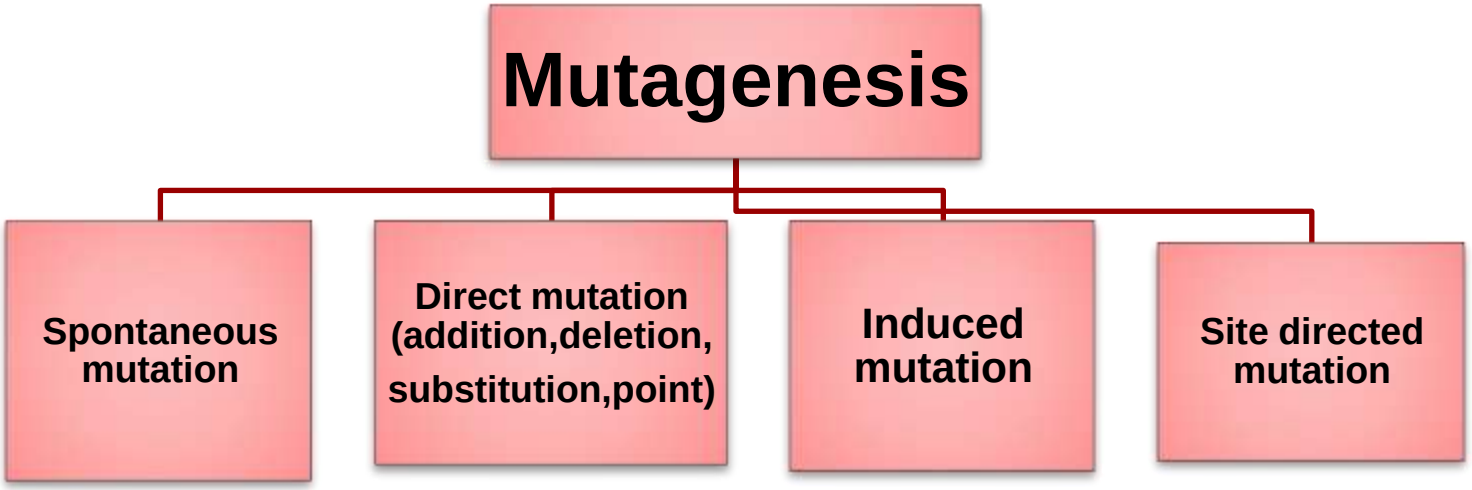


Types of mutation

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/structural changes	high
UV rays,chemicals	Pyrimidine dymerisation	Trnsversion,deletion,frameshift transitions from GC → AT	Medium
Hydroxylamine(NH_2OH	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,deletion,AT → GC/GC → AT	Medium
Phage,plasmid,DNA transposing	Base substitution,breakage.	Deletion,duplication,insertion.	high

CHOICE OF MUTAGEN

- Mutagenic agents are numerous but not necessarily equally effective in all organisms.
- Other factors--

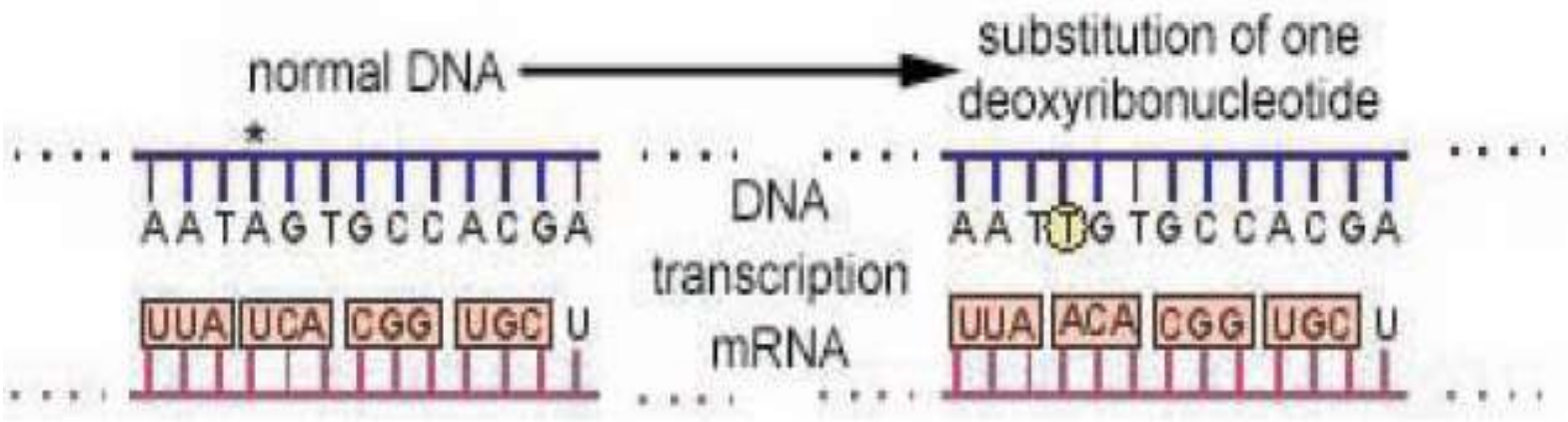
(a)the safety of the mutagen.

(b)simplicity of technique.

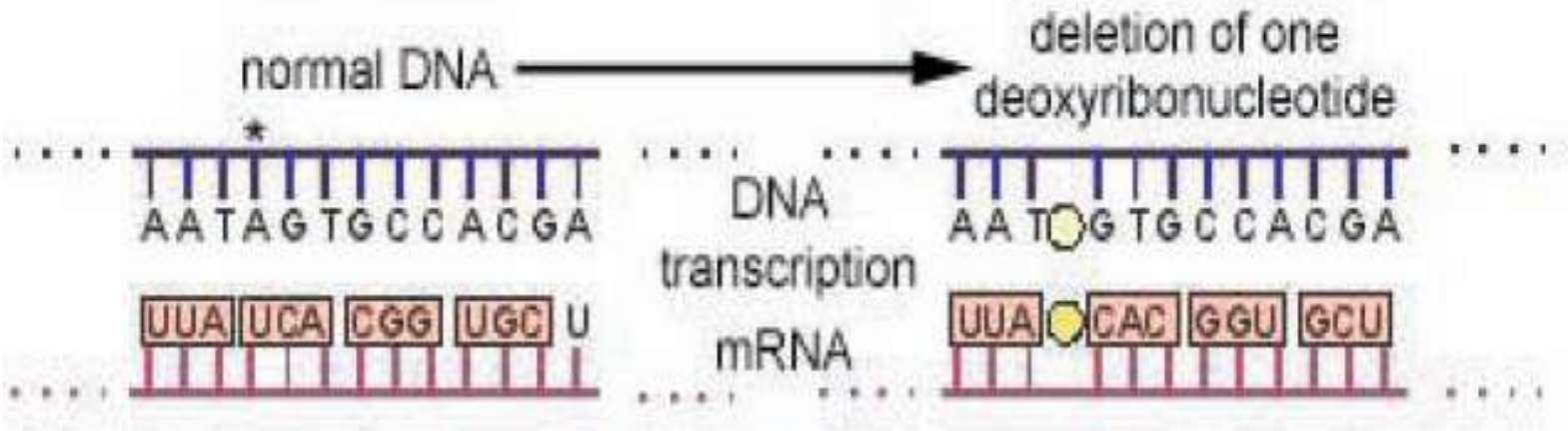
(c)ready availability of the necessary equipment and chemicals.

- 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.

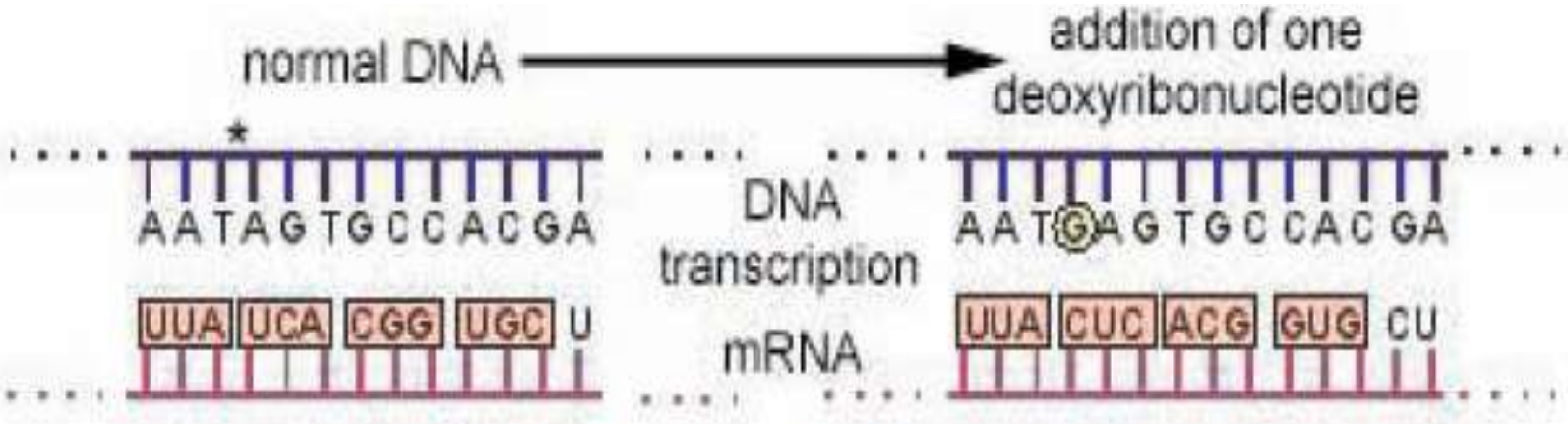
3.Direct mutations



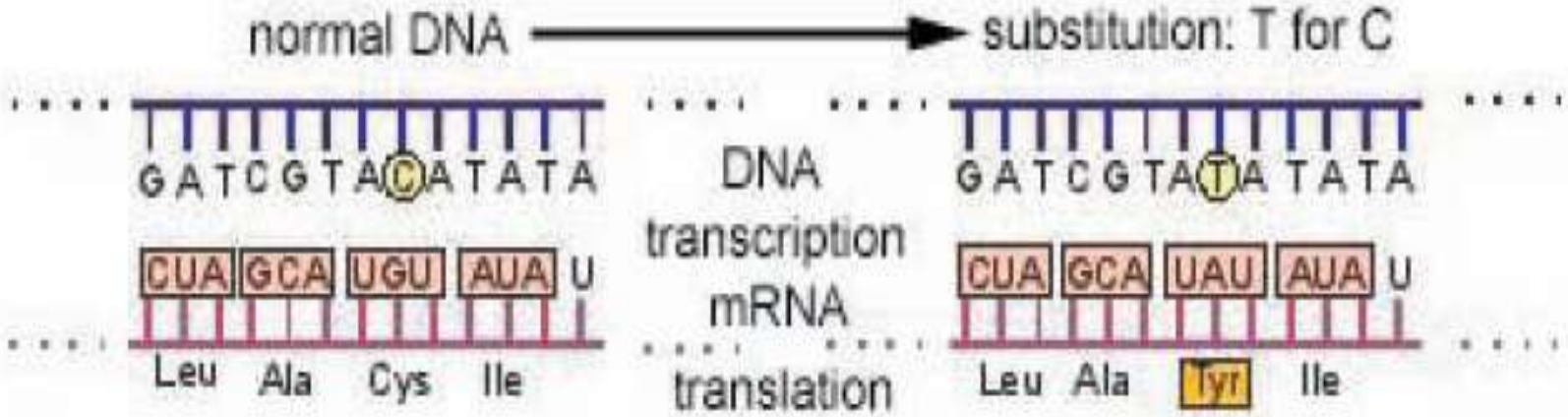
i) Point Mutation or Substitution of a Nucleotide



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 C**G**G. If the middle base is changed by SDM from G to C the codon sequence becomes C**C**G which codes for a different amino acid (Glycine).

Cont..

Mix oligonucleotide with recombinant vector ssDNA.

↓

Carried out at low temperature (0-10ºC) 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.

↓

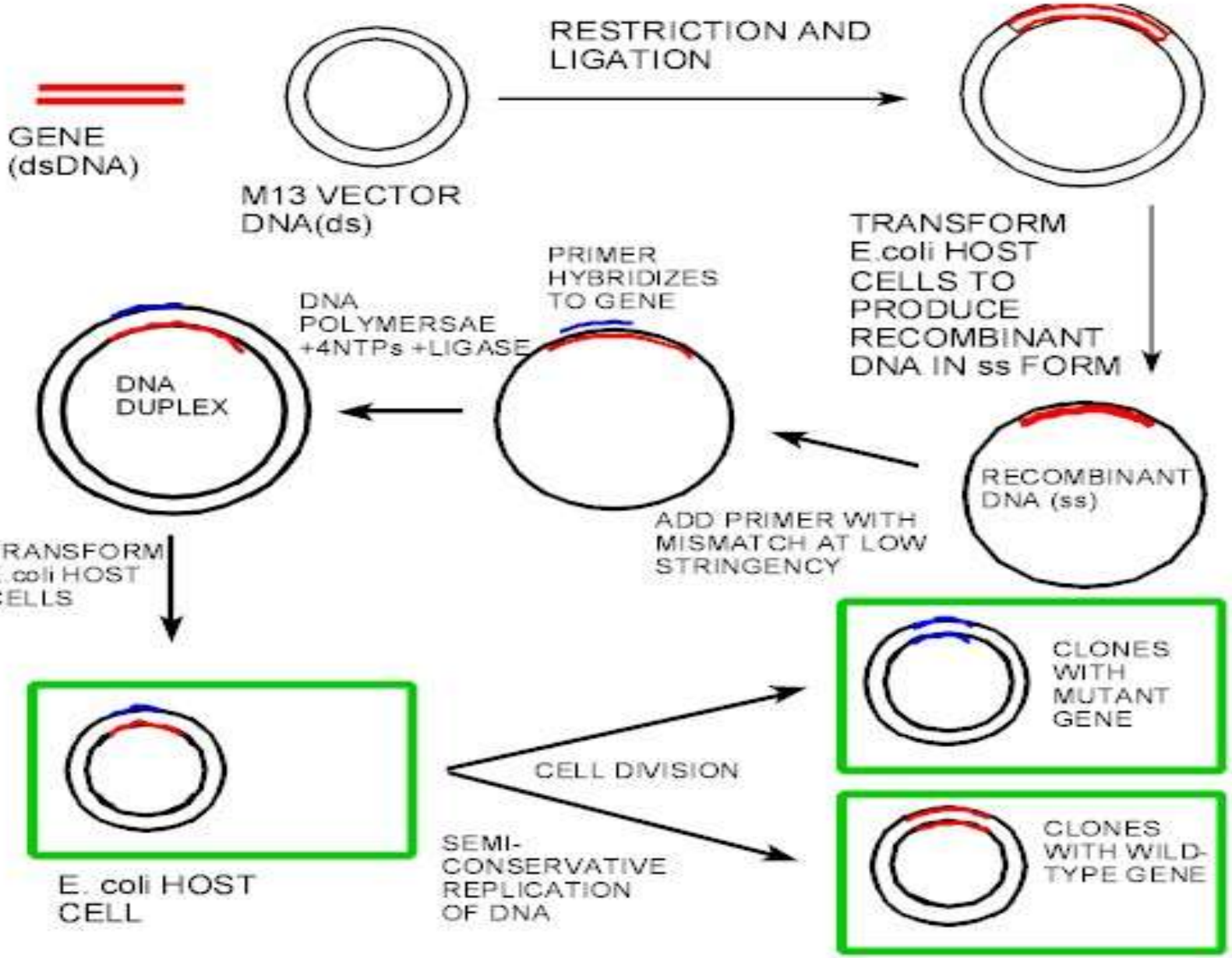
Transform *E. coli* cells and allow them to replicate recombinant vector molecule.

↓

DNA replication is semi-conservative, therefore <u>two</u> 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, HNO_2, 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 cnditions 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.

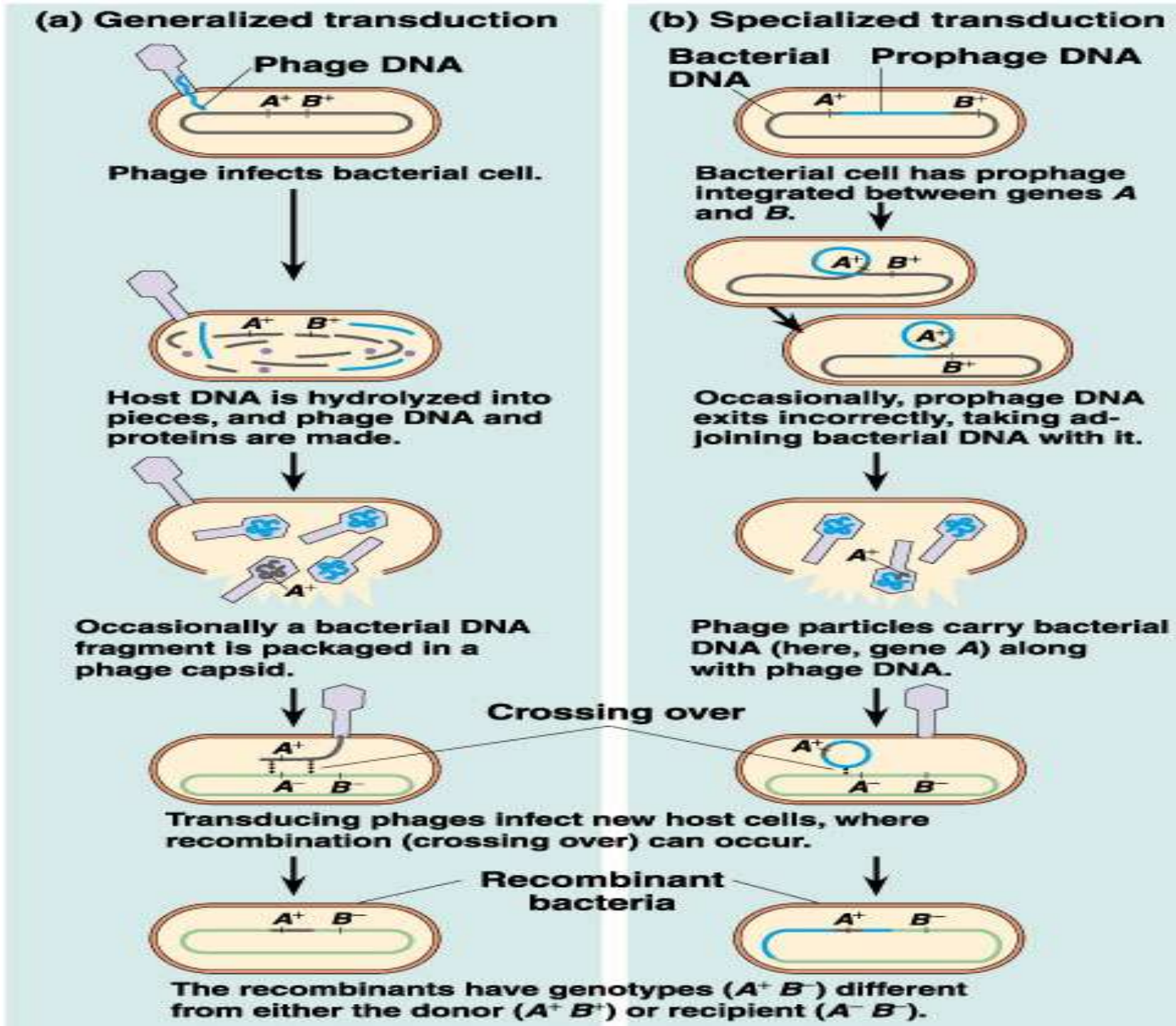
Screening of mutant:

- 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

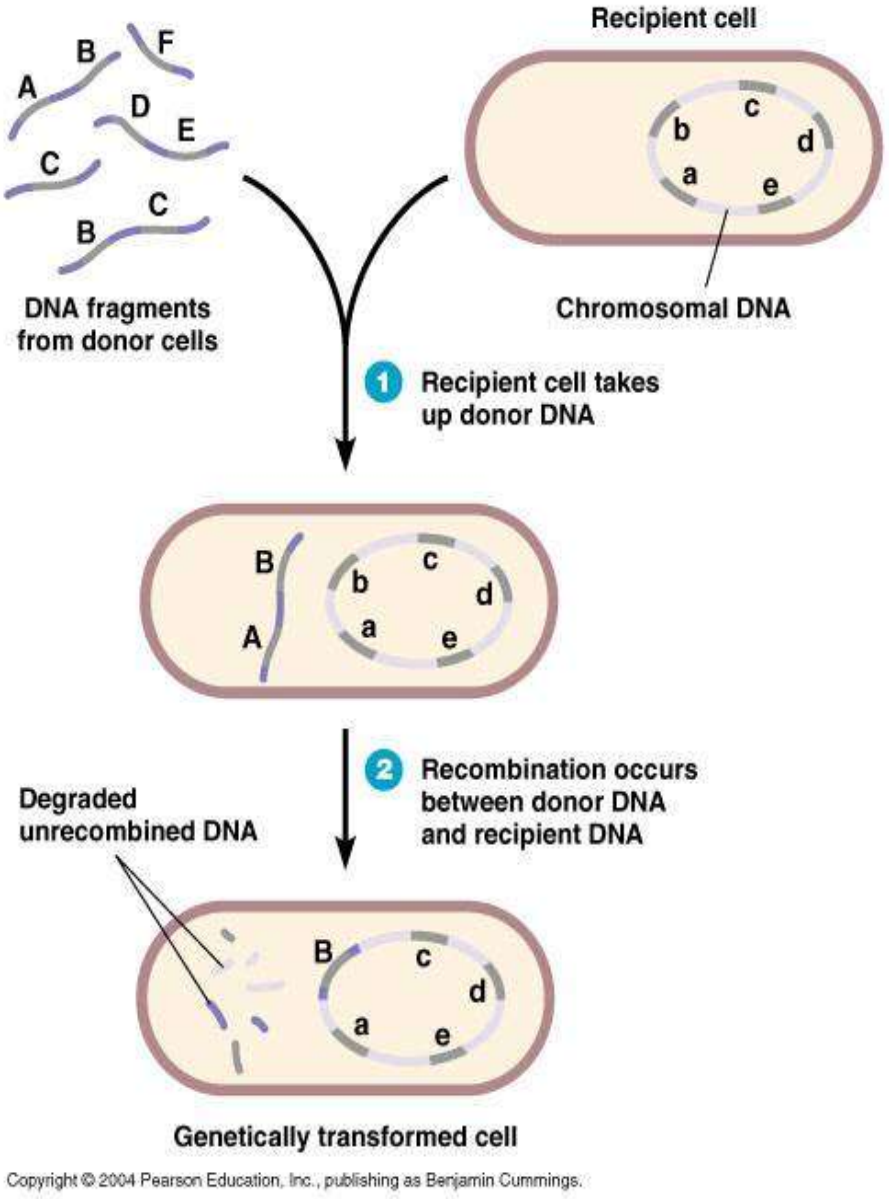
2.Transduction-

- 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
Phage DNA
A+ B+
Phage infects bacterial cell.
A+ B+
Host DNA is hydrolyzed into pieces, and phage DNA and proteins are made.
A+
Occasionally a bacterial DNA fragment is packaged in a phage capsid.
(b) Specialized transduction
Bacterial DNA
Prophage DNA
A+ B+
Bacterial cell has prophage integrated between genes A and B.
A+ B+
A+
B+
Occasionally, prophage DNA exits incorrectly, taking adjoining bacterial DNA with it.
A+
Phage particles carry bacterial DNA (here, gene A) along with phage DNA.
Crossing over
A+
A− B−
A+
A− B−
Transducing phages infect new host cells, where recombination (crossing over) can occur.
Recombinant bacteria
A+ B−
A+ B−
The recombinants have genotypes (A+ B−) different from either the donor (A+ B+) or recipient (A− B−).

3.Transformation

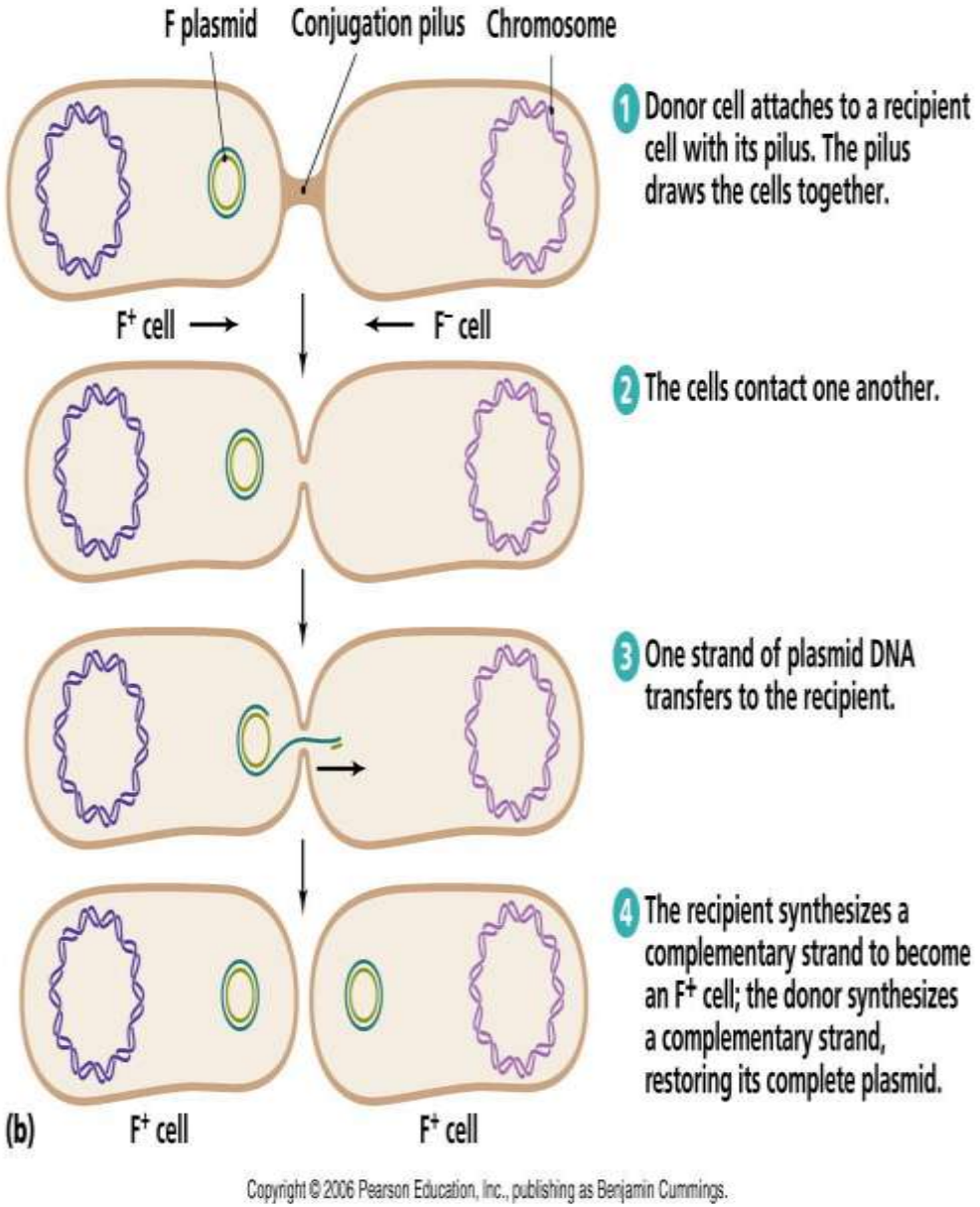
- When foreign DNA is absorbed by, and integrates with the genome of, the donor cell.
- Cells in which transformation can occur are ‘competent’ cells.



- 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 Alfoldi, 1976) among several species of *Streptomyces spp. Like S. coeli-color, 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 a*l, 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.

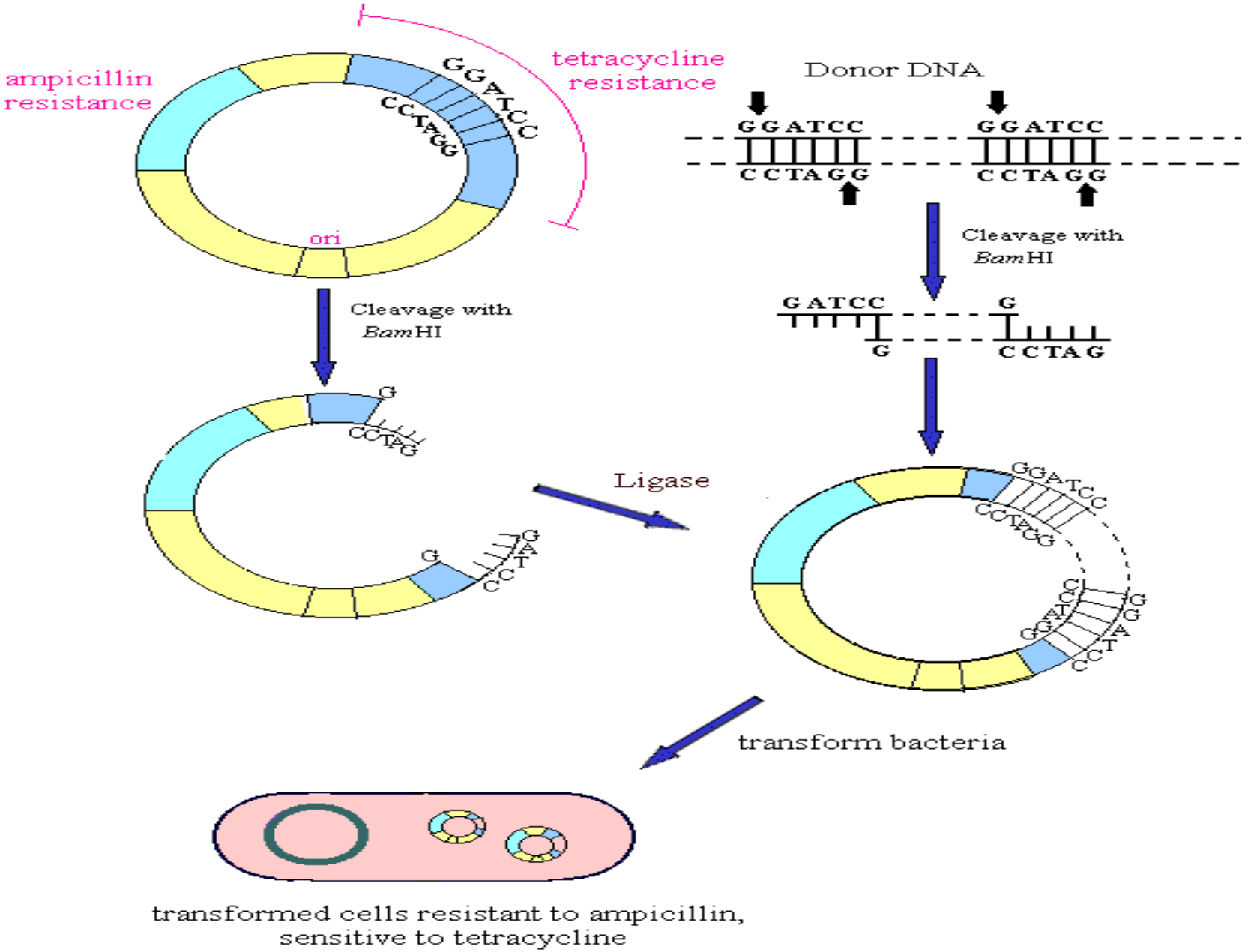
iii. Transformation : Recombinant vector is put into suitable host organism, like; bacteria, yeast, plant or animal cells, by several physical or chemical methods. Transformed cells are identified by several ways:

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

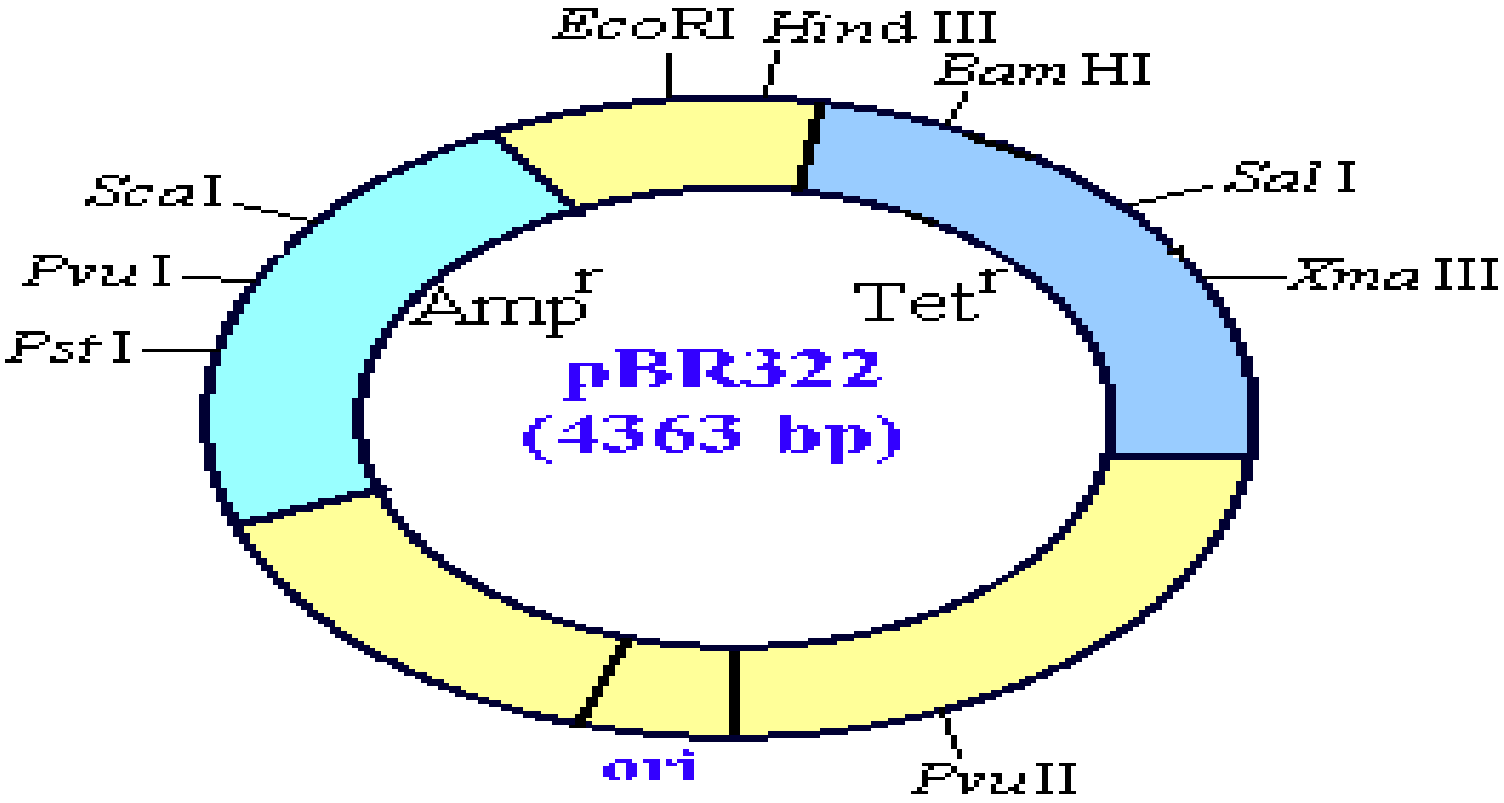
a.) Phages

- 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

Characterstics 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,
- accomodate 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).

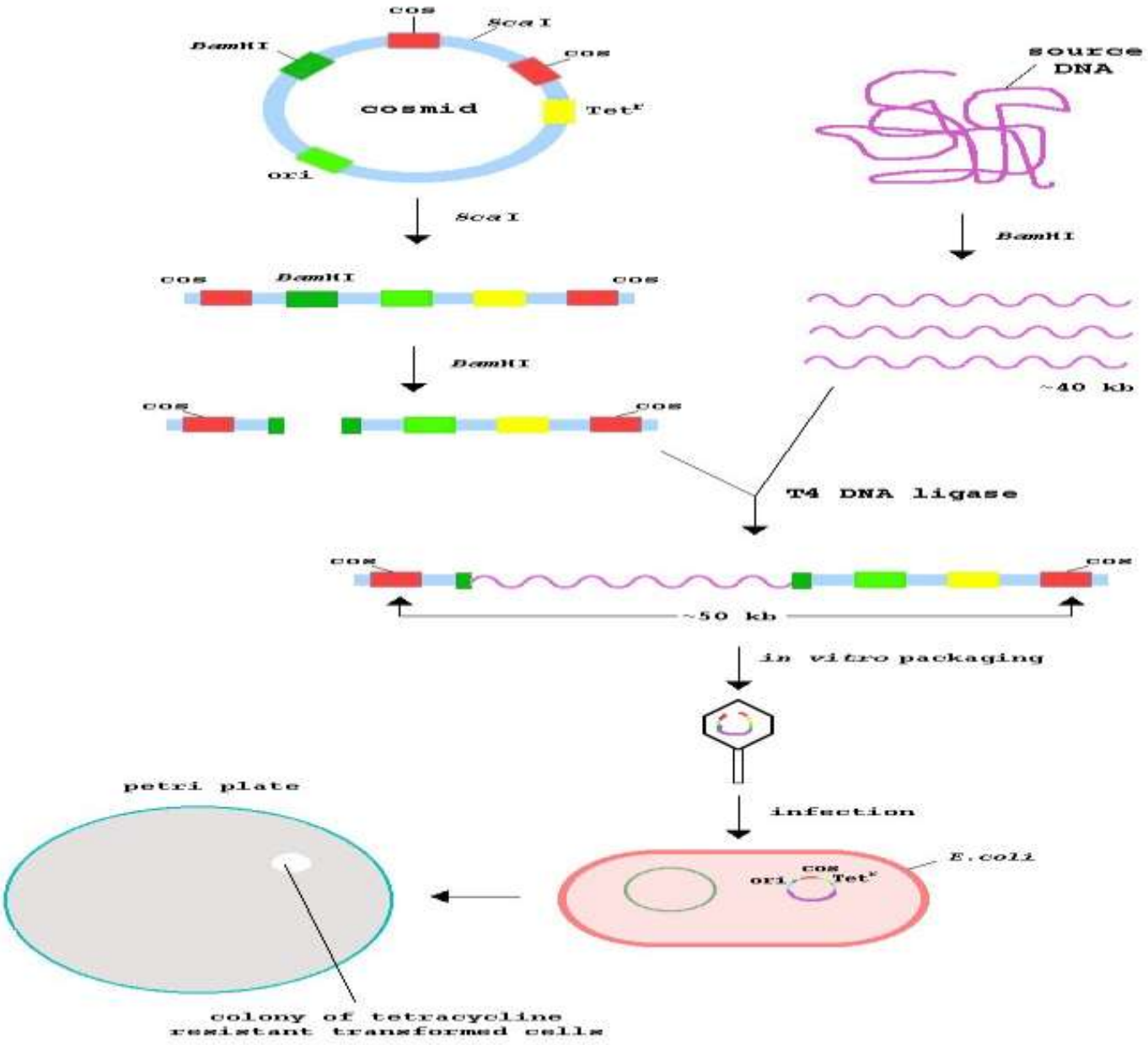
- **Phage lambda** has double stranded DNA, around 48.5 kbp,. There contain single stranded, 5' projections at each end, called as **cos** sites. These are complementary in sequence. When it is injected into host cell, phage DNA circularize by means of these sequences.

- 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.

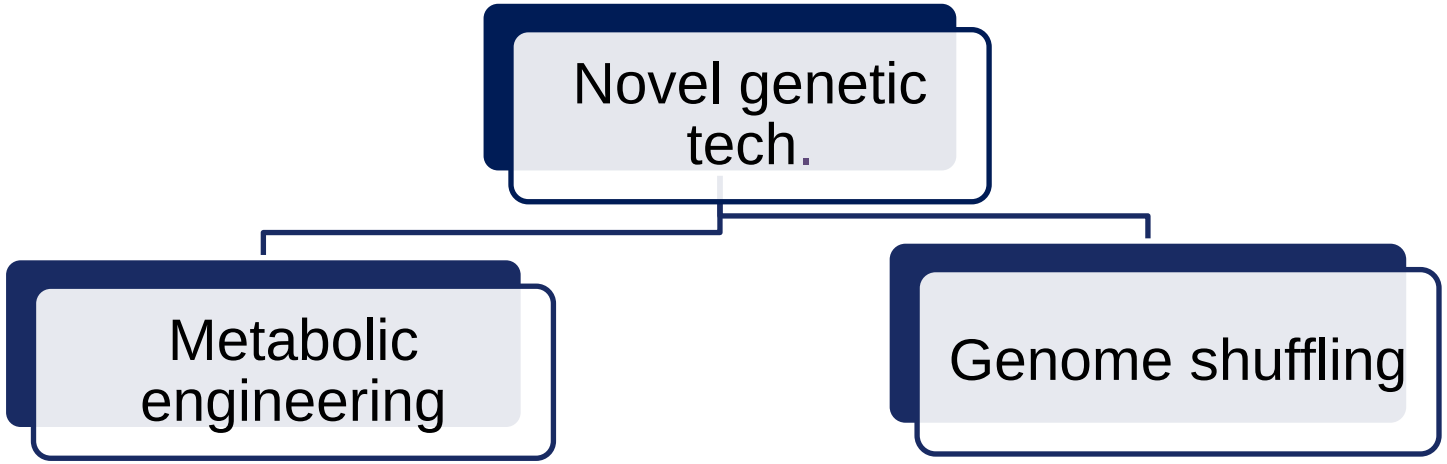
c. Cosmids--

- 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 clonning 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 isoluecine 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*.

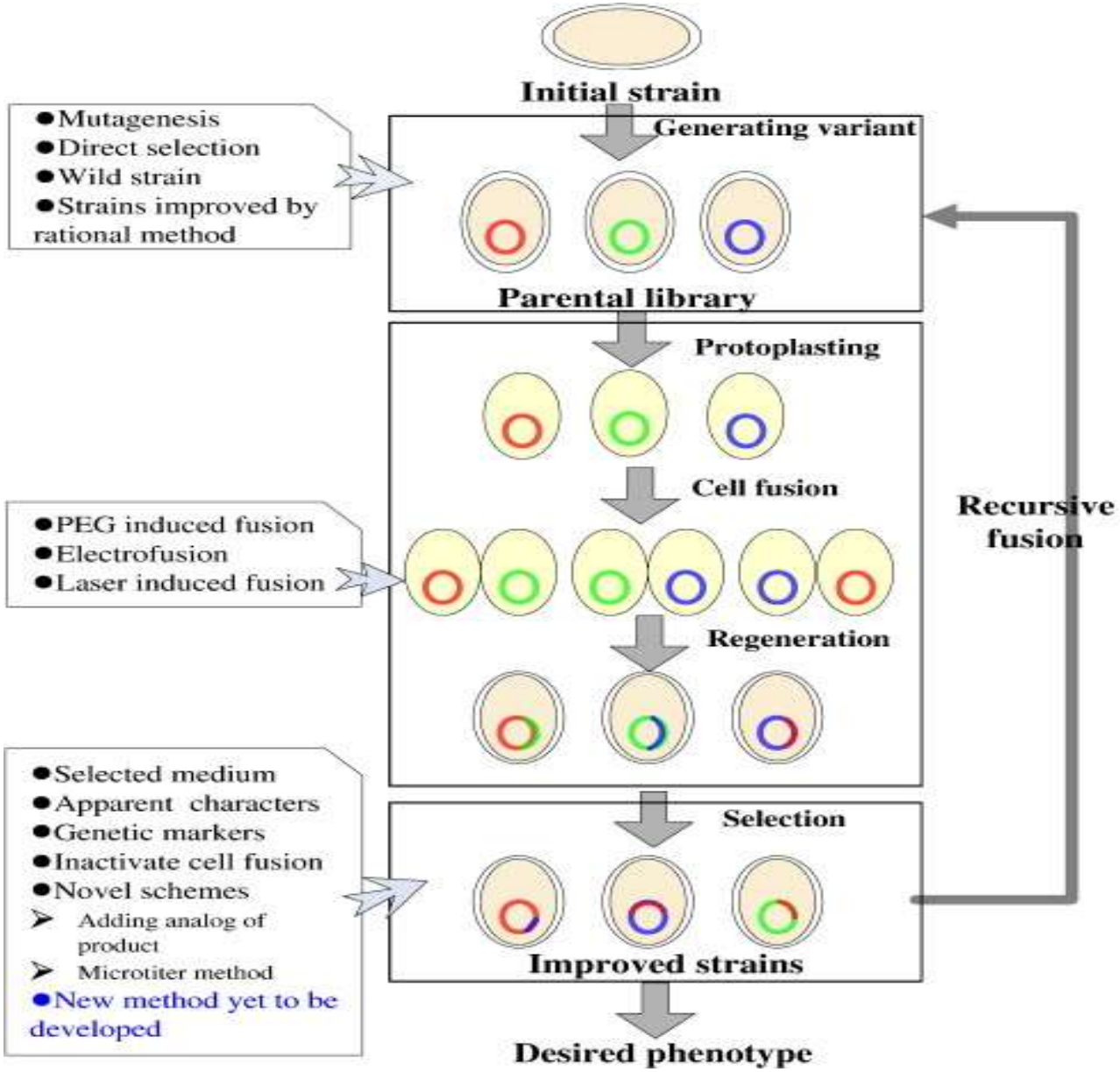
Enhance growth include enhanced substrate utilization
e.g *E.coli ;*glutamate dehyrogenase into *M.methylotrophus* ;carbon conversion increased from 4% to 7%

The Strain of *E.coli* has been engineeried 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 reccursive 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 *Lactobaccilus*(Patnaik *et al* ,2002;Wang *et al*, 2007;John *et al*,2008), improve acetic acid tolerance in *Candida krusei*(Wei *et al*,2008), enhance Pristinaamycin 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 extenive se 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.


THANK YOU....