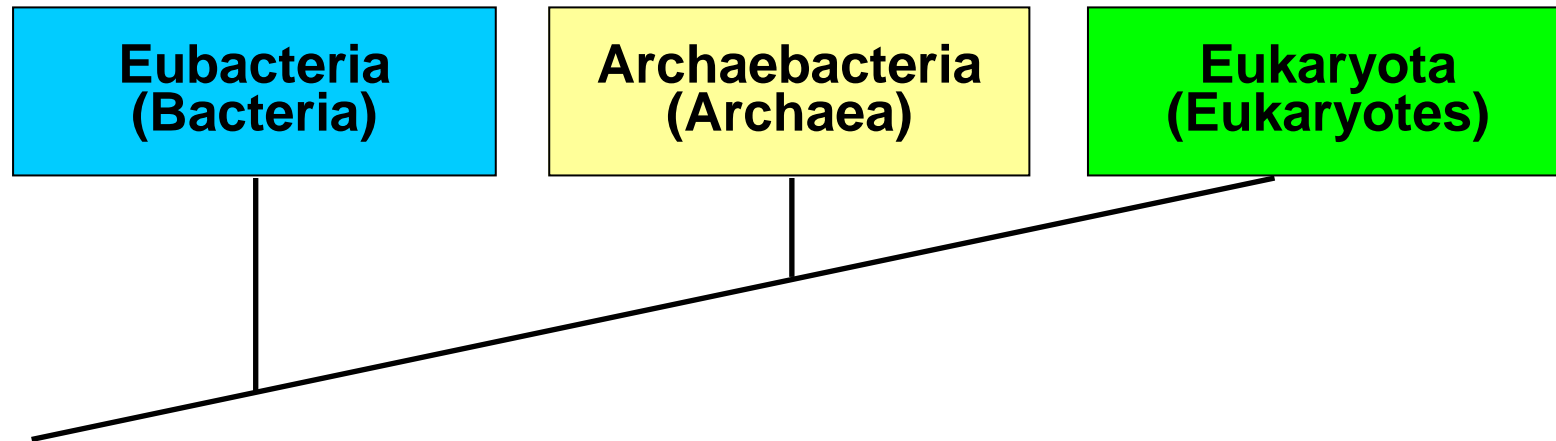


Microbial (Molecular) Biotechnology

What are microbes?

- Microbes are small single-celled organisms
- Either free-living or in colonies
- They can belong to any of the three domains

Three Domains



Eubacteria

- Gram-negative and gram-positive prokaryotes
- Either autotrophs or heterotrophs
- Can be aerobic or anaerobic
- Mesophiles
- Examples:

E. coli

Lactobacillus

Agrobacterium

Staphylococcus

Archea

- Ancient domain, but only recently identified
- Through DNA analysis they were determined to differ significantly from eubacteria
- Found predominantly in extreme environments
(Extremophiles)

Thermophiles 50- 110°C

Psychrophiles 0- 20°C

Alkaliphiles pH>9

Halophiles 3- 20% salt

Methanogens use $H_2 + CO_2$ to produce CH_4

Eukaryotes

Predominately yeasts/molds, protists, algae

Sac shaped cells that form sexual spores

Examples:

Sacchromyces

Penicillium

Aspergillus

Pichia

(Iva Pichová)

Commercial Uses of (GMO) Microbes

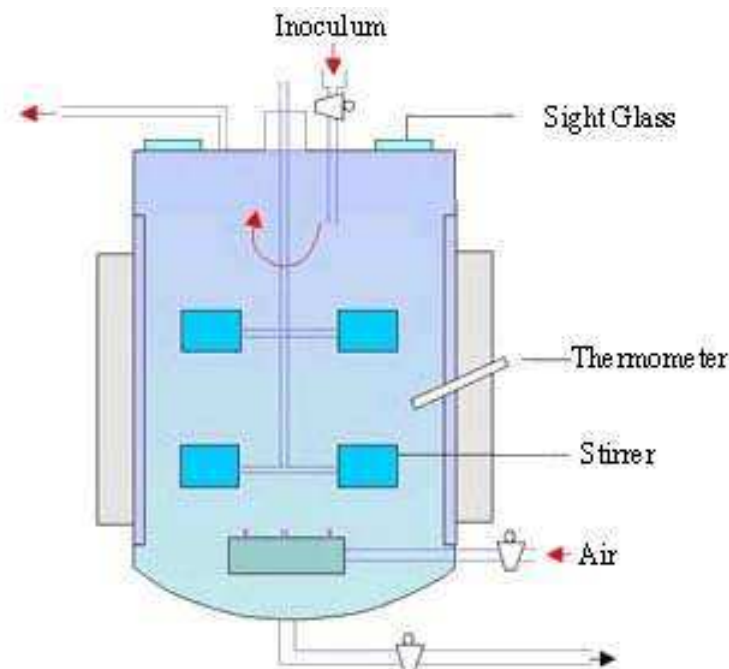
- Products
- Bioconversion/Biocatalysis
- Agriculture
- Bioremediation
- Oil/Mineral Recovery

Industrial fermentation

is the intentional use of microorganisms such as bacteria and fungi to make products useful to humans is a process for the production of useful products through mass culture of single-cells

The end products or the various intermediate products (metabolites) are siphoned off & purified for commercial use

Fermenter or
Bioreactor





15 000L Fermenter



1000L Disposable Bag

http://www.wavebiotech.com/products/wave_bioreactor/system500/index.html
<http://www.pharmaceutical-technology.com/projects/lonza/lonza1.html>

Types of Products Produced in Microbes

- Amino Acids
- Vitamins
- Food Additives
- Enzymes
- Recombinant Protein Drugs
- Antibiotics
- Fuels
- Plastics

Examples of bacterially-expressed proteins:



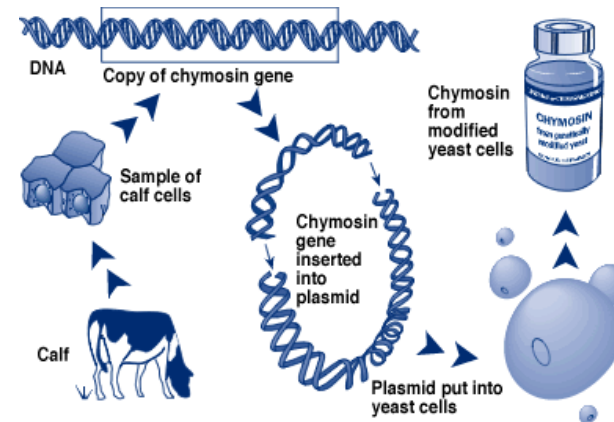
Enzyme: chymosin - the enzyme used to curdle milk products cheese production



Hormone: bST - bovine somatotropin; used to increase milk production

Chymosin

- Proteolytic enzyme (cleaves kappa casein) from the stomach of young calf – maternal milk coagulation – to stay in stomach
- The gene is found in humans (on chromosome 1), but it is not expressed pseudogene



Most hard cheese sold in the UK is made using an enzyme from GM yeast rather than animal rennet.

Chymosin

- In 1988, chymosin was the first enzyme from a genetically-modified source to gain approval for use in food. Such enzymes have gained the support of vegetarian organisations and of some religious authorities. Chymosin obtained from recombinant organisms has been subjected to rigorous tests to ensure its purity.
- Today about 90% of the hard cheese in the UK is made using chymosin from genetically-modified microbes. Notice that the cheese is NOT made using a GMO, but rather the product of a GMO (the enzyme). Consequently, ALL cheeses on sale are in fact 'GMO free'.

Bovine somatotropin

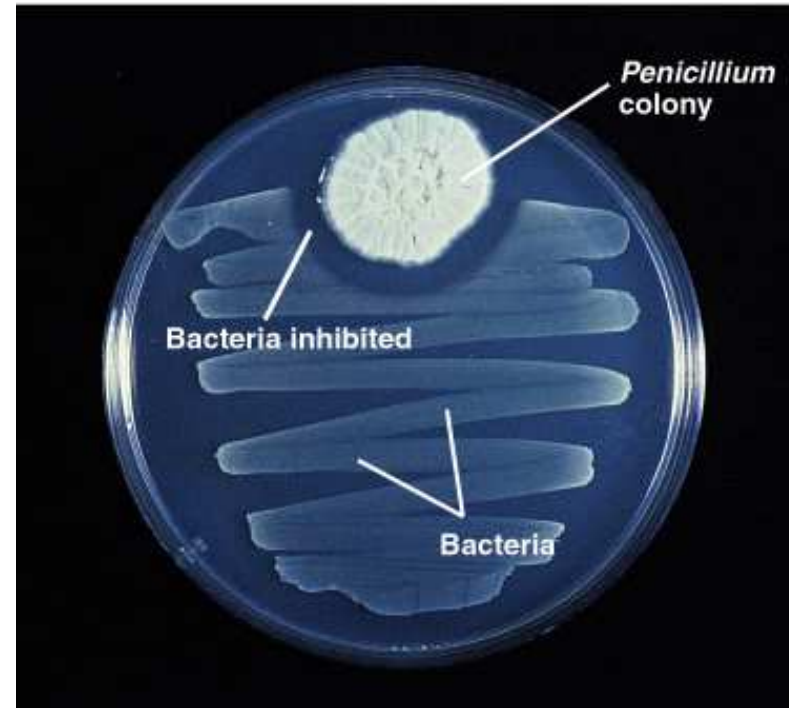
- Bovine somatotropin (abbreviated bST and BST), or BGH, is a peptide hormone produced by the cow's hypophysis. (190 aa)
- Used to increase milk production
- 1982 - Genetic engineering to clone the recombinant BST gene into *E. coli*.
- 1986 - Monsanto was first to get FDA approval for rBST
- rBST has not been allowed on the market in Canada, Australia, New Zealand, Japan, Israel and all European Union

Controversy

- Animal health – true
- Human health - one of the ways that rBGH increases milk production, is by stimulating production of insulin-like growth factor (IGF-1). Human IGF-1 and bovine IGF have an identical amino acid sequence. Well established correlation between abnormally high levels of circulating IGF-1 and the development of human cancers

But IGF-1 is denatured in the stomach

1928: Alexander Fleming discovered the first antibiotic. He observed that *Penicillium* fungus made an antibiotic, penicillin, that killed *S. aureus*. 1940s: Penicillin was tested clinically and mass produced.



Original *Penicillium* moulds produced less than 10 units of penicillin per ml of fermentation broth (1943)

By 1955 *Penicillium* strains produced 8000 units/ml

Mutation with UV and X-Ray, strain selection / culture improvement

Still it is not GMO?

How Are Microbes Modified?

- Artificial Selection
- Recombinant DNA
- Metabolic Engineering

Recombinant DNA Microbes

Transgenic microbes are created when cDNAs for the protein product are cloned into expression vectors

Human genes inserted into *E. coli*

Genes from extremophiles are moved to mesophiles due to the ease in culturing of mesophiles

Mesophiles also have 5 to 10x higher growth rates

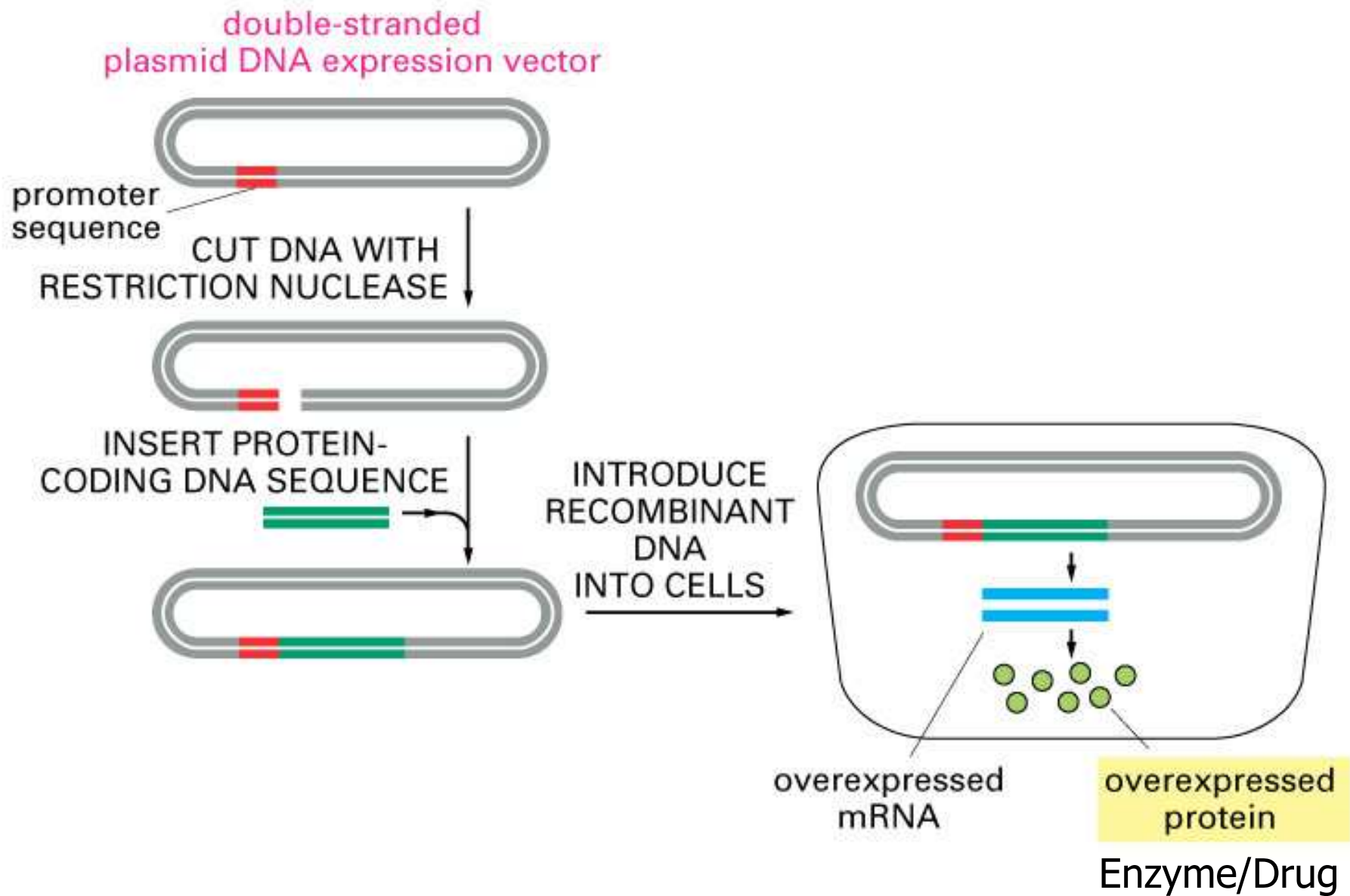
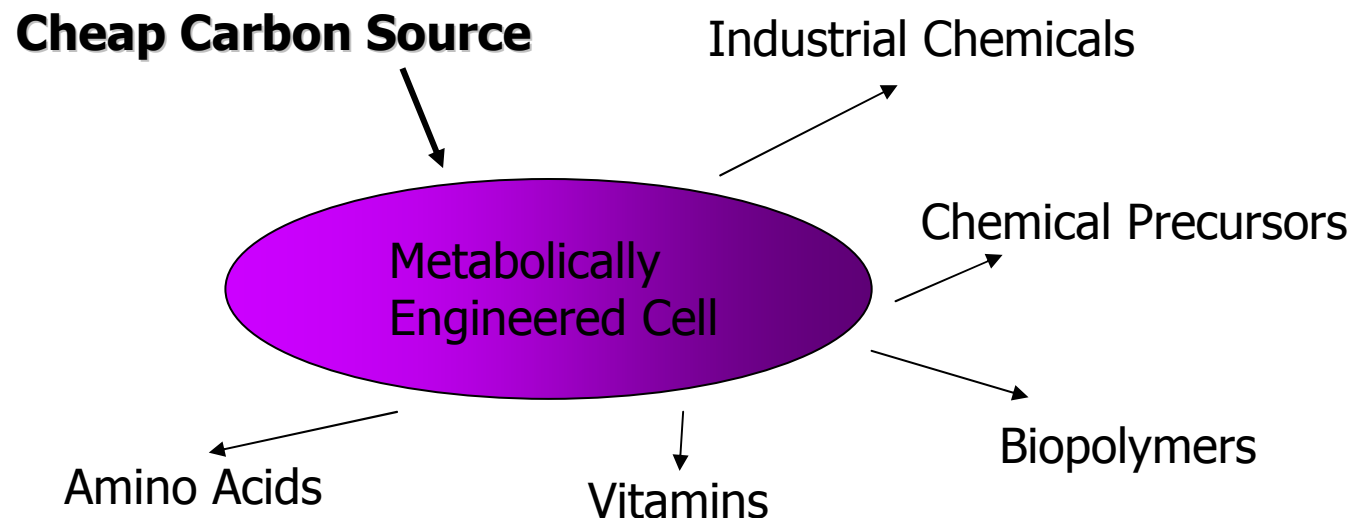
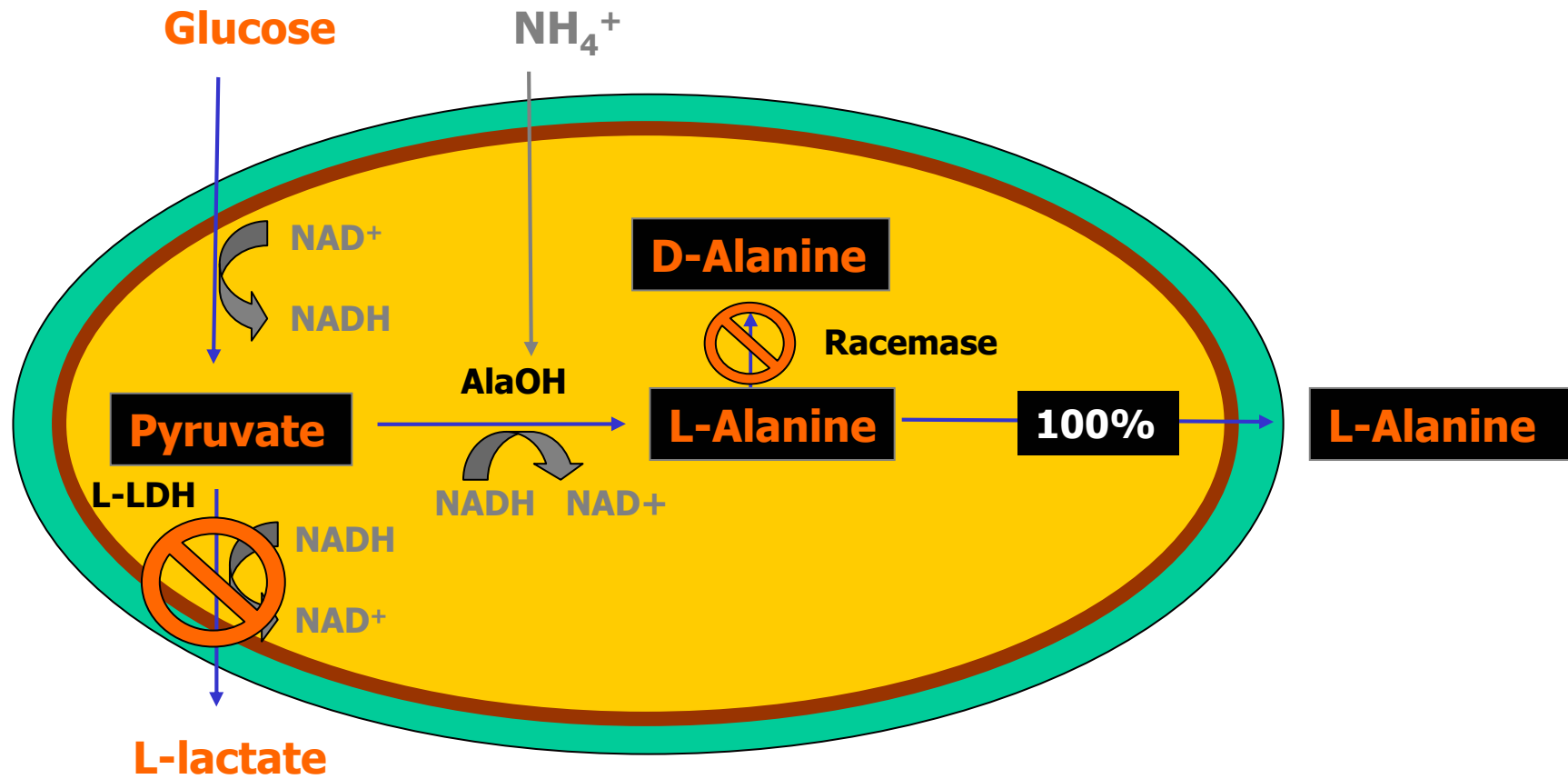


Figure 10-32 Essential Cell Biology, 2/e. (© 2004 Garland Science)

Metabolic Engineering, manipulation of pathways within an organism to optimize the production of a compound

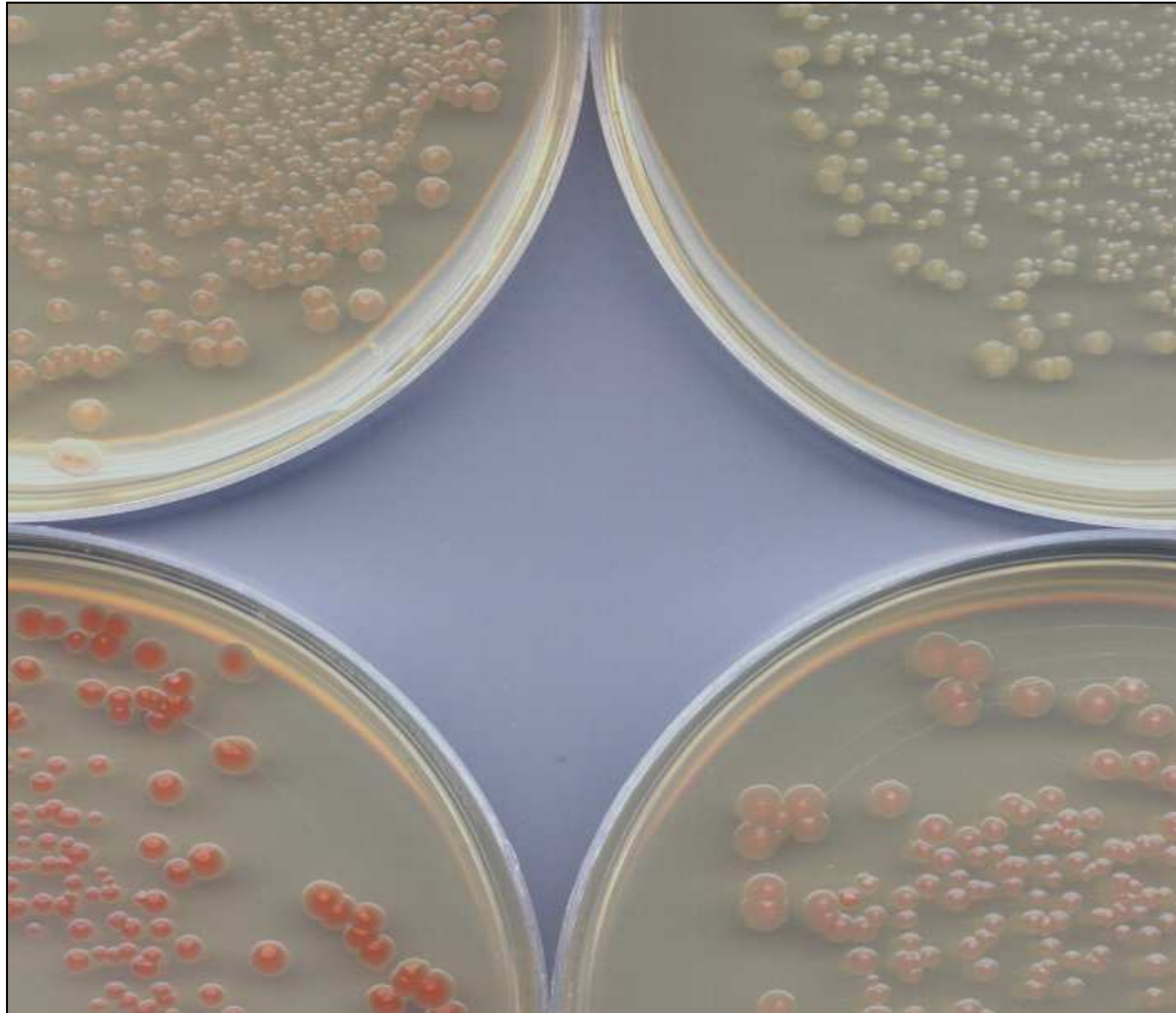
Done by turning off particular genes, either through mutation or deletion





The microbe is forced to produce alanine at higher than normal amounts

Carotenoid production in E.coli cells



Enzymes

Enzymes, the most common product produced by microbes

Overall value of industrial enzymes is about \$2.0 billion¹

They are found in many household items that you would never think to have a biotechnology component

¹ 2004 data

<i>Enzyme Name</i>	<i>GM Organism</i>	<i>Use (examples)</i>
α -acetolactate substances decarboxylase	bacteria	Removes butter aroma from beer
α -amylase	bacteria	Converts starch to simple sugar
Catalase	fungi	Reduces food deterioration
Chymosin	bacteria or fungi	Clots casein to make cheese
β -glucanase	bacteria	Improves beer filtration
Glucose isomerase	bacteria	Converts glucose to fructose
Glucose oxidase	fungi	Reduces food deterioration
Lipase	fungi	Oil and fat modification
Maltogenic amylase	bacteria	Slows staling of breads
Pectinesterase	fungi	Improves fruit juice clarity
Protease	bacteria	Improves bread dough structure
xylanase (hemicellulase)	bacteria or fungi	Bread dough, breakdown of plant cell walls

Detergent Enzymes

Detergents are the largest application of industrial enzymes

Traditionally these are **lipolases, proteases & amylases**

A recent innovation is the addition of **mannanase**

(this enzyme aids in removing stains containing guar gum -ice cream, tomato sauce or salad dressing - thickener)

These enzymes are engineered to improve stability in the presence of detergent, alkaline pH, and cold water

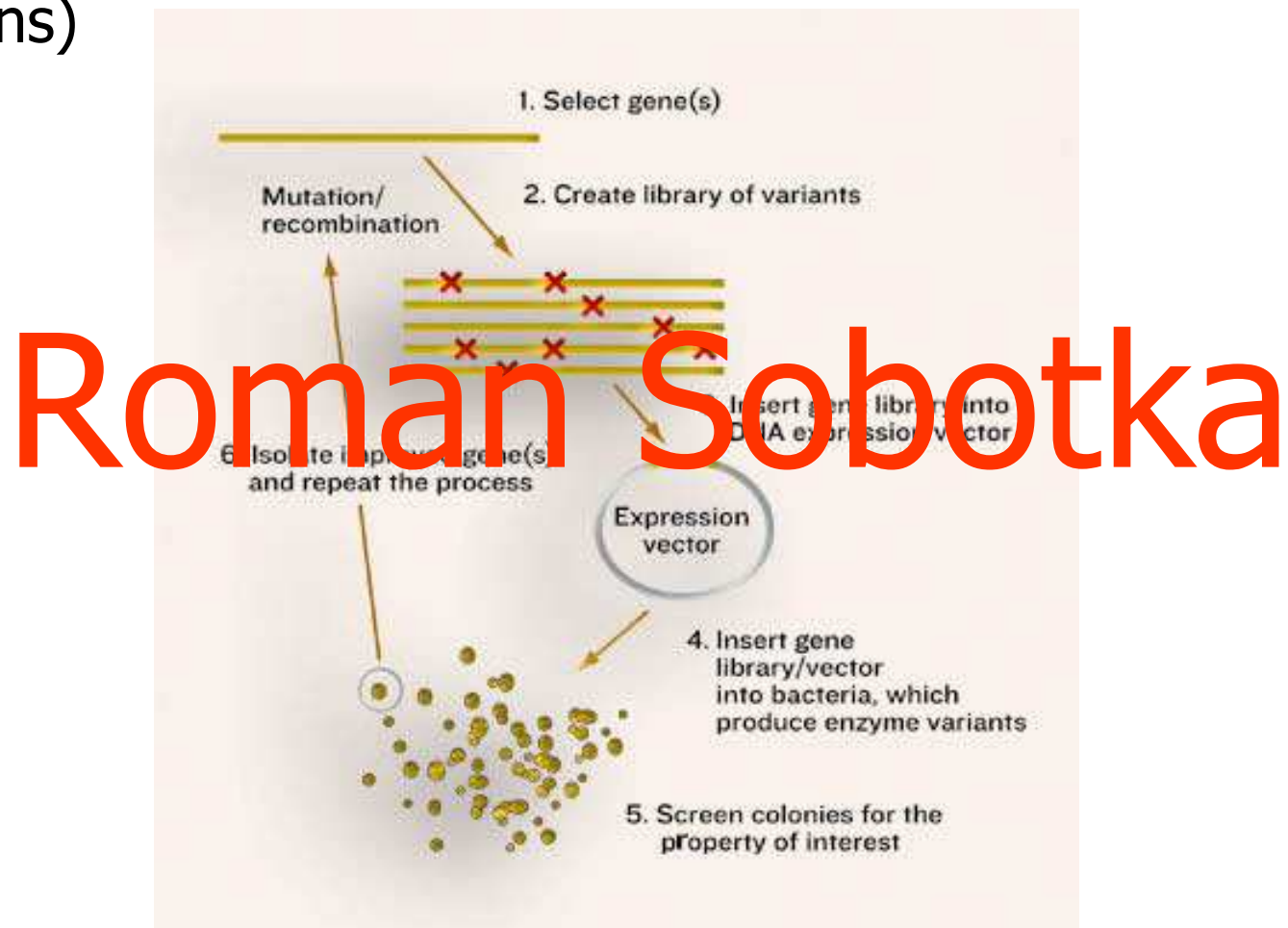
Subtilisin, a protease used in laundry detergents

The recombinant protein was engineered to remain active in the presence of bleach (Sodium hypochlorite, NaClO)

Bleach caused the oxidation of one amino acid (methionine) and the enzyme lost 90% of its activity

By replacing this amino acid with alanine, the engineered enzyme was no longer sensitive to oxidation

Directed evolution is the most recent tool utilized in the creation of new and better enzymes (& other proteins)



Products produced in Prokaryotic Systems

- Restriction Endonucleases -> produced in *E. coli*
- L- Ascorbic Acid (Vitamin C) -> recombinant *Erwinia herbicola* (gram-negative bacterium)
- Synthesis of Indigo (blue pigment -> dye cotton /jeans) -> produced in *E. coli*
- Amino Acids -> produced in *Corynebacterium glutamicum* (gram-positive bacterium)
- Lipases (laundry industry) -> from *Pseudomonas alcaligenes* produced in *Pseudomonas alcaligenes*
- Antibiotics (most of them from *Streptomyces*, other gram-positive bacteria, fungi) -> produced in recombinant *Streptomyces* and fungi (*Penicillium*)
- Biopolymers (PHB - Polyhydroxybutyrate-> biodegradable plastics) -> produced in *E. coli*

Enzymes for Feed

Enzymes are used in animal feed to breakdown cellulose
(cellulase)

New use of enzymes (phytases) which breakdown phytic acid
(phosphatase)

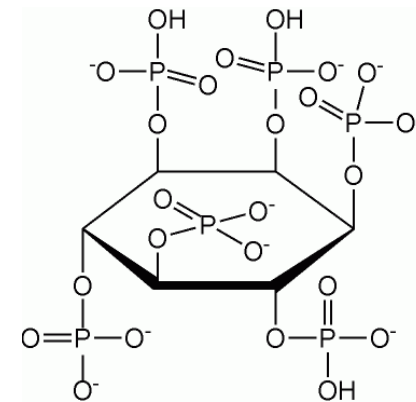
This allows better utilization of plant phosphorus stores

Allowing bone-meal to be removed from feeds

The latest generation of phytases are from fungus and have been
engineered to survive high temperatures used during food processing

65% of poultry and 10% of swine feeds contain enzymes

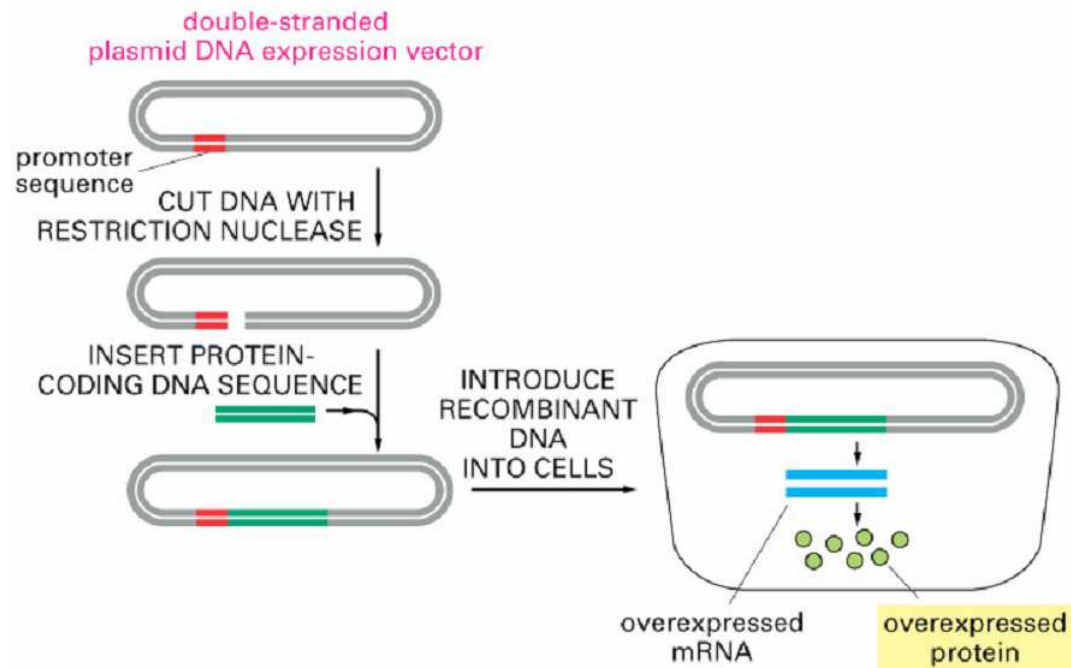
Phytic acid - myo-inositol hexakisphosphate
in grains and oil seeds



Recombinant Drugs

Besides antibiotics which are derived from microorganisms

Protein medicines are produced by inserting human genes into microbes



1982, FDA approves the first recombinant protein drug, **human insulin** produced by *E. coli* developed by Genentech

Today there are >75 recombinant protein drugs approved by the FDA with 100s more being studied

Currently the global market for recombinant protein drugs is \$47.4 billion¹

Other Products From Microbes

Fuels, Plastics, Medications

Ethanol Production

Produced via anaerobic fermentation by yeast

Corn starch is hydrolyzed to glucose monomers

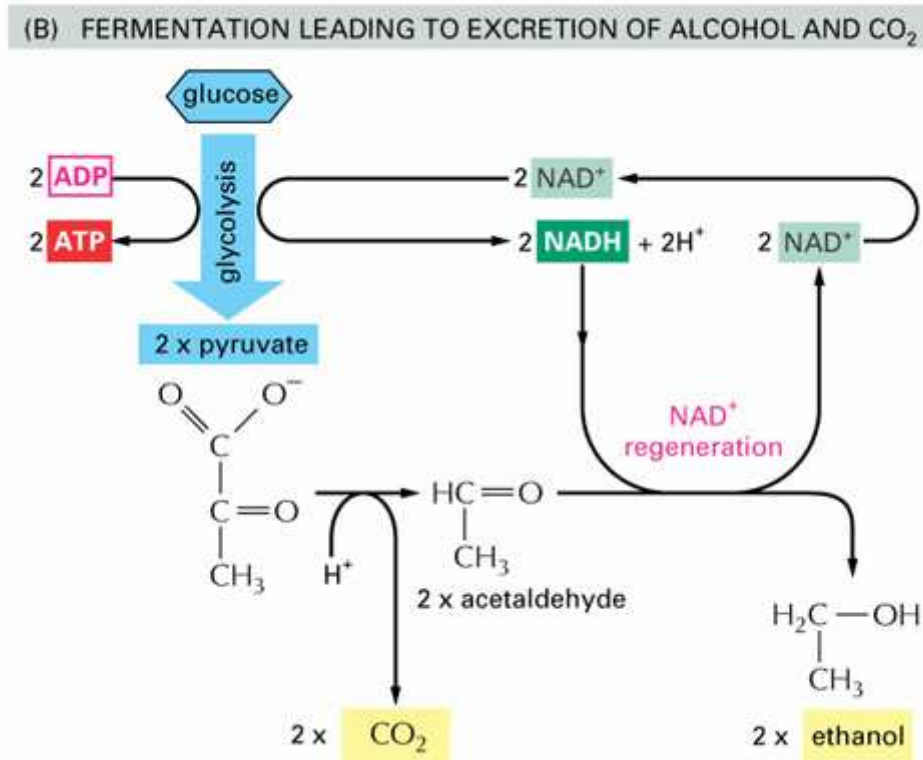


Figure 13-4 part 2 of 2 Essential Cell Biology, 2/e. (© 2004 Garland Science)

Problem with Corn Ethanol

Ethanol contains 76000BTU/gal

Takes ~98000BTU/gal to produce from corn sugar

Gasoline contains 112000BTU/gal

Costs 22000BTU/gal to extract and refine

A BTU (British thermal unit) is defined as the amount of heat required to raise the temperature of one pound of water by one degree Fahrenheit

2004 3.4 billion gallons of ethanol were produced

U.S. consumes 140 billion gallons of gasoline/yr

Plastics

Polyhydroxyalkanoates (PHAs) is a polymer made by some microbes as a way of storing carbon

PHB - Polyhydroxybutyrate-> biodegradable plastics) -> produced in *E. coli* on lactose

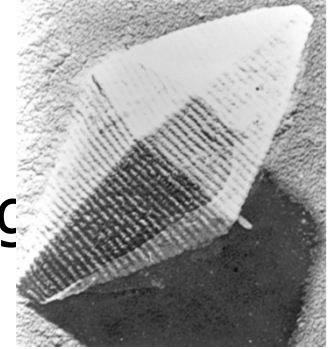
Up to 80% of the microbe's biomass is plastic

PHA is sold to make shampoo bottles in Germany, and disposable razors in Japan



Microbes and Agriculture

Microbial Pesticides



Bacillus thuringiensis (Bt) is an aerobic spore-forming bacterium

During sporulation produces insecticidal crystal protein (ICP), a toxin (Cry)

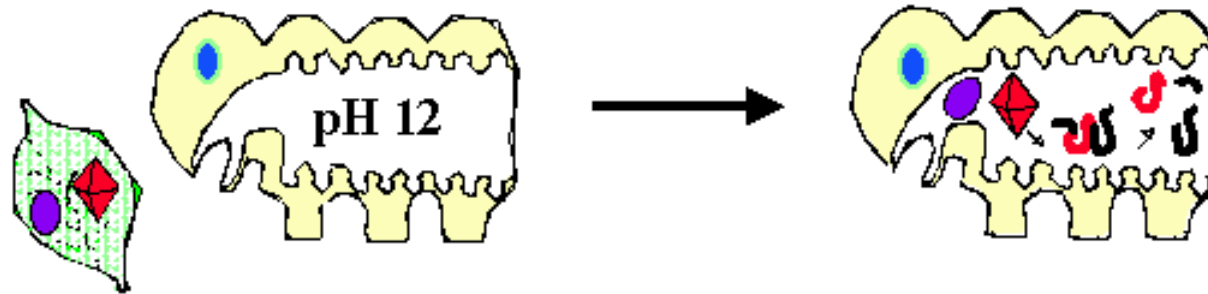
The toxin breaks down quickly in the environment

They have no toxicity to humans & there is no withholding period on produce sprayed with Bt

Cry toxins vary in their toxicity and specificity

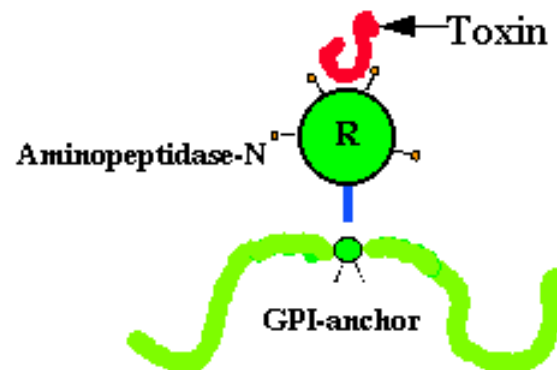
Strain engineered to produce toxin constitutively, mutated variants made in *E. coli* to back-transform *Bacillus* to broaden toxin specificity

MECHANISM OF TOXIN ACTION

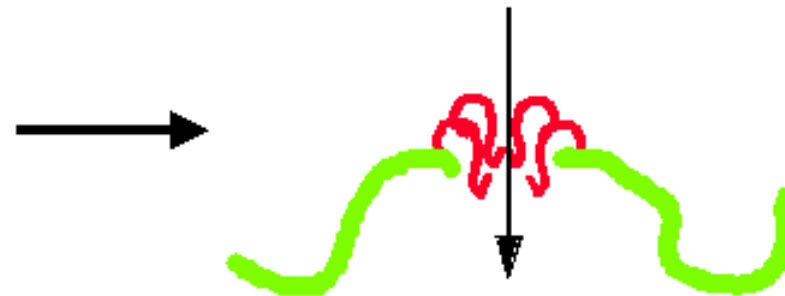


1 Crystal and spore eaten by insect

2 Crystal dissolves and protoxin is processed to smaller 'active' form by gut enzymes



3 Activated toxin binds to receptor (R) in the midgut epithelium



4 Toxin inserts into the membrane making it permeable to ions and small molecules so that the cell bursts



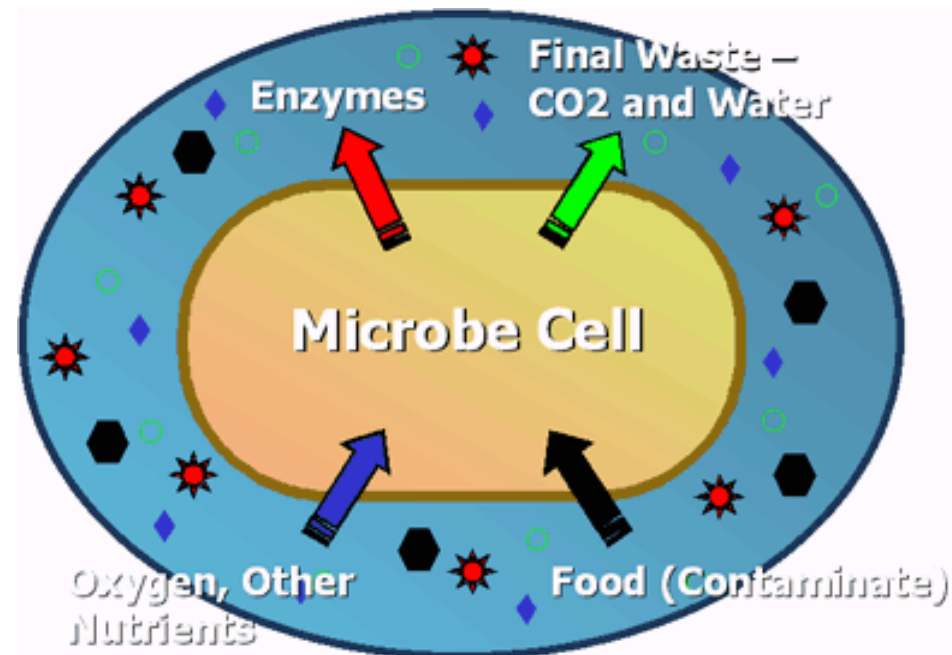
Lets make the plant to make Bt

Bioremediation

Usually not *GMOs*

Bioremediation is reclaiming or cleaning of contaminated sites using microbes or other organisms

This entails the removal, degradation, or sequestering of pollutants &/or toxic wastes



Bacteria are isolated from the contaminated site based on their efficiency at digesting & converting the waste

The bacteria are tested for performance and safety

Bacteria are placed back in the waste environment in high concentrations

The bacteria grow & in the process digest & convert the waste into CO_2 and H_2O – not a single strain but consortia

Frequently, you need to feed them

What can be cleaned up using bioremediation?

- Oil spills
- Waste water
- Plastics
- Chemicals (PCBs)
- Toxic Metals

Oil/Wastewater Cleanup

Bioremediation

Bacteria degrade organic matter in sewage.



Bacteria degrade or detoxify pollutants such as oil and mercury



Microbes that digest hydrocarbons found throughout the environment

These naturally occurring microbes are utilized during a spill to clean shore lines

Fertilizer is added to supply the nutrients phosphorus and nitrogen

This approach was used after the Exxon Valdez

Stimulated the natural rate of biodegradation by 2 to 5x

There have yet to be any other instances of this being used on a large-scale



Exxon Valdez off the Coast of Alaska

Smaller scale cleanup is feasible

For 3 months nutrients and microbes were sprayed on this field

After 11 months the site was deemed clean



Before



After

6000yards³ petroleum conc. Before 4000ppm After 100ppm

Wastewater

Treatment of domestic sewage or industrial waste

Utilizes aeration to oxygenate allowing aerobic microbes to digest solid waste



Before



After

Plastic Degradation

140 million tons of plastics are produced each year

Traditional plastics are very stable and do not degrade

Some plastics have been shown to be biodegradable

Strains of bacteria have been isolated that breakdown:

Polyurethane

Polyvinyl alcohol

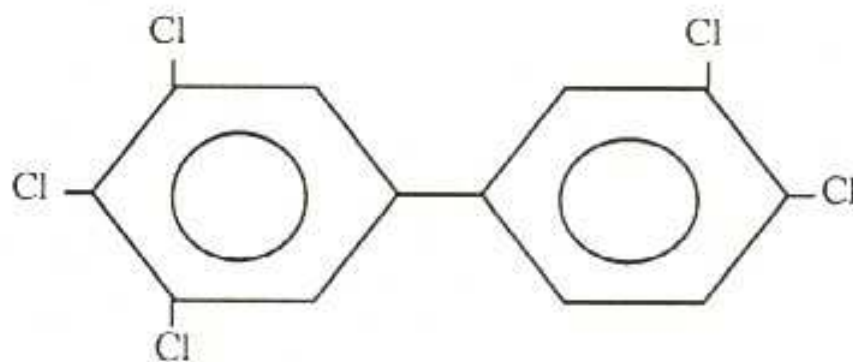
Nylon-66

Chemicals

Polychlorinated biphenyls (PCBs)

PCBs have low water solubility, good insulating properties, high boiling points and resistance to chemicals

The largest uses for PCBs was in capacitors, transformers, & as plasticizers



(b) 3,3',4,4',5-Pentachlorobiphenyl (IUPAC #126)
(A true coplanar PCB)

1977, Monsanto (main producer) stops all PCB production

Millions of lbs of PCBs are still in place around the world

The stability properties that made PCBs so useful have allowed them to persist in the environment

Most people in industrialized countries have PCBs in their tissue

Microbes that dehalogenate PCBs have been isolated

This process is referred to as [halorespiration](#)

Involves the replacement of the Cl with an –OH

This process is multi-step with four enzymes required

These enzymes are now the target of protein engineering to optimize their performance

Heavy Metal Clean up

Uranium processing has left contaminated groundwater sites across the United States and the world

Traditional “pump-and-treat” methods take decades and expose workers to toxic levels of uranium

Geobacter to convert soluble uranium (VI) to insoluble uraninite (IV)

The microbes are encouraged to multiply by injecting acetate

In ~50 days, 70% of the uranium is converted into uraninite.

Biomining

Microbe assisted mining has gone on for millennia

Early copper miners used microbes to leach copper from ore without even knowing it

Low-grade ore and mine tailings are exploited biologically

Sulfides of metals like zinc, copper, nickel, cobalt, iron, tungsten, lead are insoluble in water

These sulfides are converted to sulfate which are soluble

The sulfates leach out of the ore and are then extracted



Cu_2S not soluble



CuSO_4 is soluble



Commercial Bioleaching Tanks