# PRODUCTS AND TECHNOLOGIES IN BIOENGINEERING

Products of biochemical engineering:

1. Primary metabolites: their biosynthesis is directly connected to the growth or energy production of cell (amino acids, organic acids, ethanol)
2. Secondary metabolites: their biosynthesis is not connected to the growth or energy production of cell, the production is forced by unfavorable conditions (like substrate limit) (antibiotics, pigments).
3. Recombinant proteins, which were not coded in original genom of cell, their gene is transmitted from an other organism.
4. Bioconversion products (aspartame, steroids)

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# 1. Production of primary metabolites : metabolic engineering

The genome is changed with mutations:

- Branches of biosynthetic pathway are closed, whole substance flux is forced to form the product (auxotrophic mutants)

- Reactions transforming the end product are eliminated. (auxotrophic mutants)

- Control mechanism of overproduction is to be eliminated (antimetabolite resistant mutants)

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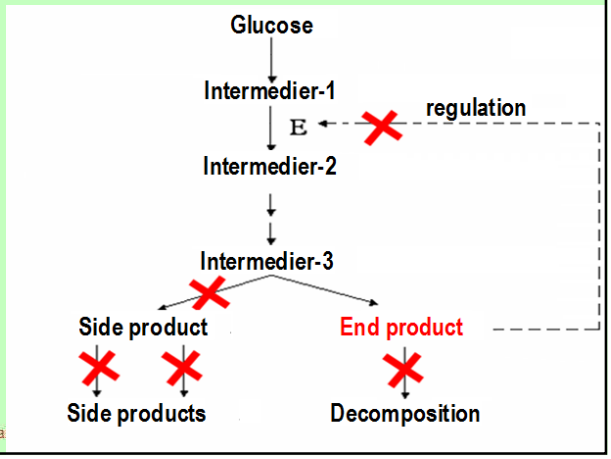
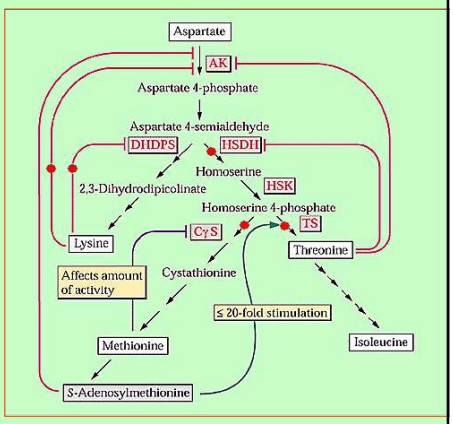


# Case study: fermentation of lysine

A lysine is produced from aspartic acid.
The following mutants were isolated:

- Hom$^-$ and Hom$^{leaky}$,
- Met$^-$, Thr$^-$ auxotrophs, and
- AEC$^r$ and ML$^r$ regulatory mutants.

In some organisms the lysine is de-carboxylated to cadaverine but this producing bacteria don't have this activity.



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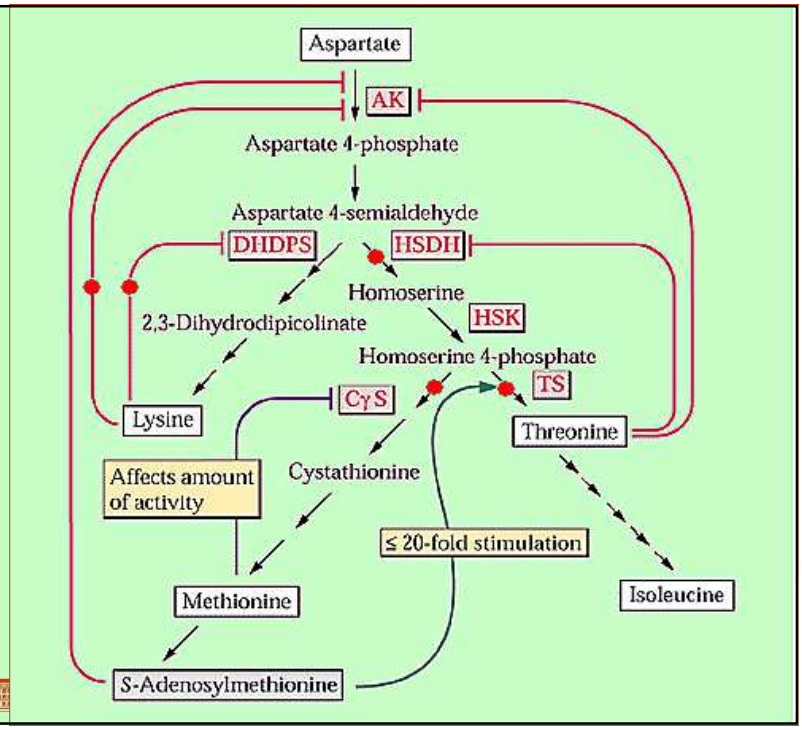
# Case study: fermentation of lysine



## Fermentation technology

*Corynebacterium* and *Brevibacterium* strains are used.
C-source: dextrose, molasses, in alternative processes acetic acid or paraffins.
Nitrogen source: ammonia, ammonium salts or urea.
Homoserine, threonine and methionine must be present in small concentration (soy meal, corn steep liquor), but if we have a leaky mutant, this can be omitted → cost reduction.
Biotin: min 30 $\mu$g/l is necessary (beat molasses)
Opt: pH = 7, T = 28°C t(ferm) = 60 hrs
Final concentration: 100-120 g/l, productivity $Y_p$ = 30-40%.

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## Secondary metabolites: antibiotics

= Secondary metabolites produced by microorganisms that can inhibit or kill other microorganisms.
In the last 80 years ~12-13 thousands antibiotics were discovered. Only ~2-300 molecules became a human medicine. From these ~10% is produced with direct fermentation, ~80 % with fermentation, after that chemical modification (= semi synthetic drug). The left 10% is produced with chemical synthesis (costs).

Why so few?
- toxicity
- low efficiency, competitors are better
- side effects
- resistance

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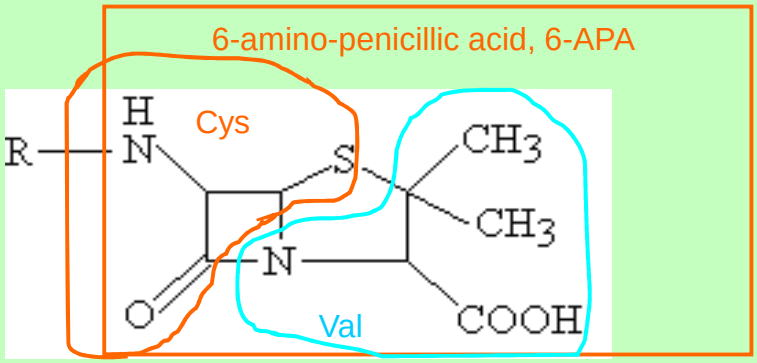
## Case study: penicillins

Structure: β-lactam ring: 6-amino-penicillic acid

Combination of two amino acids: cysteine + valine

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## Production of penicillin

Fermentation, chemical synthesis is not economic.
Main fields of technological development:

Strain development (bio):
- Screening
- Induced mutation
- Selection of mutants
- Strain conservation

Technology (engineering):
- Surface/submerged
- Precursors (4-8 x)
- Medium optimation
- Control of metabolism (sugar limit, C/N, Fe ion)
- Aeration, bioreactor
- Controlled conditions (pH, t)

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## Phases of production:

1. Strain conservation
2. Inoculum propagation steps
3. Producing (main) fermentation
   "Fed batch": batch process with regular nutrient additions
4. Downstream processing: key operation:
   extraction: removal of penicillin with non-miscible organic solvent (cooling, short contact time)

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## Main fermentation

Typical secondary metabolite fermentation, with two phases:

First phase (~40 h): cell propagation, optimal nutrient supply, intensive aeration, agitation, primary metabolism.

Nutrients in this phase:

- Carbon source: few % of sugar (dextrose, molasses), consumed till the end of propagation phase
- Nitrogen source: in this phase inorganic ($NH_4$) salts consumed till the end of propagation phase
- P: added as inorganic phosphate but it should be consumed till the end of propagation phase

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# Main fermentation

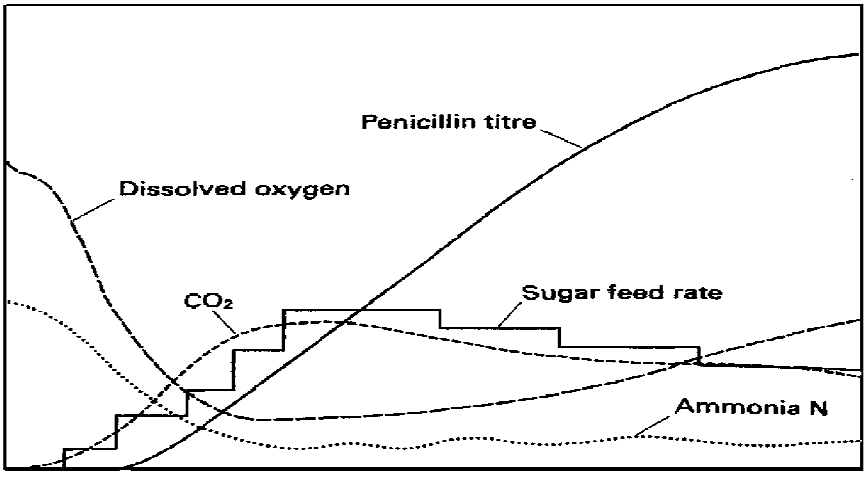
Second phase, production phase: 120-160 h, multiple substrate limitation, forced secondary metabolism.

Nutrients in the second period:

- Carbon source: sugar limited metabolism (earlier: hardly metabolisable compounds: lactose, starch; nowadays: dosage of small amounts of dextrose, according to the oxygen level)
- Nitrogen source: addition in the form of proteins: CSL, soy meal, peanut cake → daily dosage to keep a low concentration
- P: in the presence of phosphate the secondary metabolism doesn't run therefore no phosphate addition.
- Precursor: phenyl-acetic acid, regular addition to keep the concentration in the 2-4 g/l range.

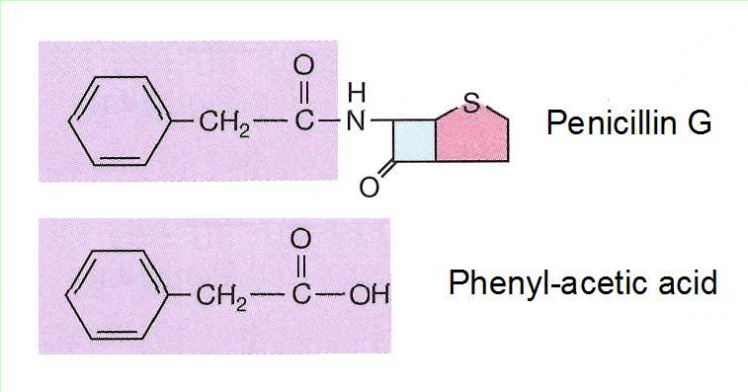


# Diagram of penicillin fermentation




# Precursor

Compound prepared with chemical synthesis given into the fermentation broth, where the microorganisms are ready to built it into the product molecule. This saves substances and energy for the microbe.

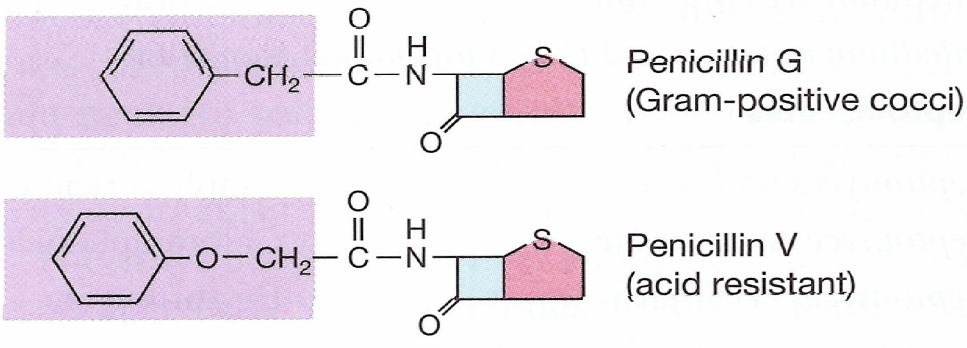




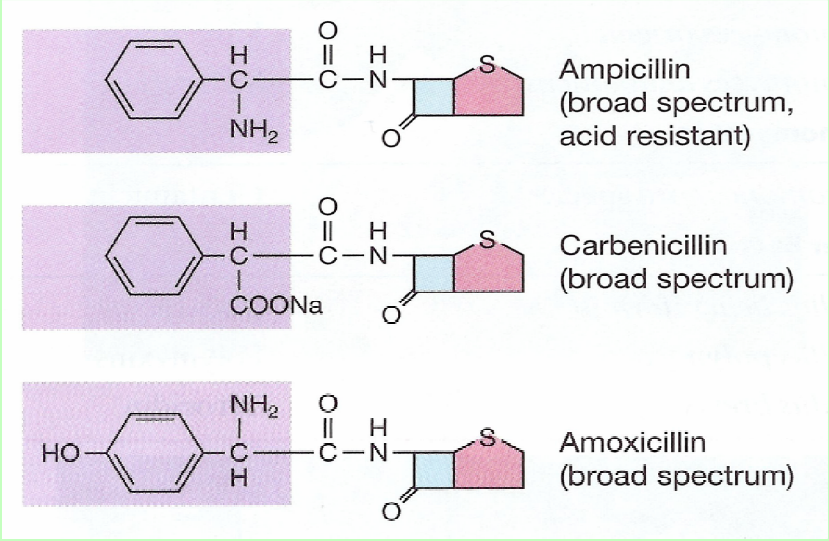

# Semisynthetic penicillins

The fermented platform molecules:

**Natural penicillins**



## Semisynthetic penicillins






## Summary of secondary metabolite production

Strain development:
The classic mutation–selection method is repeated since 60 years. Genetic manipulation is less effective because of complexity of biosynthesis and regulation.

Technology:
Two-phase fermentation, first cell propagation than product formation.

Market:
Patents run out, antibiotics became generic drugs. Hard competition, depressed prices. (China, India)

# RECOMBINANT PROTEINS

By function:

- Hormones (insulin, erythropoietin)
- Enzymes (general – medical use)
- (Monoclonal) antibodies (therapy - analysis; Herceptin, ProstaScint)
- Vaccines (active and passive immunization)

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# Production of recombinant proteins

Options:

- With prokaryotes (bacteria)
  - Quick growing, cheap media, but:
  - The product often intracellular,
  - No glycosylation → the protein has no activity
- With eukaryotes (animal cell culture)
  - Slow propagation, expensive medium, elaborated fermentation, lower product concentration, but:
  - biologically active product.

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# RECOMBINANT VACCINES

Subunit vaccines: only one immunogenic protein (=subunit) of the virus is produced as recombinant protein, and used as antigen in active immunisation. Steps of production:

1. Isolation or synthesis of the gen coding the antigen protein.
2. Transfection into a proper host cell and expression.
3. Protein production with fermentation.
4. Downstream processing



# HEPATITIS B VACCINE

HBV – hepatitis B virus – destroys the liver cells, causes liver failure, icterus, cirrhosis, rarely carcinoma. Acute illness, no spontaneous healing.

~5% of the mankind is infected → ~350 million
Transmission of virus: blood, common needle, sexual contact

Latency period: 1,5 – 3 months, also infective

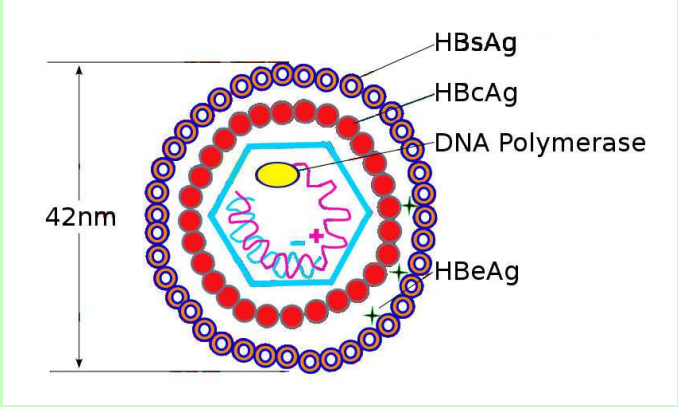
Virus particles are synthesized in liver cells and after the break up they spread in the blood. Viral proteins and antibodies can be detected in blood.

# HEPATITIS B VACCINE

The HBV (hepatitis B virus) has 3 different antigens:

s – surface,
e – endo,
c – core

+ a DNA and DNA-polymerase





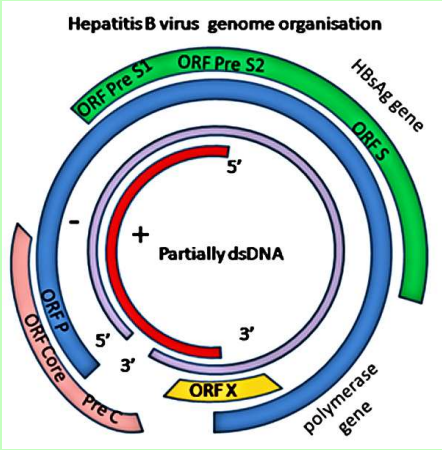

# HEPATITIS B VACCINE

The DNA of HBV is not double stranded everywhere!

The – strand has ~3200 nucleotides, the + strand has only 55-75% of it.

The HBsAg protein has 226 amino acids, sugars and lipids, too.

This gene was cloned.



# HEPATITIS B VACCINE

At first the DNA of surface antigen was cloned into *E. coli* by a plasmid. It formed the protein but it had no activity, because:

- lipid and carbohydrate parts were missing
- the proper folding (3D structure) was not formed

Later the gene was vectored into

- yeast (active protein, proper glycosylation but intracellular)
- animal cell (active protein, proper glycosylation and extra-cellular)

Both technologies give proper product but the yeast vaccine is cheaper and safer (no mammal viruses).

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# HEPATITIS B VACCINE

The structure of shuttle vector:

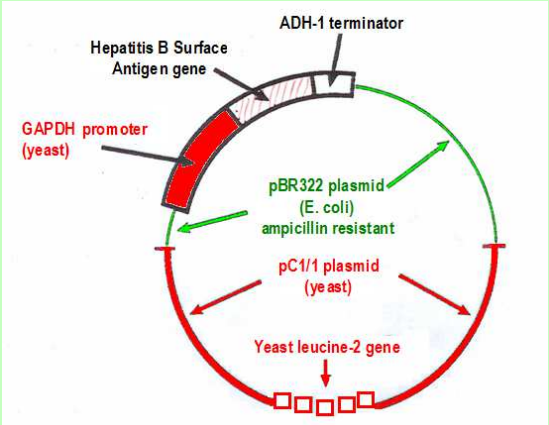
Two replication origins

Two markers:

- ampicillin resistance
- leucine enzymes (the yeast is Leu$^-$)

Expression cassette:

- constitutive promoter
- useful gene
- terminator gene

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# HEPATITIS B VACCINE

Upstream: batch fermentation
First phase: Leu-free medium (increases the plasmid number)
Second (production) phase: complete medium, with Leu

Downstream:
Centrifugation
Cell disruption (high pressure homogenizer)
…
Purification steps
….

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