# 10th lecture: Products and Technologies in Bioengineering

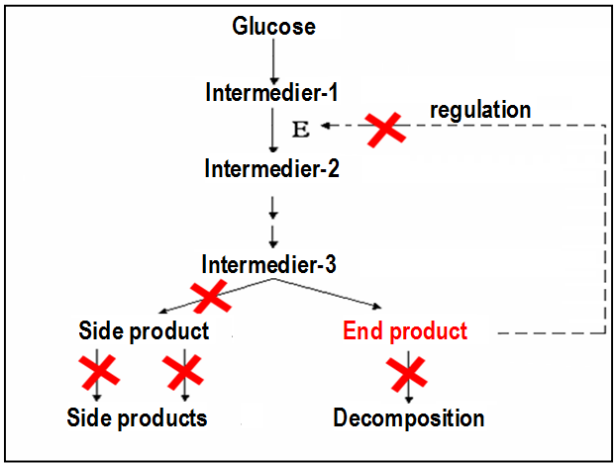
**Products of biochemical engineering**:

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 ge-nom of cell, their gene is transmitted from an other organism.
4. Bioconversion products (aspartame, steroids)

**Production of primary metabolites** :

The growing understanding of the biochemistry and genetics of microorganisms has led to the production of strains which excrete excess primary metabolites (amino acids, vitamins, purine nucleotides). Strain improvement with classical metabolic engineering: induced mutation (with radiation or chemicals) and selection of mutants.

The genome is changed with mutations:




- The whole metabolic flux should be directed to the desired product. Branches of biosynthetic pathway must be closed, whole substance flux is forced to form the product (auxotrophic mutants)
- Keep the produced substance: reactions transforming the end product must be eliminated (auxotrophic mutants)

  In the case of auxotrophic mutants the lack of the product of the blocked pathway may stop the growth of the strain. In this case a small amount of this missing metabolite must be added in the culture medium. Another way is the application of *leaky* mutants, in which enzyme activity is not eliminated totally but the mutant protein has a small activity, it is able to produce a small amount of the metabolite.
- Biochemical feed back mechanisms (inhibition or repression) of overproduction are to be eliminated. In an unbranched biosynthetic pathway the end product inhibits the activity of the first enzyme of the pathway, called feedback inhibition. A conformation change and hence inactivation (allosteric effect) occurs when an effector (end product) is attached to a specific site of the enzyme (allosteric site). This site is different than the substrate binding site, the inhibition is not competitive. In branched biosynthetic pathway, feedback inhibition of the first common enzyme by means of one of the end products would cause more than one end product to be affected. In branched biosynthetic pathways, different kinds of feedback inhibition are found:
  - The end product inhibits the first enzyme in each case *after* the branch point.
  - The first step of the common synthesis path is catalised by several isoenzymes, each of which can be regulated independently.
  - The first common enzyme in a branched biosynthetic pathway is influenced by each end product only slightly or not at all; there must be an excess of all end products for inhibition to occur (phenomenon called multivalent inhibition).

– Each end product of a branched pathway acts as an inhibitor; cumulative inhibition is the effect of all the inhibitors.

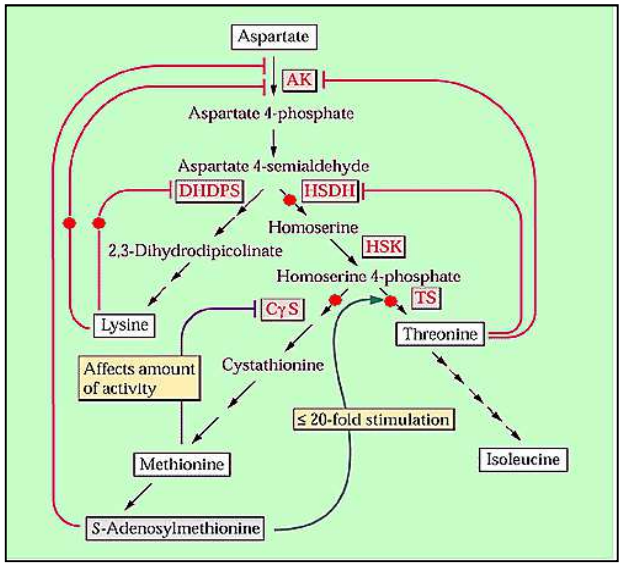
Antimetabolite resistance can be used to select mutants which exhibit defective regulation. Altered regulation may occur in such mutants. Antimetabolites, because of their structural similarity to metabolites, may cause feedback inhibition or but unable to substitute for normal metabolites. Antimetabolites cause death of normal cells, but analog-resistant mutants can form an excess of metabolites, in some cases through changed regulatory mechanisms (elimination of allosteric inhibition), causing constitutive product formation.

**Case study: fermentation of lysine**

A lysine is produced from aspartic acid. Using this metabolite microorganisms synthesize it in either via the diaminopimelic acid pathway or the aminoadipic acid pathway. Bacteria use the DAP pathway. Aspartokinase is regulated via multivalent feedback inhibition from L-threonine.

Effective L-lysine producers are found among mutants of *Corynebacterium* and *Brevibacterium* strains which are homoserine auxotrophs or among methionine –threonine double auxotrophs. High-lysine-producing strains are also found organisms resistant to the lysine antimetabolite S-(β-aminoethyl)-L-cysteine (AEC) or methyl-L-lysine (ML):

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




In some organisms the lysine is decarboxylated to cadaverine but these producing strains don't have this activity.

Fermentation technology

Carbon source may be molasses, dextrose, in alternative processes acetic acid or alkanes.
As nitrogen source ammonia, ammonium salts or urea is used (these strains have urease activity).
Auxotrophs need homoserine, threonine and methionine in small concentration (soy meal, corn steep liquor), but with leaky mutants this can be omitted → cost reduction.
Biotin: minimum 30 $\mu$g/l is necessary (sugar cane molasses contain the sufficient amount, beat molasses or starch hydrolysates need supplement).
Optimum: pH = 7, T = 28°C t(ferm) = 60 hrs
Final concentration: 100-120 g/l, productivity $Y_p$ = 30-40%.
Downstream: key step: adsorption on cationic ion exchanger.

**Secondary metabolites: antibiotics**

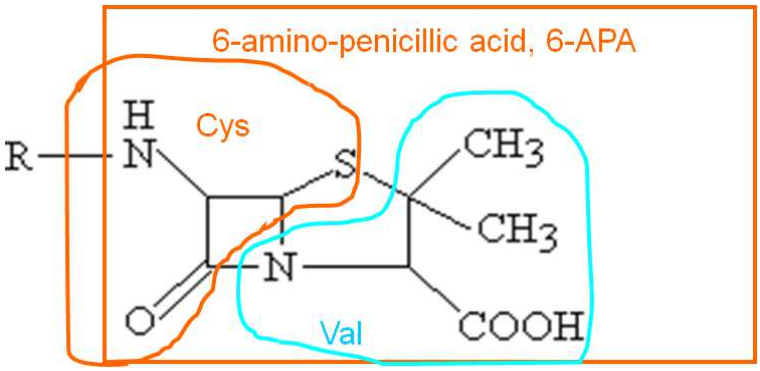
= Secondary metabolites produced by microorganisms that can inhibit or kill other microorganisms.
In the last 80 years ~12-13 thousands antibiotics were discovered. Only ~2-300 molecules became a human medicine. From these ~10% is produced with direct fermentation, ~80 %

with fermentation, after that chemical modification (= semisynthetic drugs). The left 10% is produced with chemical synthesis (costs).

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

**Case study: penicillins**

Chemical sructure: the basic structure of the penicillins is 6-amino-penicillanic acid (6-APA) which consists of a thiazolidine ring with a condensed β-lactam ring. The 6-APA carries variable acid moiety in position 6. In "natural" penicillin this amino group is acylised with benzoic acid (benzylpenicillin, penicillin G). With exchange of this acyl group semisynthetic penicillins are produced. Biosynthesis of penicillin is going on with combination of two amino acids: cysteine and valine.




<u>Production of penicillin</u>

Fermentation, because chemical synthesis is not economic.

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

<u>Main fermentation</u>

Typical secondary metabolite fermentation, with two phases:

In the typical penicillin fermentation there is a <u>growth phase</u> about 40 h duration, during which time the greatest part of cell mass is formed. The culture needs optimal nutrient supply, intensive aeration, agitation, the primary metabolism is running.

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

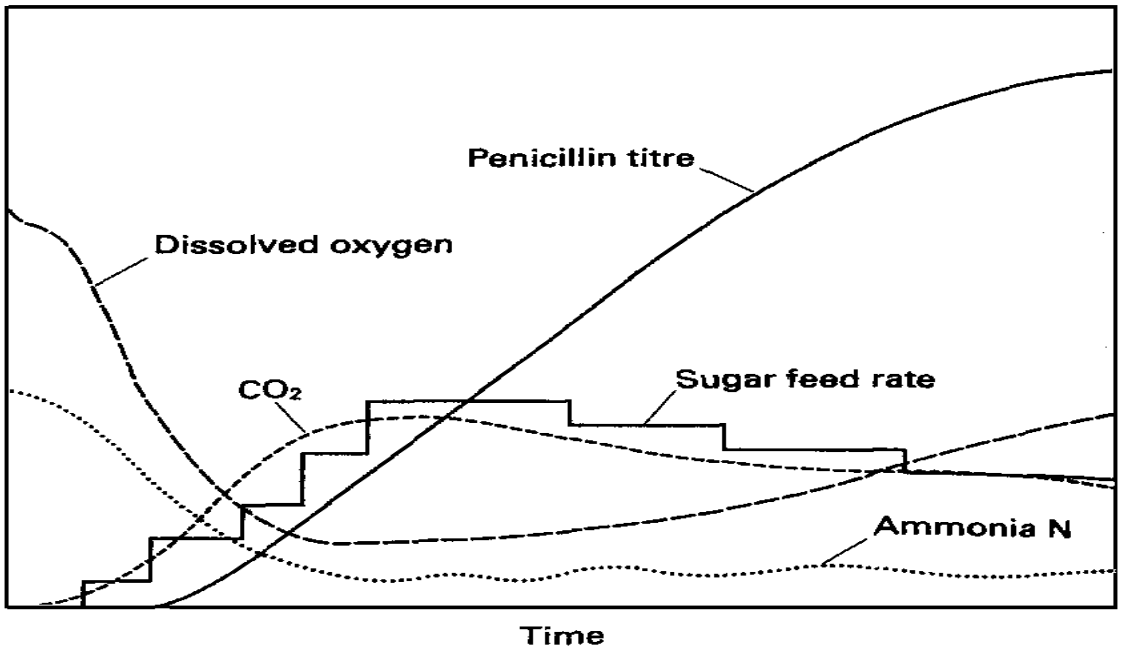
After that the culture proceeds to penicillin <u>production phase</u>. By feeding with various medium components, the production phase can be extended to 120-160 h. In this phase multiple substrate limitations force the secondary metabolism.

Nutrients in the second period:

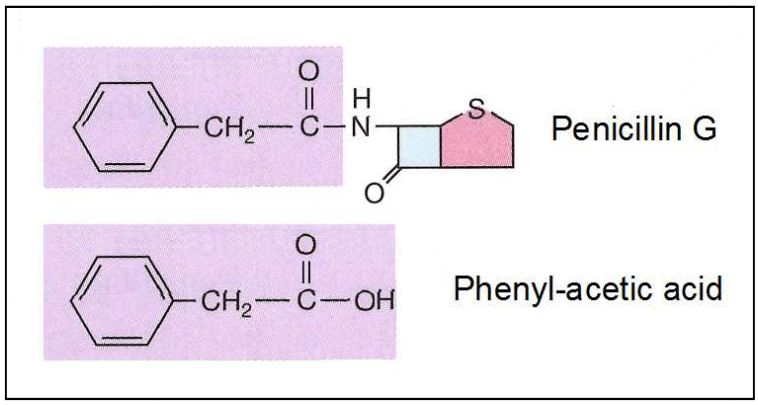
- Carbon source: sugar limited metabolism (earlier: hardly metabolisable compounds: lactose, starch; nowadays: dosage of small amounts of dextrose, according to the oxygen level)
- Nitrogen source: addition in the form of proteins: CSL, soy meal, peanut cake → daily dosage to keep a low concentration

- 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:



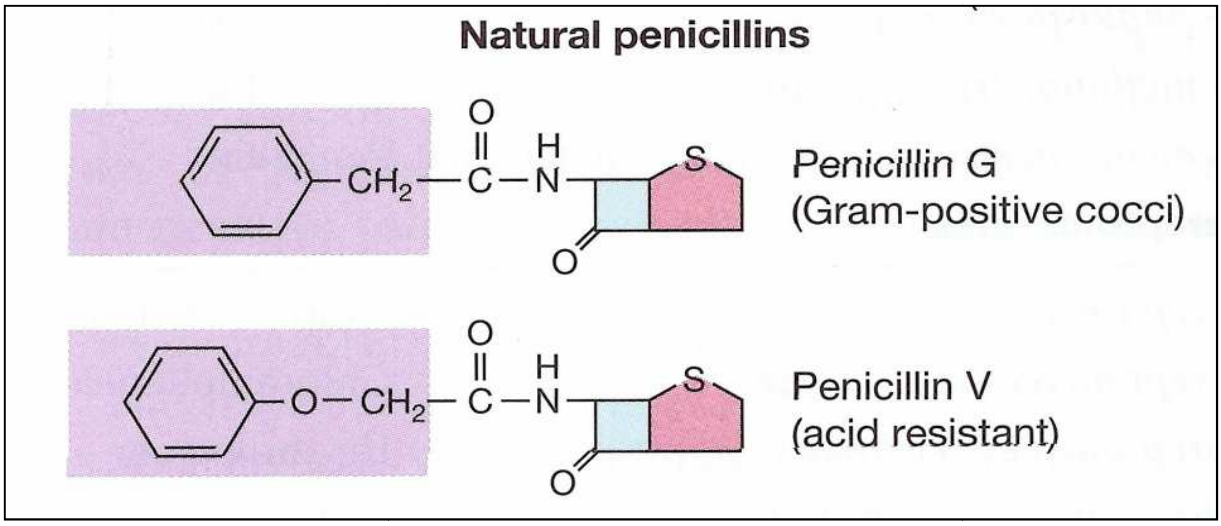

Precursors: are compounds prepared with chemical synthesis, given into the fermentation broth, where the microorganisms will built it into the product molecule. This saves substances and energy for the microbe. For penicillin G the phenyl-acetic acid is the proper precursor.



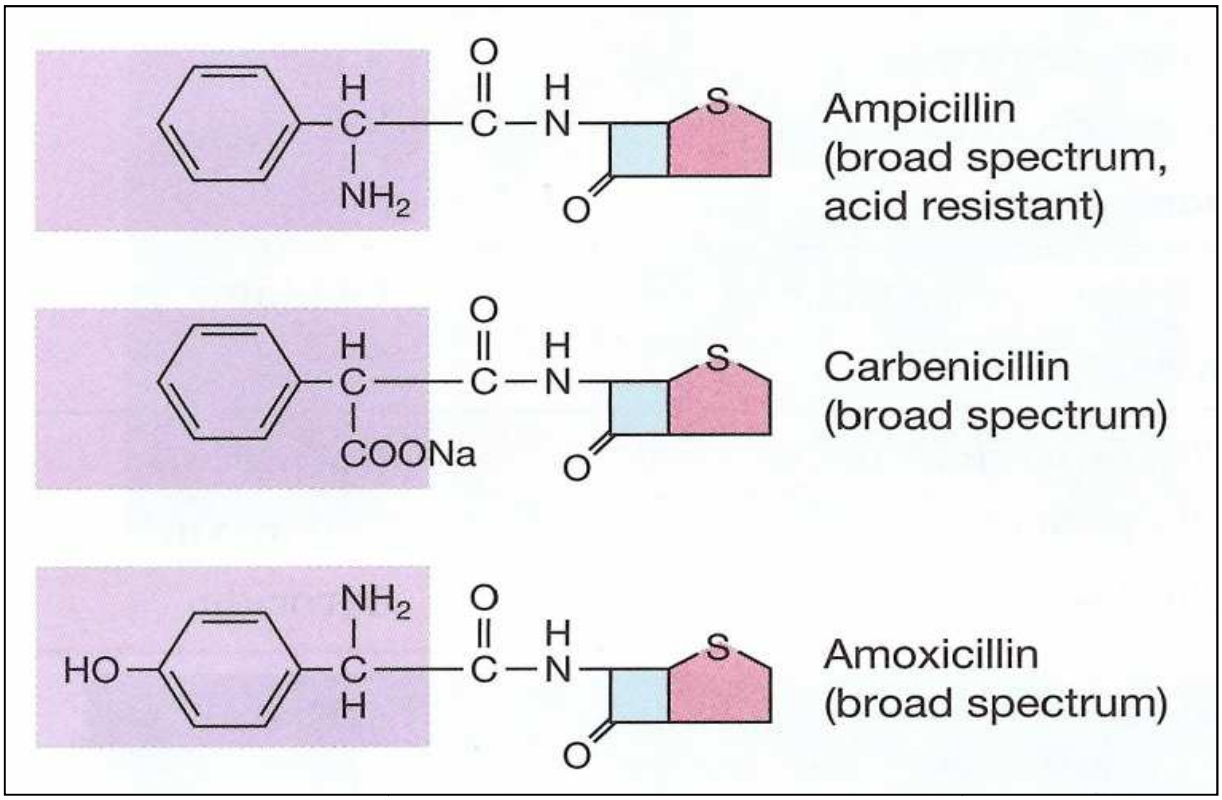

Semisynthetic penicillins

Many penicillin antibiotics used in medicine are produced semisynthetically by chemical modification of the basic penicillin ring structure. The starting material for chemical modification is the nonacylated 6-amino-penicillanic acid (6-APA). 6-APA is produced by the splitting-off of the acid side chain of penicillin G with the enzyme penicillin acylase. In the following step the desired new side chain molecule of acidic character is coupled to the amino group.

The fermented platform molecules are the “natural” penicillins:




About hundred of semisynthetic penicillins were developed and accepted in human medicine. These differ in chemical properties (stability, solubility) and antibiotic effectiveness (spectrum, resistance). Structure of some widely used molecules is presented:




Summary of secondary metabolite production:

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

Market: Patents run out, antibiotics became generic drugs. Hard competition, depressed prices. Competitors in China and India.

**RECOMBINANT PROTEINS**

Most of today's protein pharmaceuticals fall into 4 general classes: (1) hormones, (2) vaccines, (3) antibodies, (4) enzymes. Three of these classes, hormones, antibodies, and enzymes, are primarily used to treat noninfectious or endogenous diseases (i.e. genetic diseases or diseases of aging), whereas the vaccines are used to treat or prevent infectious or exogenous diseases (i.e. bacterial of viral infections).

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.

Case study: RECOMBINANT VACCINES

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

Step 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

Case study: **HEPATITIS B VACCINE**

Hepatitis B virus (HBV) is the major infective agent and it can cause either acute or chronic viral hepatitis. The virus destroys the liver cells, causes liver failure, jaundice (icterus). In acute hepatitis, the virus can be cleared, leading to recovery. In chronic hepatitis B, the virus persists, no chance for spontaneous healing. It may lead to progressive liver disease, cirrhosis and primary hepatocellular carcinoma.

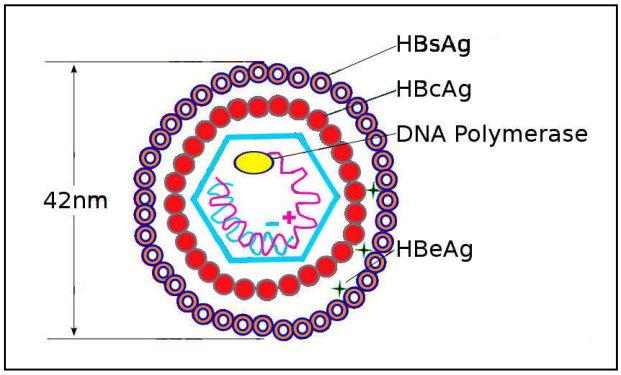
Virus particles are synthesized in liver cells and after the break up they spread in the blood. Viral proteins and antibodies can be detected in blood.

It has been estimated that about five percent of the world's population is infected which means ~350 million chronic carries. The virus maintained in the population primarily via maternal transmission. In the developed countries, infection is also transmitted by exposure to contaminated needles or instruments, by blood transfusion, drug abuse or sexual contact. The incubation period for hepatitis B varies from six weeks to three month.

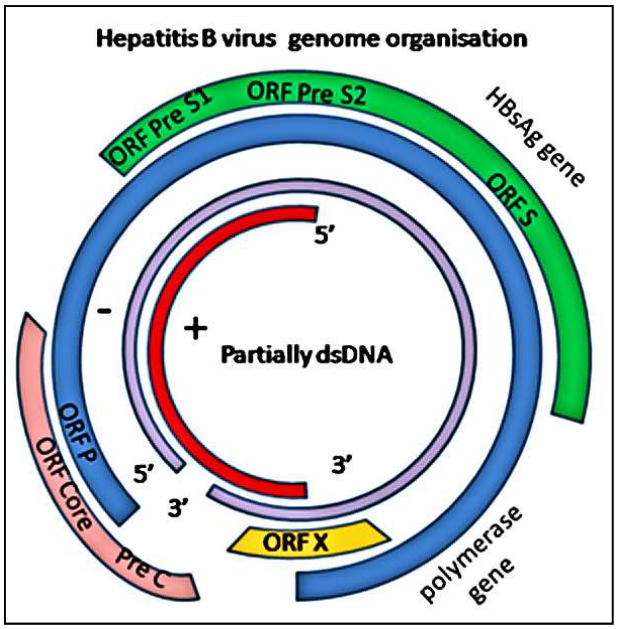
The HBV (hepatitis B virus) has 3 different antigens:

s – surface,
e – endo,
c – core
+ a DNA and DNA-polymerase

The hepatitis B viral particle has a diameter of 42 nm and contains a partially(!) double stranded DNA genome, which is enclosed within a core structure and surrounded by a lipid containing envelop in which numerous copies of HBsAg are embedded.



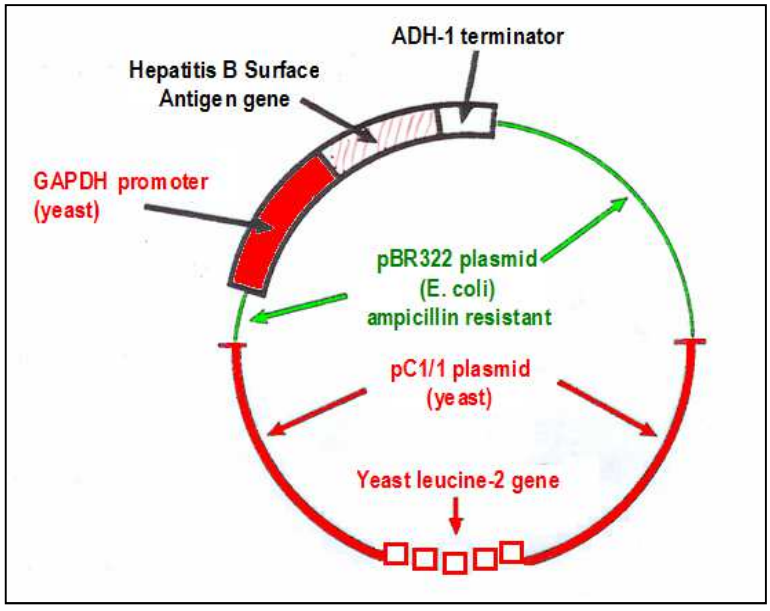
The genome of HBV is a small, circular DNA molecule, which is partially double stranded, partly single stranded in a variable ratio. The long or (-) strand has fixed length of 3200 nucleotides whilst the length of the short or (+) strand ranges from 55 to 75% of that of the minus strand.
The HBsAg protein has 226 amino acids, in human cells it is glycosylated. Out of the cells it is assembled into 22 nm lipoprotein particles which are highly immunogenic




At first the gene of surface antigen was cloned into *E. coli* by a plasmid. It formed the protein but the protein had no activity, because the formed antigen does not assume the proper folding (3D structure).

Later the gene has also been expressed in recombinant mammalian cells in culture and in baker's yeast, *Saccharomyces cerevisiae*.

Both host systems produce HBsAg, which proved to be highly immunogenic. In yeast all of the HBsAg is contained intracellularly whereas the mammalian cells secrete the antigen. All yeast-derived antigen is non-glycosylated whereas approximately 75% of the mammalian-derived HBsAg is glycosylated. Nevertheless, this biochemical difference is reflected neither in the antigenicity of the various particles nor in their ability to elicit virus-neutralising antibodies. Both technologies give proper product but the yeast vaccine is cheaper and safer (no dangerous viruses).
The structure of shuttle vector:




Two replication origo
Two markers:
- ampicillin resistance
- leucine enzymes (the yeast is Leu$^-$)

Expression cassette:
- constitutive promoter
- useful gene
- terminator gene

Upstream: batch fermentation
First phase: Leu-free medium (increases the plasmid number)
Second (production) phase: complete medium, with Leu
The fermentation takes about two days, during this the yeast continuously manufactures and stores HBsAg.
Downstream: in order to isolate the expressed antigen, the yeast is harvested by means of centrifugation and disrupted with high pressure homogenizer.
Purification steps include adsorption and chemical treatment.