Bioengineering and Fermentation

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Technische Biochemie

**What should you learn?**
- What is bioengineering?
- Aspects of system biotechnology
Bio-Engineering
The Biologist view

• Want to understand organisms and living systems
• Discover underlying mechanisms that govern how organisms work
• The knowledge is then used to develop or improve medical, industrial or agricultural processes.
• Comfortable with uncertainty
Bio-Engineering
The Engineering view

• See a problem and want to come up with a practical solution
• Apply mathematics and scientific knowledge
• Want precision and reproducibility
• Consider technical and economic constraints
Difference between Chemical and Biochemical Engineering

- Metabolic Engineering
- System Biology
- Synthetic Biology

Biochemical Engineering

Chemical Engineering
Fermentation is a process of culturing cells or other microorganisms in a container, bioreactor, or fermenter for experimental or commercial purposes.
Industrial microbiology uses microorganisms, typically grown on a large scale, to produce valuable commercial products or to carry out important chemical transformations. This process is commonly referred to as Fermentation.
# Production Systems

## Cell Types

<table>
<thead>
<tr>
<th>E. coli&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Used in production</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces cerevisiae</em>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Insulin, IFN, TNF, IL, hGH</td>
</tr>
<tr>
<td><em>Aspergillus</em>&lt;sup&gt;1&lt;/sup&gt; sp.</td>
<td>tPA, β-IFN, Erythropoetin,</td>
</tr>
<tr>
<td>CHO&lt;sup&gt;2&lt;/sup&gt; cells</td>
<td>Factor VIIa</td>
</tr>
<tr>
<td>BHK&lt;sup&gt;3&lt;/sup&gt; cells</td>
<td>Impfstoffe</td>
</tr>
<tr>
<td>VERO</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>without further specifications,  
<sup>2</sup>CHO = Chinese Hamster Ovary,  
<sup>3</sup>BHK= Baby Hamster Kidney
Requirements to production systems

• Safe and controllable fermentation
• Preferred GRAS Organismen
• Low cost production
• High production of recombinant protein (g/L not mg/L)
• Easy downstream processing
• One unit production
• No post-biosynthesis degradation, modification of product
Other interesting systems

- *Bacillus subtilis*
- *Trichoderma reesii*
- *Insect cells*
- *Nicotiana tabacum*
- *Daucus carota*
- *Mammalian cells (BHK, CHO)*
- *Goats*
Bacterial expression

**Advantages**
- Simple fermentation and transformation
- High yield
- Well studied expression systems
- Many proteins are expressed in inclusion bodies

**Disadvantages**
- Many proteins are expressed in inclusion bodies
- No post-translational modifications
- Improper folding of disulphide linked proteins
**Escherichia coli, Enterococcaceae**

**Advantages:**

- high replication rate
- Growing on cheap media
- well known fermentation technology
- Molecular Biology is well known
- Simple Scaling Up
- Intracellular accumulation of proteins
- no posttranslational modifications of proteins
- Inclusion bodies
Escherichia coli, Enterococcaceae

Disadvantages:

• complex downstream technology
• use of surfactants for protein refolding
• endotoxins (LPS)
• no Glykosilation
Use of safe strains like $K_{12}$

**Advantages**

- Easy to use
- Safe handling in the production process
- Efficient
- Fully understood,
- High diversity for strain selection and available vectors
DNA-Transfersystems for *E. coli*

- **Plasmidvektors** (Electroporation, CaCl$_2$)
- Phage vectors (Lambda-, M13-phages)
- Cosmid vectors
- Bacterial Artificial Chromosomes (BACs)
- Bakterio phages P1 Vectors (PACs)
Schematic structure of a vector

- **Therapeutic gene**
- **Marker Gene**
  - Antibiotika-Resistance gene
    - (β-Lactamse, Neomycin, Tetracylin)
- **Rop-Gene**
  - Coding RNA stability
- **Promoter gene**
  - Binding to transcription factor and RNA-polymerase
- **Origin of Replication (Ori)**
  - Start for DNA-Polymerase
- **Poly-A-Signal**
Plasmid pBr322

- first and mostly used vectors
- ca. 15 copies per cell; >1000 after plasmid-amplification
- Selection of transformed \textit{E. coli} for ampicillin or tetracyclin resistance

\texttt{rep} – Replication of plasmids
\texttt{rop} – coding Rop-Protein (RNA-stability)
\texttt{bla} - coding beta-Lactamase
\texttt{tet} – codiert Tetracyclinresistenz
β-Galaktosidase reporter gene assay
Yeast expression systems

• **Advantages**
  - Simple fermentation and transformation
  - High yield
  - Well studied expression systems
  - Some limited post-translational modifications
  - Powerful secretory pathways

• **Disadvantages**
  - Improper folding of disulphide linked proteins
  - Hypoglycosylation
Pichia pastoris and Kluveromyces sp.

**Advantages**
- Protein glycosylation
- High protein yield
- MeOH-Metaboliser
- Secretion systems
- Easy Downstreaming

**Disadvantages**
- Difficult fermentation
- Limited vectors for transformations
- Recombinant genes must be stabil incorporated
- Unusual Codons
Advantages

• GRAS Organism
• Protein folding similar to mammalians
• Protein glycosylation
• Secretion mechanisms
• Genom, Proteom, Metabolom well known
• Easy applicable downstream processes
• Extracellular space for recombinant proteins
Saccharomyces cerevisiae

Disadvantages

- Low transformation efficacy
- Limited number of vectors for transformation
- Recombinant genes must be stable incorporated
- Time dependend (slow growth in culture)
- „Hyperglyclycolisation“ (high- Mannose-type → can cause in humans allergic reactions)
Intracellular product accumulation
Protein Modification: Addition of Carbohydrates or Glycosylation

The added carbohydrates are important for **protein folding**, the **targeting** of proteins to their respective compartment and as **recognition sites** for cell-cell interactions.
Problems with glycosilation

- Natives Glykoproteins
- Transgenes Tier
- Bacterien
- Pilz
- Transgene Pflanze

Peptide
Galactose
Mannose
N-glycolyneuraminic acid
N-acetylgalactosamine
N-acetylneuraminic acid
Xylose
Fucose
Protein Processing: The Example of Proteolytic Processing of Insulin

Proinsulin

Signal sequence

Cleavage of signal sequence
Disulfide bond formation

Connecting polypeptide

Removal of connecting polypeptide

Proinsulin

Insulin
DNA-transfer systems for *S. cerevisiae*

- Integrating vectors
- Autonom replicating plasmids
- Episomal vectors
- Centromeric vectors
- Yeast Artificial Chromosomes (YACs)
Integrating vectors

- No real plasmid, because no ori-Region for yeasts
- Direct integration into host genome
- Low transformation efficacy
- High transformation stability
**YACs**

- Special centromeric vectors
- Capability of 10-1000 kBp
- Genetically stable at size of 20 kBp
- Cloning of hetero genomes and genome mapping
- Very important for metabolic engineering and synthetic biology
Yeast transformation

- Electroporation
- protoplast fusion
- problems:
  - bad freezing
  - limited storage
  - polyploid forms
## Risk assessment for GMOs

<table>
<thead>
<tr>
<th>Risks for humans</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>for enviroment</td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>not assessible</td>
</tr>
<tr>
<td>S2</td>
<td>low</td>
</tr>
<tr>
<td>S3</td>
<td>moderate</td>
</tr>
<tr>
<td>S4</td>
<td>high</td>
</tr>
</tbody>
</table>
• Process if frequently aerobic so fermentor has to be well aerated.
• The aeration will be sufficient to mix many cultures
• If the culture is thick or sticky, additional stirring is required by a motor driven paddle called an **impeller**.
Airlift and Cycle-Flow-Reaktor

Advantage: minimum of cell destruction
easy scaling up
Continuous Fermentation

Discontinuous Fermentation
Blood clotting factor VIII

- Rcombinate und Kogenate
Recombinant CBS coagulation proteins

TF-FVIIa

FX → FXa

FXa → FIXa

FIXa → FXIa

FXIa → FVIII

FVIII → PROTHROMBIN

PROTHROMBIN → FXa

FXa → FVa

FVa → FXa

FXa → FV

FV → FXIII

FXIII → FXIIIa

FXIIIa → FIBRIN

CROSS-LINKED FIBRIN

IIa
Huge (2332 amino acids, 165 – 280 kDa)
B domain is heavily glycosylated and dispensable
FVIII circulates as a heterodimer; HC varies in length

**Variable Heavy Chain**

**Light Chain**

Processed during synthesis
Batch-Refeed-Process for Recombinate®
Continuous production of Kogenate®
Precautions for biosafety

- Oxygen need
- pH
- Temperature
- Mixing velocity
- Glucose concentration
- Mycoplasma und Viruses
- Cell density
- Cell vitality
- Sterility
An approach to watch (10 years+)

- Production of recombinant proteins by genetically transformed mammalian cells in culture very $$$
- Production by transgenic livestock potentially less expensive, higher capacity
- GTC Biotherapeutics (Framingham, MA) produces recombinant antithrombin (ATryn) in the milk of transgenic goats
- ATryn approved in Europe, phase III in USA – but indications for this protein are limited
- GTC continues to pursue rFIX production via this route and may be able to avoid issues that caused AmCross to drop this approach
Cleaning in Place (CIP)
Structure of Follitropin beta

Glycosilation
Sialylic acid, Fucose (50%), Mannose

- Humane follicel stimulating hormone
- N-glycosilation
- Fucose only in β-unit

Protein unit
α/β sub unit
Perfusion bioreactor

- Medium inlet
- Cells
- Lumen
- Inner Membrane
- Outer Membrane
Biosynthesis of Follitropin-beta

• Cloning into CHO-cells
• yield >50%

Rekombinant plasmid

CHO.FSH.30-Klon

replikation

Up to 150 copies / cell
Biosynthesis of Follitropin-beta

- Fermentation
- Produktion im perfusion bioreactor
Biosynthesis

CHO-Masterseed

Puregon®-Bioreactor
Downstream-Process of Follitropin-beta

• Chromatographic purification
  – Anion exchange chromatography
  – Cation exchange chromatography
  – Gel chromatography
  – Hydrophobic Interaction chromatography