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Studienbrief

Cell Line Engineering

Modul 3.2b

Im Studiengang Biopharmazeutisch-Medizintechnische Wissenschaften

(Master of Science)



Gefördert vom Ministerium für
Soziales und Integration Baden-
Württemberg aus Mitteln des
Europäischen Sozialfonds sowie vom
Ministerium für Wissenschaft, Forschung
und Kunst Baden-Württemberg

Modulnummer	3.2b
Modultitel	Cell Line Engineering
Leistungspunkte	3 ECTS
Sprache	Englisch
Modulverantwortlicher	Prof. Dr. Chrystelle Mavoungou
Dozenten	Dr. Lothar Steeb
Studiengang	Biopharmazeutisch-Medizintechnische Wissenschaften (M.Sc.)
Voraussetzungen (inhaltlich)	Keine
Voraussetzungen (formal)	Keine
Lernziele	<p>Beim Modul Cell Line Engineering lernen die Studierenden die wichtigste(n) Zelllinie(n) kennen und können deren Bedeutung erklären.</p> <p>Die Studierenden können wichtige molekularbiologische Methoden nennen und erläutern, kennen wichtige zellbiologische Aspekte, die die Produktion von Biopharmazeutika in eukaryotischen Zellen beeinflussen.</p> <p>Außerdem erlernen die Studierenden verschiedene Vorgehensweisen der Zelllinien-Entwicklung und können anhand vorgegebener Fragestellungen die Prinzipien der Zelllinien-Entwicklung erläutern.</p>
Inhalte	<p>Basics & molecular biology methods</p> <ul style="list-style-type: none"> - CHO cell line - Introduction of genes & overexpression - Gene knock-out: ZFN, TALENs, meganucleases, CRISPR/CAS9 - Gene silencing: siRNA, miRNA <p>Cell line engineering regarding the following subjects:</p> <ul style="list-style-type: none"> - Protein expression - Cell proliferation - Cell death - Metabolism - Secretory pathway - Glycosylation
Literatur	<ul style="list-style-type: none"> - Fischer, S., Handrick, R., & Otte, K. (2015). The art of CHO cell engineering: a comprehensive retrospect and future per-spectives. <i>Biotechnology advances</i>, 33(8), 1878-1896. - Aktuelle Forschungsliteratur
Lehrveranstaltungen und Lehrformen	<p>Präsenzveranstaltungen:</p> <ul style="list-style-type: none"> - Modulprüfung <p>E-Learning</p> <ul style="list-style-type: none"> - Online-Sprechstunde - Skripte und selbstständige Nachbereitung <p>Summe: 180 h</p>
Prüfungsform	<p>60 Min Klausur</p> <p>Prüfungssprache ist Deutsch.</p>

Biopharmaceuticals, such as monoclonal antibodies (**mAbs**), cytokines or peptide hormones (Fig. 1), became increasingly important for the treatment of diseases during the past decades (about one quarter of all new drugs).^{1,2} This is also reflected in the amount of drug approvals (Fig. 2A). The total number of licensed biopharmaceutical products has reached 246 in 2014 and it can be expected that the demand for biopharmaceuticals will further increase during the upcoming years.^{3,4}

#	Product	Sales \$ billion	Manufacturer	Expression system	Indication
1	Humira (adalimumab; anti-TNF)	11,00	AbbVie & Eisai	Mammalian	Rheumatoid arthritis
2	Enbrel (etanercept; anti-TNF)	8,76	Amgen, Pfizer Inc., Takeda Pharmaceuticals	Mammalian	Rheumatoid arthritis
3	Remicade (infliximab; anti-TNF)	8,37	J&J, Merck & Mitsubishi Tanabe Pharma	Mammalian	Rheumatoid arthritis
4	Lantus (insulin glargine)	7,95	Sanofi	<i>E. coli</i>	Diabetes
5	Rituxan/MabThera (rituximab; anti CD20)	7,91	Biogen-IDEC, Roche	Mammalian	NHL and CLL
6	Avastin (bevacizumab; anti-VEGF)	6,97	Roche/Genentech	Mammalian	Cancer
7	Herceptin (anti-HER2)	6,91	Roche/Genentech	Mammalian	Breast cancer
8	Neupogen/Neulasta (pegfilgrastim)	4,39	Amgen	<i>E. coli</i>	Myelosuppressive in chemotherapy
9	Lucentis (ranibizumab; anti-VEGF)	4,27	Roche/Genentech, Novartis	<i>E. coli</i>	Macular degeneration
10	Epogen/Procrit/Eprex/ESPO (epoetin alfa)	3,35	Amgen, J&J, KHK	Mammalian	Anemia

Figure 1: The 10 top-selling biopharmaceuticals.³⁻⁵ CLL: Chronic Lymphocytic Leukemia; HER2: Human Epidermal growth factor Receptor 2; J&J: Johnson & Johnson; NHL: Non-Hodgkin's Lymphoma; TNF: Tumor Necrosis Factor; VEGF: Vascular Endothelial Growth Factor.

Due to this commercial significance and the high clinical dosage requirements, there is a great volume demand for biopharmaceuticals. For that reason, pharmaceutical companies are seeking for a fast, controllable and cost inexpensive way of manufacturing, as well as the constant improvement of manufacturing capacities.

¹ Cf. Browne, S./ Al-Rubeai, M. (2007): Selection methods for high-producing mammalian cell lines. in: *Trends in biotechnology*, 25 (9), S.425f.

² Cf. Walsh, G. (2013): *Biopharmaceuticals: Biochemistry and biotechnology*, John Wiley & Sons

³ Cf. Walsh, G. (2014): Biopharmaceutical benchmarks 2014. in: *Nature Biotechnology*, 32 (10), S.992f.

⁴ Cf. Fischer, S./ Handrick, R./ Otte, K. (2016): Pushing the limits of protein expression with miRNAs. in: *Drug Target Review* (3), S.20f.

⁵ Cf. Zhu, J. (2012): Mammalian cell protein expression for biopharmaceutical production. in: *Biotechnology Advances*, 30 (5), S.1158f.



Biopharmaceuticals are pharmaceuticals, which are produced by means of biotechnological manufacturing.

The base material of biologic pharmaceuticals derives from a biological source. This can be microorganisms, organs and tissues from plants and animals, as well as cells or fluids (including blood) from humans

[Walsh, G. (2013)]

Different **expression systems** are available for the production of therapeutic proteins, including prokaryotes, yeast/fungi, plants, insect and mammalian cells.⁶ Nevertheless, yeast/fungi, plants and insect cells are less frequented.

Prokaryotes, such as *Escherichia coli*, have shown to be efficient expression systems, because they possess a short generation time, are easy to handle, there is great know-how about the fermentation process and the yield may be up to 20% of the total cellular protein (gram/liter scale).^{7,8} The recombinant proteins may be excreted into the periplasm of the bacterial cell or they are stored in so called **inclusion bodies**.⁹ In case of the latter, the protein product may be insoluble and misfolded, causing additional steps regarding solubilization and refolding.⁹

Bacteria display a limited potential for **posttranslational modifications**, such as N- and O-linked glycosylation, fatty acid acylation, phosphorylation, and disulfide-bond formation. However, these modifications are required for the structure (secondary, tertiary and quaternary) and functionality of a protein and can obviously affect the solubility, stability, half-life and protease resistance of therapeutic proteins. Therefore, the usage of prokaryotes is restricted to the production of biopharmaceuticals, which are not naturally glycosylated, and small peptides, e.g. insulin. Additionally, proteins expressed in *E. coli* tend to retain their N-terminal methionine, which may evoke **immunogenicity** (the ability to trigger an immune response) in the patient.¹⁰

On the basis of these disadvantages, mammalian cells continue to be the system of choice, because they perform posttranslational modifications, which are:

- 1) **similar to human cells**, thus lowering the immunogenicity
- 2) **important for target interaction**, consequently increasing the efficacy of the biopharmaceutical

⁶ Cf. Browne, S./ Al-Rubeai, M. (2007)

⁷ Cf. Schmidt, F. (2004): Recombinant expression systems in the pharmaceutical industry. in: *Applied Microbiology and Biotechnology*, 65 (4), S.363f.

⁸ Cf. Yin, J./ Li, G./ Ren, X. et al. (2007): Select what you need: A comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. in: *Journal of Biotechnology*, 127 (3), S.335f.

⁹ Cf. Yin, J. et al. (2007)

¹⁰ Cf. Daly, R./ Hearn, M. (2005): Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. in: *Journal of molecular recognition*, 18 (2), S.119f.


Already 60 - 70% of all biopharmaceuticals are produced in mammalian cell based processes (Fig. 2B).¹¹ Among the cells lines used, the **Chinese hamster ovary (CHO)** cells are by far the most frequently used (~33% of all product approvals) (Ch. 2).

The choice of an expression system depends on the required quality and quantity of the protein product, as well as the costs (Fig. 3).¹⁰

A selected high producing animal cell line is the pivotal point in the development of a cell culture bioprocess (Fig. 4). **Cell line development** starts by cloning the gene of interest (**GOI**) for the desired product into an expression vector with a respective selection marker and then transfecting the vector into eukaryotic cells. The selection marker ensures to obtain stable transfected cells by integration the GOI into the chromosome. Afterwards a high producing clonal cell line is selected from this heterogeneous cell pool by applying cell selection methods, e.g. limiting dilution. Upon this, the selected cell clones are characterized regarding their growth and titer of recombinant protein. This process is followed by expansion processes and further screening, such as productivity, stability of transgene expression and the quality of the product.¹² A lead clone is chosen for cell banking. The average time for developing a production cell line takes about 12 month.¹³

Host system	E. coli	Yeast	Insect cells	Mammalian cells	
Cell growth	Rapid (30 min)	Rapid (90 min)	Slow (18–24 h)	Slow (24 h)	
Expression level	High	Low-high	Low–high	Low–moderate	
Protein folding	refolding usually required	refolding may be required	correct folding	correct folding	
Post-translational modifications	N-linked glycosylation	-	High mannose (mostly)	Simple, no sialic acid	+
	O-linked glycosylation	-	+	+	+
	Phosphorylation	-	+	+	+
	Acetylation	-	+	+	+
	Acylation	-	+	+	+
	Gamma-carboxylation	-	-	-	+
	Advantages	- easy scale-up - low cost - fast - easy operation	- easy to culture - low cast - fast - eukaryotic protein processing - protein secretion	- eukaryotic protein processing - higher yield than mammalian cells	- posttranslational modifications - immunogenicity lower
Disadvantages	- protein solubility - posttranslational modifications - immunogenicity	- optimization of growth conditions	- culture conditions - glycosylation - bioactivity - immunogenicity	- low yield - culture conditions - optimization of culture	

Figure 2: Comparison of expression systems.¹⁴ -: no; +: yes.

 The term “clonal cell line” describes a cell line derived from a single cell. This implies that all cells are genetically and phenotypically identical. However, an entirely homogeneous cell line does not exist.

¹¹ Cf. Wurm, F. (2004): Production of recombinant protein therapeutics in cultivated mammalian cells. in: *Nature Biotechnology*, 22 (11), S.1393f.

¹² Cf. Barnes, L./ Moy, N./ Dickson, A. (2006): Phenotypic variation during cloning procedures: Analysis of the growth behavior of clonal cell lines. in: *Biotechnology and Bioengineering*, 94 (3), S.530f.

¹³ Cf. Bandaranayake, A./ Almo, S. (2014): Recent advances in mammalian protein production. in: *FEBS letters*, 588 (2), S.253f.

¹⁴ Cf. Yin, J. et al. (2007)

When a production cell line has been selected, large scale production begins (upstream processing, **USP**). For this reason, a cryovial is taken from the cell bank and expanded by successive expansion into larger culture vessels, e.g. spinner flasks, wave bags and bioreactors. In the end the cells are cultured in a production bioreactor (e.g. eukaryotic cells are cultured in up to 15.000 L at Boehringer Ingelheim and up to 2000 L at Rentschler Biotechnologie). Finally, the product is harvested by separating the biomass (cells) from the product material (within the culture medium), e.g. by centrifugation or filtration.

The USP is followed by the downstream processing (**DSP**). This term refers to the purification of the fermentation product. Initially the collected medium is concentrated. If the product is intracellular (only in the case of bacterial production), the cells are disrupted and the medium and cell debris are discarded. The next steps involve the isolation of the product from the fermentation broth, e.g. precipitation, and the purification, e.g. affinity and size exclusion chromatography. Finally, the product is polished and mixed with excipients (**formulation**).

Even though, mammalian cell lines show the highest similarity to human cells, the system still exhibits bottlenecks regarding growth capacity, cultivation time and product yield.¹⁵ Eventually this leads to tremendous developmental costs for a manufacturer.

Different strategies like media and bioprocess optimization lead to accelerated cell proliferation and higher cell density.¹⁵ A different approach, **cell line engineering**, utilizes genetic engineering to overcome production bottlenecks. Cell line engineering, which will be the topic of this lecture, is aiming at the optimization of mammalian cell based production processes by implementing approaches regarding:

- Productivity → Ch. 4
- Cell proliferation → Ch. 5
- Cell death → Ch. 6
- Metabolism → Ch. 7
- Secretion → Ch. 8
- Glycosylation → Ch. 9

The overarching goal of cell line engineering is the maximization of product yield and the minimization of costs.

For further information on this chapter, please refer to the following literature:

Yin, J./ Li, G./ Ren, X. et al. (2007): Select what you need: A comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. in: *Journal of Biotechnology*, 127 (3), S.335f.; Schmidt, F. (2004): Recombinant expression systems in the pharmaceutical industry. in: *Applied Microbiology and Biotechnology*, 65 (4), S.363f.

Wurm, F. (2004): Production of recombinant protein therapeutics in cultivated mammalian cells. in: *Nature Biotechnology*, 22 (11), S.1393f.

Bandaranayake, A./ Almo, S. (2014): Recent advances in mammalian protein production. in: *FEBS letters*, 588 (2), S.253f.

The present script is meant to be a support for studying. Therefore, it is indispensable, that the students refer to the provided videos as well as current scientific literature. A list of web links will be provided for literature, which is highly recommended and free of charge.

¹⁵ Cf. Fischer, S. et al. (2016)

Ansprechpartner

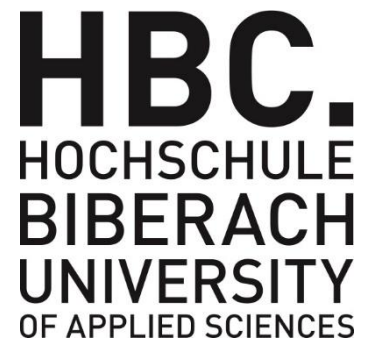
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„Pharmazeutische Grundlagen und Antikörper- Engineering“ im Studiengang „Biopharmazeutisch-Medizintechnische Wissenschaften (BM-Wiss)“ wurde entwickelt im Projekt Cross-Over, das aus Mitteln des Ministeriums für Wissenschaft, Forschung und Kunst des Landes Baden-Württemberg gefördert und aus dem Europäischen Sozialfonds der Europäischen Union kofinanziert wird (Förderkennzeichen: 696606). Dabei handelt es sich um ein Vorhaben im Programm „Auf- und Ausbau von Strukturen der wissenschaftlichen Weiterbildung an Hochschulen in Baden-Württemberg“.
