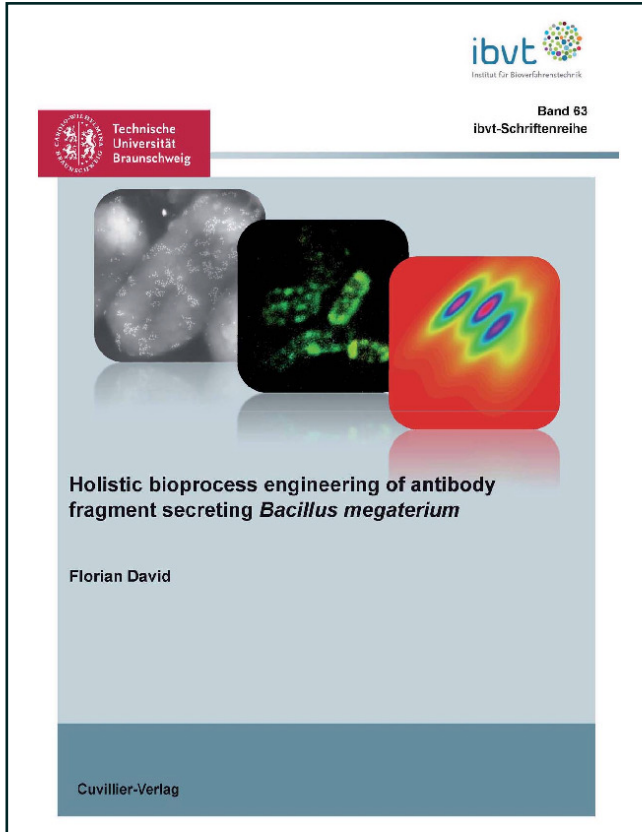




Florian David (Autor)

# Holistic bioprocess engineering of antibody fragment secreting *Bacillus megaterium*



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Telefon: +49 (0)551 54724-0, E-Mail: [info@cuvillier.de](mailto:info@cuvillier.de), Website: <https://cuvillier.de>



## 1 Introduction

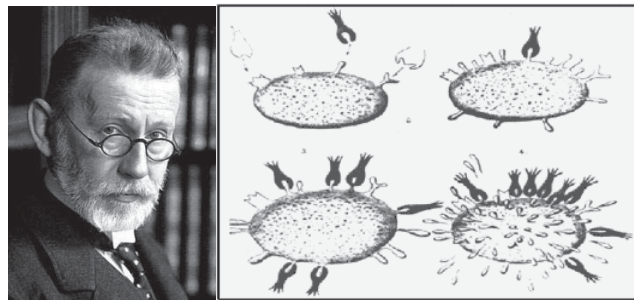
Antibodies (AB) and antibody fragments (ABF) are some of the most promising therapeutic and diagnostic tools of the 21st century. Less side effects and high specificities make them to ideal drugs for treating cancer, autoimmune, cardiovascular and infectious diseases. Their mechanism of action goes back to natural principles of the human immune system and uses its autologous defense devices to cope with health threatening diseases. The production of these multifunctional drugs is done by the key technology of biotechnology. Microbial and mammalian cells undergo genetic modifications to specifically produce the desired AB formats. The current challenges are to reduce the high production costs and to meet the increasing demands of these potent therapeutic tools. Therefore creating new production systems with lesser costs and higher productivity are most desirable.

As an alternative to expensive mammalian production platforms beneficial microbial systems can be used to efficiently produce and secrete ABFs. In this work the Gram positive bacterium *Bacillus megaterium* was used to thoroughly characterize production and secretion of a model ABF regarding process characterization and transfer to industrial scale. A contemporary holistic approach of bioprocess control, monitoring and optimization was followed. Traditional methods improving the process performance like optimization of culture medium and bioprocess development towards high cell densities were combined with cutting-edge technologies of flow cytometry (FCM) and transcriptome analysis. With these two new technologies at hand new insights on the ongoing processes in the model organism could be found on single cell level (by FCM) and on the overall gene expression profile (transcriptome analysis). These will help to identify possible bottlenecks of the overall bioprocess' performance and product secretion of ABFs with *B. megaterium* as production platform.

## 1.1 Antibodies

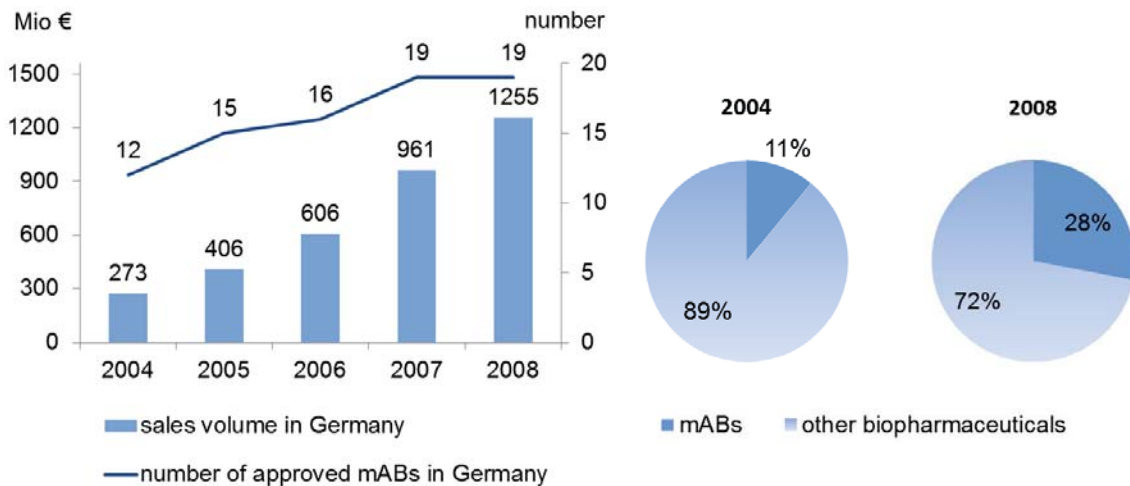
### 1.1.1 Benchmark analysis

More than 100 years ago Nobel Prize winner Paul Ehrlich proposed in 1908 the creation of “magic bullets” (**Fig. 1**) used to fight against human diseases which nowadays have become highly specific cancer therapeutics in form of monoclonal AB (mAB) constructs. The first Food and Drug Administration (FDA)-approved mAB appeared 25 years ago and today 28 mAB-based drugs are safe and effective therapeutic agents in the treatment of cancer, inflammation, cardiovascular and infectious diseases [1].



**Figure 1:** Nobel prize winner Paul Ehrlich (© The Nobel Foundation); magic bullets, side chain theory (1890) [2].

It has been projected, that in the next 10 years 135 mABs will be approved by the US FDA [3]. Being the major proportion of almost 50% of the therapeutic protein market they present the fastest growing sector in pharmaceutical industry. Predicted sales of mABs will reach \$56 billion dollars by 2012 with a compound annual growth rate (CAGR) of 13% [4]. **Figure 2** gives an overview about approved mAB products in Germany and their overall sales in the last years [5]. The increasing economic impact in Germany of mAB based products is highlighted at the corresponding growing fraction of overall biopharmaceutical products sales from 2004 to 2008.



**Figure 2:** Sales volume and number of mABs based products in Germany from 2004 to 2008 [5].

Besides the therapeutic usage, mABs are intensively used for diagnostic purposes and revealing total new insights in the field of cell biology and single cell analysis. Analytical *in vitro* methods such as Enzyme-Linked-Immunesorbent-Assay (ELISA), Radio-Immuno-Assay (RIA), blotting techniques, FCM, confocal imaging, immunochemistry, diagnostic biochip sensors, bio-imaging and protein purification are highly dependent upon the use of polyclonal or monoclonal ABs [6]. As a future perspective, recombinant mABs may even be used in consumer products e. g. for toothpaste to protect against tooth decay related to caries [7-9].

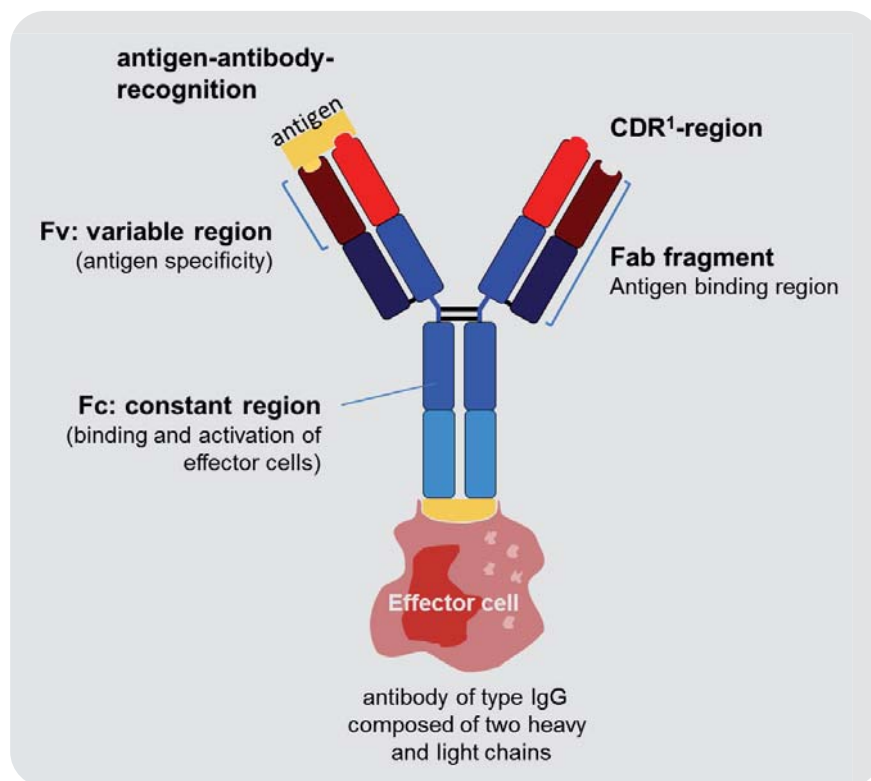
To meet the need of the high demand of mAB based therapeutics and diagnostics of more than 1000 kg/year [10] host cell engineering, optimization of cultivation and purification processes are of great importance.

### 1.1.2 Antibody formats

ABs are a class of flexible molecular adaptors playing a crucial role in the adaptive immune systems of vertebrates [11]. Using them for therapeutic purposes is a nearby approach as they naturally function in vertebrates to protect the organism against infections, malignant cells and toxic molecules. They were originally discovered by Behring and Kitasato in 1890 [12] but it took another 70 years until the basic structure was determined [13]. Due to their diverse and heterogeneous structures, ABs mediate diverse humoral and cellular immune responses. Thereby they execute various biochemical mechanisms such as antigen recognition, AB-dependent cellular cytotoxicity (ADCC) and complement-dependent cellular cytotoxicity (CDC). Five distinct classes of ABs exist in most higher mammals (IgG, IgA, IgM, IgD and IgE) differing in form and function based on variances in amino acid composition, charge distribution and carbohydrate content [14].



Immunoglobulins exhibit a symmetrical Y-structure and consist of pairs of identical heavy and light chains linked together through disulfide bridges (**Fig. 3**) [15]. The heavy chain type determines the subclass of AB linked to different physiological functions. The particular chains form globular domains that are either related to the specific antigen binding (variable region) or show Fc-related properties like complement activation and lymphocyte binding being essential for the cellular immune response [16]. **Figure 3** gives an overview on such a typical whole size AB IgG structure.



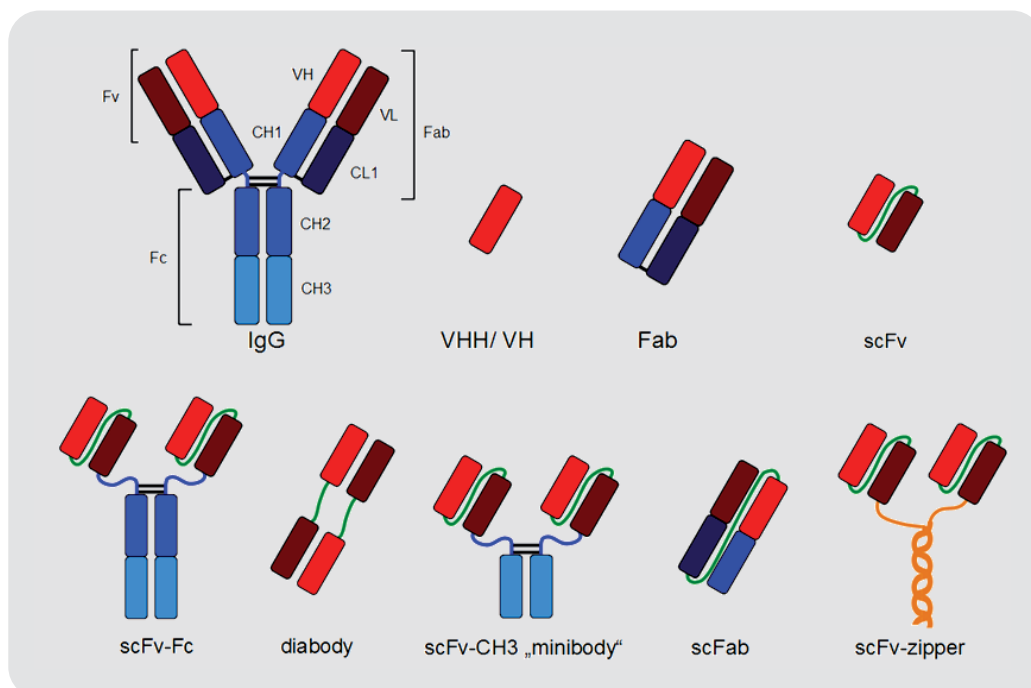
**Figure 3:** Functional parts of a whole size IgG mAb with specific effector binding and antigen binding functions, CDR<sup>1</sup> = complementarity determining region ([17] modified).

In general one has to distinguish between monoclonal and polyclonal ABs. The latter are used for detection reagents in research and are created from animal immunization activating an unspecific *in vivo* AB response. These ABs display unknown specificities and are very immunogenic, restricting their therapeutic application [6]. Monoclonal ABs instead are created by the so-called “hybridoma technology” which is based on the fusion of AB producing spleen cells from immunized mice or rats with immortal myeloma cell lines [18]. To generate fully human ABs, transgenic mice are used in which the mouse AB genes were replaced with the human equivalents [19]. In contrast to these *in vivo* techniques there are also *in vitro* AB selection methods available. Methods like phage display, yeast surface display or ribosome display are used to directly link the phenotype to the genotype and screen for higher affinity, stability and solubility of ABs derived from recombinant libraries of human VH and VL genes [6, 20, 21]. The phage display technology for



instance has been used for generating 30% of all human ABs currently in clinical development [22].

Besides natural whole size IgGs (~150 kDa) other AB formats are also developed for various applications including Fab fragments, single chain ABs (scFv) and single domain ABs (sdAB) (**Fig. 4**). AB molecules were developed exhibiting a certain biological activity e. g. at particular therapeutic and diagnostic applications [23]. The structures can be divided into two groups those being subject of major protein engineering modifications and those consisting of native components of the original IgG. The Fv fragment can be stabilized by linker polypeptide creating a single chain Fv (scFv) ABF, allowing an expression from a single gene thereby producing a single chain polypeptide [24, 25]. Additionally multimeric variants of the scFv such as dia-bodies and tri-bodies were created. An alternative approach describes the fusion of Fv fragments with leucine zipper forming amphipathic helices with leucine residues lining up on the hydrophobic face of a helix [26]. A so-called "Fab fragment" consists of the variable domain of the heavy and the light chain linked by a disulfide bond (**Fig. 4**). Different linker constructs at the hinge region facilitate various formats like single chain Fabs (scFab) [27], dimeric or even trimeric Fab constructs. Also bispecific ABs were generated at which one domain binds e. g. to a cancer specific surface and recruiting with the other domain cytotoxic T-cells inducing T-cell-dependent cytotoxicity [28]. Other AB formats like VHH/VH naturally existing in llamas and camels are lacking the light chain of ABs but still exhibiting high stabilities and affinities [29]. They display the smallest format of ABFs with a molecular weight of 15 kDa.



**Figure 4:** Different designs of ABFs consisting either of antigen specific (red), effector recruiting parts (blue) or both, stabilized by linker peptides (green), (Figure adapted from [6]).



### 1.1.3 ABF – key benefits

Currently most therapeutic ABs on the market are whole size IgG mABs produced in mammalian systems. They have the advantage to be fully human origin, to exhibit glycosylation patterns which are important for effector function and to have no immunogenic effects. However the production process in mammalian cell lines is rather time consuming, expensive and complex with high Cost of Goods (COG), so that new expression platforms producing highly specific, adjustable AB formats are most desirable. ABFs like Fab and scFv have multiple benefits compared to full-length IgG Abs, as the complete AB glycoprotein is not always necessary for its therapeutic function. The optimal composition might not be the whole AB structure but a distinctive fragment containing the specific antigen-binding domain. ABFs not possessing any Fc part can be advantageous for therapeutic application as they do not lead to the recruitment of effector cells or the activation of the complement system. In particular during inflammation processes only the neutralizing antigen-binding activity is desired. Due to their smaller size they show certain pharmacokinetic advances like the increased penetration into solid tumors, the possibility of local applications [30] or rapid clearance from circulating blood serum. Clearance from the blood stream is mediated by the renal pathway thus reducing the AB-half-life to hours rather than weeks [3, 31]. This may be on the one hand advantageous to avoid unspecific binding and on the other hand is beneficial for acute indications such as myocardial infarction, acute infections or intoxications. Modifications of half-life can be specifically adjusted by PEGylation (conjugation with polyethylene glycol) determining circulation time, biodistribution, immunogenicity, solubility, proteolytic degradation and storage stability [31].

A drawback of ABFs is that in some cases they consist of major protein engineering parts like peptide linker or purification tags. These may be associated with potential immunogenicity making these fragments unlikely to be used for repeated dosing therapies. However humanized ABFs and post-translational modifications (PTM) facilitate the optimal design of AB-based drugs with advanced pharmacokinetic and therapeutic function so that in contrast to standard IgG formats a greater flexibility can be achieved [32].

### 1.1.4 Specific examples

Therapeutic ABs have two modes of action. They can either work as antagonists by blocking interactions of receptor molecules or they can function as agonists by e. g. binding to cell surface receptors leading to the activation of downstream signaling cascades [31]. When recruitment of effector cells by the Fc part is not requested then AB domains containing antigen-binding properties (sdAB, scFv, Fv, Fab) are sufficient.

Possible applications of specific mABs and ABFs are being presented in the next paragraph.



- 1) Herceptin (*Trastuzumab*, Genentech (US), Roche (EU)) is one of the most prominent therapeutic mAb therapeutically used for destroying specific breast cancer tumor cells. Its development marks the beginning of a new era of designed target drugs and diagnostic tests [33]. It is a humanized monoclonal IgG AB targeting to the human epidermal growth factor receptor 2 (HER2). This receptor is overexpressed in in 20-30% of human breast cancers [34, 35]. Herceptin is produced in mammalian chinese hamster ovary (CHO) cells with a Fc part and distinctive glycosylation pattern. The potential mechanisms of how Herceptin avoids further tumor growth are versatile and comprise the following facts: degradation of HER2 receptors from the cell membrane [36], recruitment of immune cells by effector function [37], antagonizing uncontrolled growth signaling [38] and interaction with other signaling pathways [39].
- 2) The Fab fragment Cimzia (*Certolizumab Pegol*, Nektar Therapeutics, UCB Pharmaceuticals) is a humanized and PEGylated ABF recently approved by the FDA for therapeutic usage against TNF- $\alpha$  -related diseases. It is an anti TNF- $\alpha$  Fab fragment which was initially approved in 2008 for treatment of Crohn's disease but since May 2009 it is also indicated for the treatment of rheumatoid arthritis [31, 40]. It is composed of a light chain with 214 amino acids and a heavy chain with 229 amino acids and is produced in a microbial system of *Escherichia coli* cells [3]. By binding to TNF- $\alpha$  as a key proinflammatory cytokine it selectively neutralizes it and thereby inhibits further stimulation of TNF- $\alpha$  induced inflammatory reactions. The PEGylation extends the plasma half-life of the product, enabling its once-monthly subcutaneous administration [41]. As a benefit Fab units do not exhibit immunogenic parts as they consist of natural AB domains when being produced by humanized AB generation methods.
- 3) So-called "single domain ABs" (sdAB) were also shown to exhibit certain therapeutic usage possibilities. It was reported that daily oral administration of an untagged sdAB with specificity for *Staphylococcus mutans* reduced dental caries development of rats [7, 8]. Another interesting application in the field of "functional food" is an genetically engineered *Lactobacillus paracasei* strain expressing a sdAB on the surface which was shown to bind rotavirus' and thereby shortens virus-induced diarrhea in a mouse model [42, 43].



In this study, the focus of investigation is on a scFv fragment. Amongst others these formats already were shown to be effective for the antidote treatment of intoxications by ricin [44] and for neutralizing the lethal factor of *Bacillus anthracis* by inhibiting protective antigen-LF complex formation [45]. *B. megaterium* was used in the current work as the expression system for the ABF D1.3 scFv and to study the production and secretion by the bacterium itself. The ABF is directed against hen egg lysozyme which was chosen as an inexpensive antigen-model. Other fragments like anti-CRP-scFv and anti-lysozyme-Fab fragments [46-48] were also reported to be successfully produced by *B. megaterium*.



### 1.1.5 Antibody production systems

The expression of ABs and ABFs can be realized in several prokaryotic and eukaryotic production systems. All production platforms have certain advantages and disadvantages which are summarized in **Tables 1** to **3**. It has to be taken into account that optimal production hosts and parameters may vary for each generated particular AB format due to different requirements related to protein folding and posttranslational modifications, COG and regulatory acceptance [49]. The presented particular yield data is AB format and host specific and should give an idea about the efficiencies of the particular production platforms.

**Table 1:** Prokaryotic cells as productions hosts for mAB.

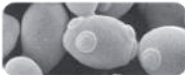
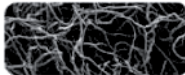
Organisms	Prokaryotic Cells	
	Gram Negative 	Gram Positive 
<b>Example</b>	<i>Escherichia coli</i>	<i>Bacillus subtilis</i> , <i>B. brevis</i> , <i>B. megaterium</i> , <i>Lactobacillus</i>
<b>Antibody Formats</b>	scFv, Fab, IgG	scFv, Fab
<b>Yield</b>	0.8 mg/L - 10 g/L (cytoplasmatic)	15 mg/L
<b>Advantages</b>	<ul style="list-style-type: none"> <li>• high cell density bioprocess with high yields</li> <li>• established host</li> <li>• minimal media</li> <li>• disulfid bond formation in periplasmatic space</li> </ul>	<ul style="list-style-type: none"> <li>• direct functional secretion</li> <li>• GRAS status</li> <li>• no endotoxins</li> <li>• reduced downstream processing</li> </ul>
	<b>Disadvantages</b>	<ul style="list-style-type: none"> <li>• inclusion body formation</li> <li>• less secretion</li> <li>• no glycolisation</li> <li>• endotoxin contamination</li> </ul>
<b>Improvements</b>	<ul style="list-style-type: none"> <li>• coexpression: <i>GroEL/ES</i>, trigger factor, <i>DanKJ</i>, <i>FkPa</i></li> <li>• coexpression: periplasmatic chaperones <i>DsbC</i>, <i>Skp</i></li> </ul>	<ul style="list-style-type: none"> <li>• gene knock out <i>htrA</i>, <i>wprA</i> (proteases)</li> <li>• process and media optimization</li> </ul>
<b>Literature</b>	[50-56]	[47, 48, 57-61]

Prokaryotic systems like the well-established Gram negative production host *E. coli* display high production yields of up to 10 g/L heterologous produced proteins as cytoplasmatic inclusion bodies (**Tab. 1**). In the last 60 years the system was optimized to produce heterologous proteins up to 50% of the total cell protein [6]. Moreover microbial cells are inexpensive, easily grown and quickly produce small amounts of target proteins for evaluation [62]. Also systems of periplasmatic secretion and thereby functional generation of disulfide bonds and modified “leaky” *E. coli* cells were developed [63]. However those leaky strains do not provide so far enough robustness for high cell density cultivations [64].



In contrast to *E. coli*, Gram positive cells like *Bacillus subtilis* and *B. megaterium* have the big advantage of being naturally high secretors. As they lack a second outer membrane, the AB is directly secreted and functionally folded through the membrane related SecA pathway [65] and finally released by diffusion through the cell wall. Although Gram positive expression systems exhibit relatively low production titers they still display a good secretion alternative to *E. coli*. Their potential for optimizing secretion is far from being exploited yet and even may lead to concentrations in g/L range as shown for other intracellular [66] and extracellular proteins [67]. However prokaryotic systems do not display any functional glycosylation patterns which are indispensable for therapeutic effector function of ABs. Therefore other production systems like eukaryotic cells play a predominant role.

**Table 2:** Eukaryotic microbial cells as production hosts for mAB.

Organisms	Eukaryotic Cells (microorganism)	
	Yeast	Fungi
		
Example	<i>Saccharomyces cerevisiae</i> , <i>Pichia pastoris</i>	<i>Aspergillus</i> , <i>Trichoderma</i>
Antibody Formats	scFv, llama V <sub>HH</sub> , Fab, scFv-Fc fusions	IgG, Fab, scFvs, llama V <sub>HH</sub>
Yield	70 mg/L - 1.2 g/L	1 mg/L - 1.2 g/L
Advantages	<ul style="list-style-type: none"> <li>• short generation time</li> <li>• secretion, no endotoxin or virus</li> <li>• robustness, simple medium</li> <li>• postranslational modifications, effector functions</li> </ul>	<ul style="list-style-type: none"> <li>• high secretion capacities</li> <li>• GRAS status</li> </ul>
Disadvantages	<ul style="list-style-type: none"> <li>• incomplete proteolytic processing</li> <li>• low transformation efficiency</li> <li>• insufficient and hyper-glycosylation</li> </ul>	<ul style="list-style-type: none"> <li>• proteolytic active</li> </ul>
Improvements	<ul style="list-style-type: none"> <li>• chaperone overexpression</li> <li>• coexpression: <i>BiP</i>, <i>PDI</i></li> <li>• bioprocess optimization</li> </ul>	
Literature	[68-76]	[72, 77, 78]




Microorganisms like yeast cells and fungi were found to efficiently secrete and at the same time glycosylate ABs and ABFs (**Tab. 2**). As a main drawback these systems sometimes show insufficient glycosylation or even hyper-glycosylation patterns. They also display increased proteolytic activity thus making production processes less reproducible and more difficult to handle. The same is true for insect cells which are based on the baculo-virus-infection system. In some cases they display a high diversity of post-translational modified products and a strong intracellular protein aggregation [31].

The current systems of choice especially for therapeutic AB production are mammalian cells like chinese hamster ovary (CHO), baby hamster kidney (BHK) or human embryonic



kidney (HEK) cell lines (**Tab. 3**). The predominant use of mammalian cell cultures has been driven by the need to obtain proteins with complex biochemical structures and resulting superior activity with native structures and function, e. g. proper folding, formation of disulfide bridges, oligomerization, proteolytic processing, phosphorylation and the addition of specific and complex carbohydrate groups [31, 79]. High extracellular product titers and an advanced secretion and folding apparatus for human glycosylation pattern make them an ideal production platform for mAB. However the overall costs for development and production are quite high compared to the other systems. As an example suspension cultures grown for 10-15 days can be used to inoculate a 10.000 L reactor followed by a subsequent cultivation of 6-14 days [3]. Here a typical cultivation process lasts at least around 25 days requiring large amounts of energy and resource consuming costs. A further challenge is the development of stable high producing cell lines and serum free media to reduce the risk of contamination. Additionally the production costs are not simply reduced by up-scaling the process. As an example for a whole cell system transgenic plants are mentioned as a most scalable production system with reduced production costs (**Tab. 3**).

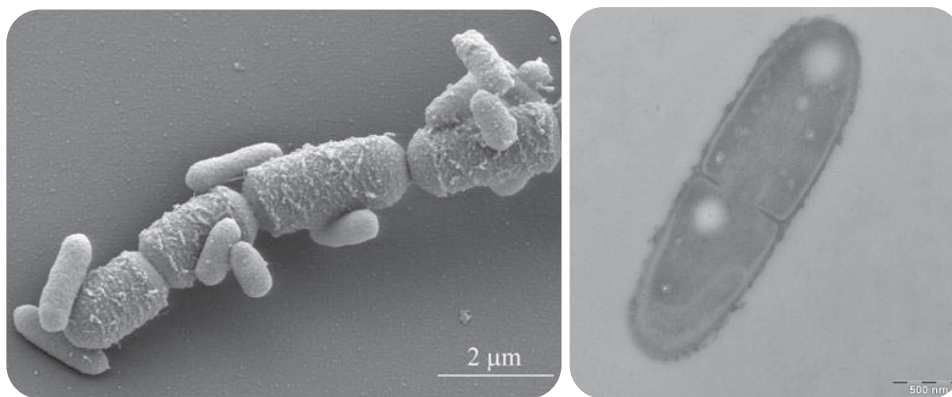
**Table 3:** Eukaryotic cells and cell systems as productions hosts for mAB.

Organisms	Eukaryotic Cells		
	Insect Cells 	Mammalian Cells 	Transgenic Plants 
<b>Example</b>	<i>Drosophila melanogaster</i> "Baculo Virus" System	CHO, BHK, HEK	<i>Nicotiana tabacum</i> , <i>Arabidopsis thaliana</i>
<b>Antibody Formats</b>	IgG, scFv	IgG, scFv-Fc	IgG, scFv, Fab, V <sub>HH</sub>
<b>Yield</b>	0.4 - 25 mg/L	1.4 - 1.8 g/L	28 - 136 mg/kg
<b>Advantages</b>	<ul style="list-style-type: none"> <li>mediate effector function</li> <li>human tolerance to baculo</li> <li>secretion, correct folding</li> </ul>	<ul style="list-style-type: none"> <li>advanced folding, secretion, post translational apparatus</li> <li>highly productive</li> <li>suitable for large and complicated proteins</li> <li>established system (60-70% of all antibodies)</li> </ul>	<ul style="list-style-type: none"> <li>1-10% of hybridoma production costs</li> <li>simple scale-up</li> </ul>
<b>Disadvantages</b>	<ul style="list-style-type: none"> <li>protease inhibitors recommended</li> <li>expensive media</li> <li>virus contamination risk</li> <li>strong intracellular protein aggregation</li> <li>high diversity of post translational modified products</li> </ul>	<ul style="list-style-type: none"> <li>high production costs</li> <li>may require animal derived media components</li> <li>extensive characterization (mycoplasma, virus testing)</li> <li>long term screening for high producers</li> <li>long process times</li> </ul>	<ul style="list-style-type: none"> <li>limits at glycosylation</li> <li>containment issues</li> <li>long development times for transgenic plants</li> </ul>
<b>Improvements</b>	<ul style="list-style-type: none"> <li>overexpression of <i>BiP</i>, <i>PDI</i></li> <li><i>hsp70</i> coexpression</li> </ul>	<ul style="list-style-type: none"> <li>serum free media (avoiding e. g. viral contamination)</li> <li>efficient chromosome integration</li> <li>optimized handling and bioprocesses</li> </ul>	
<b>Literature</b>	[80-83]	[84-88]	[89-91]

Alternative expression platforms in contrast to the cost- and time-intensive production in mammalian cells are microbial systems which are already used for production of all kinds of recombinant proteins. However, in most of the microbial systems the production itself is very efficient, though being also associated with high downstream processing costs [6, 31]. The steps for getting access to the intracellularly stored products, e. g. expressed in *E. coli*, usually comprise cellular disruption, product separation and purification. Therefore *B. megaterium* is a promising alternative with it being an efficient and less cost intensive expression host with high secretion capacities. Due to its lack of the outer membrane which is well known for Gram negative bacteria like *E. coli*, produced ABFs can directly be harvested from the culture supernatant.

## 1.2 *Bacillus megaterium* as production host

The Gram positive soil bacterium *B. megaterium*, which was discovered in 1884 from Anton de Bary, got its name from “big beast“ greek „megatherium“ related to its big size of up to 4 x 1.5  $\mu\text{m}$  (**Fig. 5**) [92]. The pronounced magnitude of the bacterium classifies it as an ideal model organism to study cell structures and protein localization. In the past *B. megaterium* has been intensively used to analyze sporulation, bacteriophages and biochemistry of Gram positive bacteria [93-96]. Also more than sixty years ago, Maurice Lemoigne discovered the polyester polyhydroxybutyrate in *B. megaterium* as an important storage compound in bacteria, today being a potential resource for generating bio-plastic [97].



**Figure 5:** Scanning electronic microscope (SEM) pictures of *B. megaterium* (4x1.5  $\mu\text{m}$ ) and *E. coli* (2 x 0.5  $\mu\text{m}$ ) (left); Ultra-thin section of a dividing *B. megaterium* cell (right) [98].

*B. megaterium* can be isolated from a various habitats like soil, water, sediments and also from honey or dry food products [99], due to its high osmotic tolerance and its ability to metabolize a vast spectrum of carbon sources.

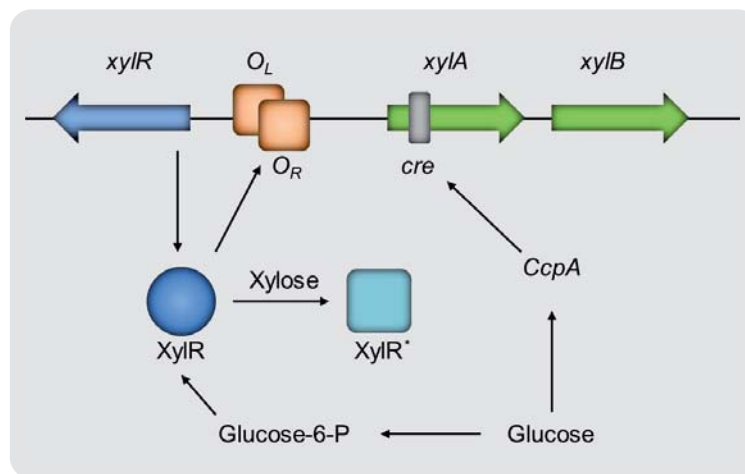
As part of the Collaborative Research Center SFB 578 the genome of *B. megaterium* strain DSM319 was sequenced and annotated [100, 101]. Thereby a phylogentic classification was done showing, according to the NCBI taxonomy database, a close relation to the *B. cereus*/*B. anthracis* group of the genus *Bacilli*.

A big advantage of *B. megaterium* compared to Gram negative organisms like *E. coli* is that it is an expression host with high secretion capacities [99]. The lack of an outer membrane allows secreted products to be directly harvested from the culture supernatant. It does not produce alkaline proteases and also has higher plasmid stability during growth [102]. To ensure the secretion of recombinant proteins signal peptides have to be added to the N-terminal end. These peptide chains are recognized by the type II secretion apparatus [65] of the SecA pathway and the protein is functionally folded upon release through the cell membrane [103, 104]. In the last years it has been shown that *B. megaterium* is able to intracellularly produce and secrete high amounts of functional



proteins [105, 106] including industrially important products like penicillin-G-acylase [107], different amylases [108], glycosyltransferases [109], dextranucrase [110], vitamin B<sub>12</sub> [111, 112], cytochrom monooxygenases [113],  $\beta$ -galactosidase, glucose dehydrogenase, formate dehydrogenase, toxin A [114, 115] and hydrolases [116, 117]. Its non-pathogen status qualifies it as an ideal industrial production strain. Besides the mentioned recombinant proteins, *B. megaterium* was shown to efficiently secrete ABFs in the surrounding medium [47, 48, 59].

The heterologous plasmid-based protein production is under the control of a xylose inducible promoter system [118] (**Fig. 6**) characterized and developed by Rygus and Hillen in 1991 [119] and is depicted in the following scheme.



**Figure 6:** Regulation of the xylose-operon from *B. megaterium*; **CcpA**: Catabolite control protein A, (transcription factor), **cre**: DNA-sequence (“catabolite response element”), **O<sub>L</sub>/O<sub>R</sub>**: operator region of the *xyl* promoter, **xylA**: xylose isomerase gene, **xylB**: xylulokinase gene, **xylR**: xylose repressor gene, **XylR**: active xylose repressor, **XylR\***: inactive xylose repressor.

In the absence of xylose the xylose repressor protein XylR is binding to the operator region O<sub>L</sub> and O<sub>R</sub> of the xylose promoter thereby inhibiting the initiation of transcription. In presence of xylose the repressor protein conformation is changed, enabling the operator region and facilitating the RNA-polymerase mediated transcription of the controlled gene. Xylose addition increases transcription efficiency up to 150 times [120]. Furthermore transcription of the xylose operon is also controlled by other mechanisms. When glucose is present the binding affinity of CcpA (Catabolite control protein A) to an inside the *xylA* gene located *cre*-sequence (catabolite responsive element) is increased, thereby inhibiting an effective transcription. The other controlling mechanisms comprises the competition of glucose-6-monophosphat, usually generated upon glucose uptake, with xylose for binding to the active site of xylose repressor. At the presence of glucose-6-monophosphat the activation of the promoter by Xylose is being blocked and transcription cannot occur. Thereby the xylose related activation of the promoter is hindered and the actual transcription is repressed [119].