Expression of recombinant antibodies

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Recombinant antibodies are highly specific detection probes in research, diagnostics, and have emerged over the last two decades as the fastest growing class of therapeutic proteins. Antibody generation has been dramatically accelerated by *in vitro* selection systems, particularly phage display. An increasing variety of recombinant production systems have been developed, ranging from Gram-negative and positive bacteria, yeasts and filamentous fungi, insect cell lines, mammalian cells to transgenic plants and animals. Currently, almost all therapeutic antibodies are still produced in mammalian cell lines in order to reduce the risk of immunogenicity due to altered, non-human glycosylation patterns. However, recent developments of glycosylation-engineered yeast, insect cell lines, and transgenic plants are promising to obtain antibodies with "human-like" post-translational modifications. Furthermore, smaller antibody fragments including bispecific antibodies without any glycosylation are successfully produced in bacteria and have advanced to clinical testing. The first therapeutic antibody products from a non-mammalian source can be expected in coming next years. In this review, we focus on current antibody production systems including their usability for different applications.

Keywords: recombinant antibody, procaryotes, yeast, fungi, insect cells, mammalian cell, transgenic organisms

INTRODUCTION

Today, antibodies are used for several applications in research, diagnostics, and therapy. They are used in many standard assays such as immunoblot, flow cytometry, or immunohistochemistry. In addition this, the emerging field of proteome research has a huge need of binders against different protein antigens and splice variants (1, 2). Moreover, recombinant antibodies are used for the diagnosis of different pathogens (3–5) or toxins (6, 7). In the past decade, several antibodies for therapeutic applications have been developed (8, 9), primarily targeting inflammatory or tumor diseases (10). In 2010, sales of approved therapeutic monoclonal antibodies in the USA and EU reached 50 billion US dollars (11).
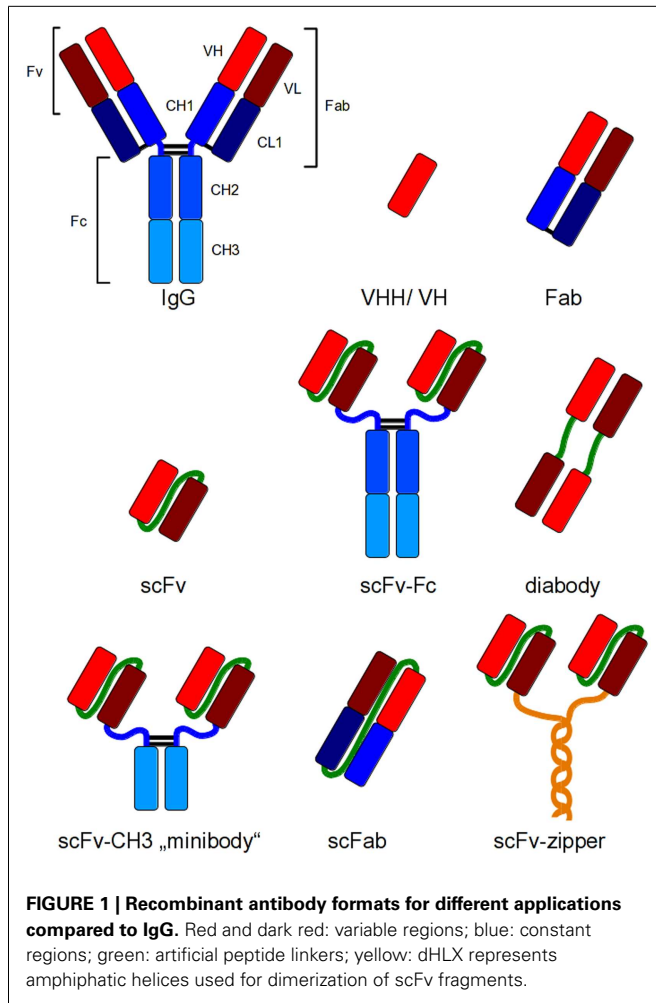
For the detection of different antigens, polyclonal antibodies are widely used in research and diagnostics. These sera contain a large and diverse amount of different antibodies with unknown specificities. However, polyclonal non-human antibodies may exhibit an immune response in human beings that hampers the therapeutic use for example after snake bites (12). Therefore, the production of monoclonal antibodies (mAbs) by hybridoma technology was a significant milestone (13) for the generation of antibodies for therapeutic use. As this technology is based on the fusion of antibody producing spleen cells from immunized mice or rats with immortal myeloma cell lines, its main obstacle is the inefficient immune response to highly toxic or conserved antigens. In addition, nearly all antibodies which are currently in clinical development are of human-origin or at least humanized in some aspect (9, 14, 15) to prevent immunogenicity. Consequently,

transgenic animals, especially mice, have been developed which contain a human immunoglobulin gene repertoire (16, 17) solving the problem of immunogenicity but not the need of an efficient immune response after immunization. Finally, *in vitro* selection technologies such as antibody phage display or ribosomal display provide a solution for the generation of human antibodies (18–22).

These new antibody generation technologies have increased the amount of antibodies for different applications and, therefore, also the need of efficient production systems. Immunoglobulin G (IgG) is a heterotetrameric molecule consisting of two heavy and two light chains, respectively, which are connected via disulfide bonds. Heavy and light chains (HC and LC) also contain intramolecular disulfide bonds for stabilization (23). These structural properties require a sophisticated folding apparatus as well as an oxidizing environment for the generation of disulfide bonds. Consequently, many traditionally expression hosts do not provide these mechanisms for efficient production of IgGs. Therefore, smaller antibody fragments have been developed which combine easier production with full antigen binding capacity of an IgG (**Figure 1**). In addition, the development of smaller fragments was the basis for most of the *in vitro* antibody generation systems (18–22). These antibody fragments can be used for applications, where epitope binding is sufficient for the desired effect including therapeutic applications such as virus neutralization or receptor blocking.

The smallest antigen binding fragment of immunoglobulins maintaining its complete antigen binding site is the Fv fragment, which consists only of variable (V) regions. A soluble and flexible amino acid peptide linker is used to connect the V regions to a scFv (single chain fragment variable) fragment for stabilization of the molecule (24), or the constant (C) domains are

Abbreviations: IgG, immunoglobulin G; mAb, monoclonal antibody.

FIGURE 1 | Recombinant antibody formats for different applications compared to IgG. Red and dark red: variable regions; blue: constant regions; green: artificial peptide linkers; yellow: dHLX represents amphiphatic helices used for dimerization of scFv fragments.

added to the V regions to obtain a Fab fragment (**Figure 1**). Today, scFv and Fab are the most widely used antibody fragments which are produced in prokaryotes. Other antibody formats have been produced in prokaryotic and eukaryotic cells, for example, disulfide-bond stabilized scFv (ds-scFv) (25), single chain Fab fragments (scFab) combining scFv and Fab properties (26) as well as di- and multimeric antibody formats like dia-, tria-, or tetra-bodies (27, 28) or minibodies (miniAbs) comprising different formats consisting of scFvs linked to oligomerization domains like immunoglobulin CH_3 domain (28), leucin zipper, helix turn helix motif streptavidin, or scFv-scFv tandems (29–31). Bispecific antibody formats combine two different antigen binding domains in one molecule (32–34). The smallest antibody fragments are V_{HH}s of cameloide heavy chain antibodies (35) and single domain antibodies (dAb) (36, 37).

For most therapeutic applications, the Fc moiety of an immunoglobulin is essential for the method of action as it mediates the effector functions such as cellular dependent cytotoxicity or the activation of the complement system. Therefore, antibody fragments have been fused to the Fc domain to regain effector functions and avidity (38, 39). **Figure 1** depicts some of these antibody formats that have been developed for different applications.

ANTIBODY PRODUCTION IN PROKARYOTIC HOSTS
GRAM-NEGATIVE BACTERIA

Escherichia coli is the most important production system for recombinant proteins reaching volumetric yields in the gram per liter scale for extracellular production (40–42). For production of functional antibody fragments, the key to success was the secretion of both V chains into the periplasmic space of *E. coli* where the oxidizing environment allows the correct formation of disulfide bonds and the assembly to a functional Fv fragment (43). This strategy also allowed the first expression of functional Fab fragments in *E. coli* described in 1988 (44).

The production of recombinant antibodies in the reducing cytoplasmic compartment results mostly in non-functional aggregates (45). Recovery of functional antibody fragments from cytoplasmic inclusion bodies by complete denaturation and refolding (46) is often not efficient. Stable cysteine free mutants of some scFvs were successfully produced in the cytoplasm of *E. coli* (47, 48). *E. coli* strains with mutations in the glutathione and thioredoxin reductase in combination with coexpression of cytoplasmic chaperones GroEL/ES, trigger factor, DnaK/J as well as signal sequence-less variants of periplasmic chaperones DsbC and Skp increased the yield of functional Fab (49).

For the production of camelid single domain antibodies (VHH), coexpression of Erv1p sulfhydryl oxidase increased the yield in the cytoplasm (50).

Despite these efforts, most antibody fragments are produced in the periplasm of *E. coli* using N-terminal leader sequences targeting the periplasmic *Sec* pathway (51), for example signal peptides derived from outer membrane protein A (*OmpA*), alkaline phosphatase A *(PhoA)*, or pectate lyase B (*PelB*) (52–54). Also the SRP pathway can be used for antibody fragment production (55). After expression, recombinant antibodies are usually isolated from the periplasmic fraction (56, 57) but also from the culture supernatant (58–61).

The yield of functional scFv fragments has been improved by co- or overexpression of GroES/L, peptidyl prolyl-cis,trans-isomerase FkpA, or other folding helper proteins (62–66). Functional expression can also be increased by optimization of cultivation parameters, such as temperature, media, or additives. Here, the optimal parameters are dependent on the individual antibody fragment (58, 67). The production system itself influences the production rate. Very high yields of antibody fragments produced in *E. coli* are mainly provided by high-cell density fermentation in bioreactors: the expression of a hapten-specific scFv produced in a bioreactor (68) lead to yields up to 1.2 g/L compared to 16.5 mg/L yield of the same antibody obtained by optimized shake flask production (69), which can be mostly addressed to the over 100-fold higher cell density in the bioreactor. A recent production system is the LEX bubble column bioreactor. Yields in the LEX system of an anti-MUC1 scFv was ∼30–40× higher and yields of an anti-lysozyme antibody was about 2× higher compared to shake flask incubation (67, 70). *E. coli* strain optimization, e.g., plasmid stability, can additionally improve production yield (71).

The Fab format requires expression, periplasmic transport, correct folding, and assembly of two different polypeptide chains. Among the different vector formats and arrangements, bicistronic vectors with the first cistron encoding the light chain and the

second cistron encoding the Fd fragment are optimal (56). Even aglycosylated full-size IgGs were successfully produced in *E. coli* (72, 73). In our view, the raison d'être of complete IgG production in *E. coli* is doubtful.

Cell wall-less L-forms of the Gram-negative bacterium *Proteus mirabilis* were used for the production of miniAbs and scFv (30, 74), but yield of total scFv and of functional scFv were different and ranged from 83 to 127 mg/L of total scFv to just 9–12 mg/L of functional scFv (74). However, quite recently scFv were produced successfully in *Pseudomonas putidas* with a yield of 0.5–3.6 mg/L. Interestingly, production yields were decreased by using scFv genes codon optimized for *P. putidas* (75).

GRAM-POSITIVE BACTERIA

Gram-positive bacteria directly secrete proteins into the medium due to the lack of an outer membrane which could facilitate production of antibody fragments. The Gram-positive bacteria *Bacillus brevis* (76, 77), *Bacillus subtilis* (78, 79), and *Bacillus megaterium* (80–85) have already been successfully used for the production of different antibody fragments. In addition, *B. megaterium* does not produce alkaline proteases and provides high stability of plasmid vectors during growth allowing stable transgene expression during long term cultivation in bioreactors (86).

Lactobacilli are also tested for antibody production and are "generally regarded as safe" (GRAS) microorganisms. To date, two lactobacillus strains were used for the production of scFvs, *Lactobacillus zeae/casei* (87, 88), and *Lactobacillus paracasei* (35, 89). The GRAS status of lactobacilli allows their direct use for oral application for example for production of anti-*Streptococcus mutans* antibody fragments to prevent tooth decay (88).

EUKARYOTIC HOSTS USED FOR ANTIBODY PRODUCTION
YEASTS

Eukaryotic cells have developed an advanced folding, post-translational, and secretion apparatus which enhances the secretory production of antibodies, including full immunoglobulins compared to bacteria. Yeasts combine the properties of eukaryotic cells short generation time and ease of genetic manipulation with the robustness and simple medium requirements of unicellular microbial hosts. Moreover, yeasts have been used for fermentation in food production for several millennia in human history; they do not produce bacterial endotoxins and have gained the GRAS status paving the way toward production of therapeutic proteins (90, 91). *Pichia pastoris* represents the major yeast strain used for recombinant antibody production (92). Other yeasts like *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Schizosaccharomyces pombe* (93, 94), *Schwanniomyces occidentalis*, *Kluyveromyces lactis*, and *Yarrowia lipolytica* (95) have also been described for protein production but have played only a minor role. *P. pastoris* shows overall optimal capacity for the production and secretion of heterologous proteins than *S. cerevisiae* and does not secrete large amounts of its own protein which simplifies the downstream processing. Moreover, *P. pastoris* prefers respiratory growth resulting in high-cell densities of more than 100 g/L dry weight (96). Probably the most prominent feature of *P. pastoris* is the metabolization of methanol as sole carbon source. The alcohol oxidase 1 (AOX1) promoter is strictly controllable by methanol and commonly used

for recombinant protein expression. The secretory production of heterologous proteins including antibodies requires an aminoterminal signal sequence targeting the yeast's secretory pathway. *S. cerevisiae* mating factor alpha (alpha-factor) pre-pro peptide is the most commonly used secretory signal sequence and is followed by appropriate proteolytic cleavage sites sensitive for the Golgi resident endoprotease KEX2 for efficient release of antibodies during secretion, which is often used in combination with ST13 exoprotease sites (97).

Expression of scFv antibody fragments in *P. pastoris* was first shown by Ridder et al. in 1995 (98). Yields for different scFvs ranged from 70 mg/L (99) to 250 mg/L (100). Up to 8 g/L functional scFv were obtained under optimized conditions in bioreactors with coexpression of BiP (101). Llama VHHs achieved over 100 mg/L yield in *S. cerevisiae* even in shake flask cultivation (102). Production of more complex, yet still single-gene-encoded formats such as dimeric scFv-Fc antibodies in *P. pastoris* achieved production levels of 10–30 mg/L (103). Antibody formats encoded by two genes such as Fab and IgG required the fusion of the two different antibody chains to the aminoterminal secretory signal sequence and their cotransformation. The yield of Fabs produced in yeast ranged from 1 to 50 mg/L by shake flask cultivation and up to 0.5 g/L in bioreactors (96).

Limited data concerning full-sized IgG expression in yeast is available. In an early study, a mouse-human chimeric antibody and its Fab fragment were produced in *S. cerevisiae* with a yield of 50–80 µg/L IgG and 200 µg/L Fab, respectively. The chimeric IgG mediated tumor specific binding and ADCC (antibody dependent cellular cytotoxicity) but no CDC (complement dependent cytotoxicity) (104). Using *P. pastoris* up to 1.4 g/L of a human IgG1 could be expressed in a 40-L bioreactor (105).

Lower transformation rates compared to *E. coli* must be considered for antibody library generation rather than for antibody production. Moreover, the frequency of homologous transformation in yeast is higher compared to higher eukaryotes facilitating the process of making stable expression clones. Specific issues of heterologous protein expression in yeast can be circumvented by optimizing gene sequences, for example by avoiding AT-rich stretches which can cause premature transcriptional termination. The productivity of antibody fragments in yeasts was increased by DNA shuffling (106).

Inefficient secretion of larger heterologous proteins (>30 kDa), proteolysis of secreted proteins during high-cell density fermentation, and inappropriate glycosylation of human glycoproteins are serious issues which required engineering of yeast strains. Overexpression of the chaperone immunoglobulin binding protein (BiP) or protein disulfide isomerase (PDI) in *S. cerevisiae* increased scFv secretion titers twofold to eightfold, with an average yield of 20 mg/L in shake flask culture (107). Yeasts tend to hyperglycosylate heterologous proteins even at positions not glycosylated in the native mammalian host, which can influence activity of antibodies and is a potential source of immunogenicity or adverse reactions in human patients. *P. pastoris* exhibits much lower hyperglycosylation than *S. cerevisiae,* and its *N*-linked carbohydrate structures are already similar to the mammalian high-mannose core unit $Man_{5-6}GlcNAc_2$ (108). Moreover, genetically modified glyco-engineered *P. pastoris* strains have been generated which

produce humanized glycosylation patterns (109–113). The therapeutic IgG antibodies produced in glyco-engineered yeast achieved results that were comparable to its counterpart Trastuzumab that has been produced in mammalian cells (114). Unlike IgGs produced in wildtype yeast, those produced in glyco-engineered yeasts were able to mediate antibody-mediated effector functions. Production processes employing glyco-engineered yeasts are currently optimized for commercial antibody production (115) as well as for high throughput screening (116).

FILAMENTOUS FUNGI
Filamentous fungi of the genera *Trichoderma* and *Aspergillus* have the capacity to secrete large amounts of proteins and metabolites into the medium (117). They are widely used in the food and biotechnological industry, for example *A. niger* for citric acid production. Moreover, *A. niger* (subgenus *A. awamori*) and *Aspergillus oryzae* gained obtained GRAS status. Two promoters are typically used for the expression of antibodies in fungi: the glucoamylase promoter (glaA) (118) and the endoxylanase A promoter (exlA) (119). Antibody chains are usually fused to the aminoterminus of glucoamylase in *Aspergillus* and cellobiohydrolase I in *Trichoderma spec.*, respectively, in order to obtain optimal secretion (120). Moreover, protease cleavage sites like KexB are introduced to release the antibody from glucoamylase before secretion (118). Yields of up to 1.2 g/L IgG were achieved in *A. niger* when both antibody chains were fused to glycoamylase. In *Trichoderma reesei*, 150 mg/L of a Fab fragment was obtained when both chains were fused with cellobiohydrolase I increasing yields 100-fold higher than with its natural signal peptide (121). *A. awamori* was used for the production of several scFvs, llama VHHs and antibody enzyme fusion proteins (117, 119, 122). A yield of 73.8 mg/L of an anti-EGFR-VHH was achieved in *A. oryzae* by using a Taka-amylase A signal sequence and 28 amino acids from the aminoterminal region of *Rhizopus oryzae* lipase (123).

Fungal proteases can result in protein degradation which was addressed by deletion mutants. *Chrysosporium lucknowense C1* contains a triple protease deletion (Delta-alp1, Delta-pep4, Delta-alp2) and was successfully used in small-scale productions for screening as well as in high scale bioreactor productions (124).

PROTOZOA
Recently, the eukaryotic parasite *Leishmania tarentolae* has been explored as an expression system for different recombinant proteins (125, 126). One major advantage of this expression system is the mammalian-like glycosylation pattern: this protozoa is able to perform O-glycosylation as well as N-glycosylation, which is highly conserved in mammalians (127). Consequently, *L. tarentolae* has been begun to be used for the production of recombinant antibodies: analysis of different signal peptides lead to a protein yield of 2–6 mg/L purified scFv (128).

INSECT CELLS
Insect cells represent a very versatile eukaryotic expression system. They can be efficiently transfected with insect-specific viruses from the family of *Baculoviridae*, particularly the *Autographa californica* nuclear polyhedrosis virus (AcNPV). Baculoviruses are highly species-specific and are considered as safe for humans,

mammalians and plants. Infection of human hepatocytes and mammalian cell lines including stable transduction has been demonstrated in cell culture without evidence of viral replication or gene expression under the control of baculoviral promoters (129, 130). Non-essential baculovirus genes involved in the viral life cycle, like *Polyhedrin*, *P10*, or *Basic* can be replaced by heterologous genes. The flexible viral envelop allows packaging of large heterologous gene sequences of more than 20 kb. Heterologous genes under the control of the strong polyhedron promoter are expressed at levels ranging from 0.1 to 50% of the total insect cell protein. Baculoviral protein expression is normally performed in insect cell lines like Sf-9 and Sf-21 of *Spodoptera frugiperda*, DS2 cells of *Drosophila melanogaster*, or High Five cells (BTI-TN-5B1-4) of *Trichopulsia ni*. High Five cells have certain advantages over Sf-9 cells for recombinant protein expression because they secrete up to 25-fold higher protein levels (131), have a more rapid doubling time, allow quick adaptation to serum-free medium and grow in suspension culture. In contrast, Sf-9 and Sf-21 cells are recommended for producing high-titer viral stocks due to higher transfection efficiency. Recombinant protein production can be performed in small-scale using plates or shake flasks as well as in large scale using Spinner flasks or bioreactors. Important parameters for optimizing baculoviral protein production are multiplicity of infection (m.o.i.), production length (usually up to 96 h), addition of protease inhibitors due to the release of viral proteases, temperature (usually 25–30°C), and media pH (pH 6.0–6.4).

Secreted monomeric anti-phOx scFv were obtained at levels of up to 32 mg/L in a 6-L bioreactor with 10^9 cells per liter after 72 h with an m.o.i. of 1 (132). Production yields of 6–18 mg/L have been achieved for various IgGs (133). Immunoglobulins produced in High Five cells showed mammalian-like terminal galactosyl residues $\beta(1,4)$-linked to the biantennary GlcNAc residues. In contrast, the absence of sialylation, the formation of paucimannosidic structures and the presence of potentially allergenic $\alpha(1,3)$-fucose linkages are different to mammalian glycosylation (134). Nevertheless, IgGs produced in insect cells were able to mediate effector functions like complement binding (135, 136) and ADCC (137). Insect cell protein expression was improved using protease deficient baculovirus strains or cell lines with additional glycosyltransferase gene modifications to obtain glycosylation patterns comparable to mammalian cell lines (138–141).

Expression of IgGs in insect cells under control of the strong *Polyhedron* promoter resulted in an extensive aggregation, probably caused by overloading the cellular folding and post-translational processing apparatus (142).

Overexpression of the ER resident chaperone binding protein (BiP) significantly enhanced levels of soluble and secreted IgGs in *T. ni* cells (143). Enhanced secretion of IgGs was also achieved by coexpression of protein disulfide isomerase (PDI) or the human cytosolic chaperone hsp70 in *T. ni* cells (138).

Due to strong usage of the cellular metabolism during baculoviral protein expression a high diversity in the post-translational modification was observed. Alternatively to baculoviral expression, insect cells can also be transfected with expression plasmids in a transient or stable manner. Here, usually Schneider 2 (S2) cells of *D. melanogaster* are used. Secretory production requires a signal sequence like the honeybee melittin leader. Stable transfection

of *Drosophila* cell lines with monomeric and dimeric antibody fragments resulted in yields of up to 25 µg/mL (144).

Immunoglobulin G production using the baculovirus expression system demonstrated IgG effector function such as complement binding (135, 136). 10 µg/mL of anti-Rhesus D antibody produced in Sf-9 cells mediated lysis of Rh+ red blood cells by ADCC (137).

MAMMALIAN CELLS

Today, 60–70% of all recombinant protein pharmaceuticals and 95% of the currently approved therapeutic antibodies are still produced in mammalian cell lines despite relatively high production costs and difficult in handling. However, the advanced mammalian folding, secretion and post-translational apparatus is capable of producing antibodies indistinguishable from those in the human body with least concerns for immunogenic modifications. Moreover, it is also highly efficient for secretion of large and complex IgGs and in combination with the folding and post-translational control it results in high product quality which reduces efforts and costs in the subsequent and more expensive downstream processing steps. The risks of contamination by pathogens or bovine spongiform encephalopathy (TSE/BSE) agents have been eliminated by well-documented Good Manufacturing Practice (GMP) compliant designer cell substrates and chemical defined media without the need of supplementing animal serum components (145). In 2004, mammalian cell culture technology reached production levels of approximately 5 g/L IgGs in Chinese hamster ovary (CHO) cells (146). Today, industrial IgG production levels often exceed 12 g/L as the result of a steadily ongoing progress in mammalian cell culture technology, which is mainly due to improved high producer cell lines, optimized production media, and prolonged production processes at high-cell densities. The highest reported IgG production titer we found was obtained in the human embryonic retinal cell line Per.C6 [Crucell, Leiden, Netherlands, (147)] with 27 g/L. Generally, the productivity of recombinant mammalian cell lines increased from initially 10 pg antibody per cell per day (pcd) in 1986 to about 90 pcd in 2004. Today, the antibody production levels only rarely exceed 100 pcd because higher cellular productivity usually corresponds to lower maximum cell densities in the production process. Producer cell lines have also been genetically engineered regarding product homogeneity, improved metabolism, reduced apoptosis, and inducible cell cycle arrest (148, 149) which allows prolonged production times for almost 3 weeks at high-cell viability and cell densities.

Chinese hamster ovary (CHO) cells are the most common cells applied in the commercial production of biopharmaceuticals. This cell line isolated in the 1950s gave rise to a range of genetically different progeny, such as K1-, DukX B11-, DG44-cell lines and others which differ in protein product quality and achievable yield. In addition, Per.C6 cells, mouse myeloma NS0 cells, baby hamster kidney (BHK) cells and the human embryonic kidney cell line HEK293 received regulatory approval for recombinant protein production. Although glycosylation patterns of mammalian glycoproteins are very similar to that in humans (150), even small differences can influence pharmacokinetics and effector functions of antibodies. Alternative designer cell lines with improved glycosylation patterns have been generated, for example human neuronal precursor cell line AGE1.HN (Probiogen, Berlin, Germany) supporting specific and complex glycostructures for the production of antibodies which require specific post-translational modifications or suffer from instability or susceptibility for proteolysis (151). CHO cell variant Lec13 (Glycotope) also produces human IgG1 with *N*-Linked glycans lacking fucose which improves on Fc-gammaRIII binding and ADCC (152).

Stable production of antibodies in mammalian cells

The generation of stable master cell lines is a prerequisite for GMP compliant IgG production in the therapeutic sector in order to guarantee long term production stability. Here, the antibody gene expression cassettes have to be stably integrated into the host cell genome.

Strong promoters like the immediate early cytomegalovirus (CMV) or the cellular elongation factor (EF) 1-alpha promoter and polyadenylation sites from the simian virus (SV) 40 or the bovine growth hormone (BGH) for improved mRNA stability and translation efficiency are usually implemented into the expression vector. Furthermore, splicing of mRNA is known to promote mRNA packaging and transfer into the cytosol in order to stabilize and enhance gene expression as well as to reduce silencing of heterologous transgenes (153, 154). For IgG expression, two different genes must be stably transfected into one cell clone, either by cotransfection or by using bicistronic expression vectors. Bicistronic vectors employing internal ribosomal entry sites (IRES) allow the translation of two or more cistrons from the same transcript (155). The encephalomyelitis virus (ECMV) IRES has shown the highest efficiency in various mammalian cell lines. Mutated IRES derivatives allow the control of translation efficiency in relation to the cap-dependent cistron. The ratio between light and heavy chain has great impact on the secretion level of functional IgGs (156). The long term stability of ECMV IRES containing bicistronic constructs has been demonstrated even in the absence of selection pressure over months (157).

There are different methods to enhance antibody expression by increasing the number of antibody gene copies in the genome through gene amplification. The two major systems on the market are based on dihydrofolate reductase (DHFR) or glutamyl synthetase (GS) selection. Yield and functionality of an IgG1 produced in *dhfr*− CHO and GS-NSO are equivalent (158) and reached 1.8 g/L in GS-NS0 cells (159). However, gene amplification also causes genetic instability, and after removing the selection pressure the yield of antibodies can be reduced again. Moreover, high producer cell lines often contain only a few copies of the antibody genes. For example, up to 2.7 g/L final antibody concentration were obtained from NS0 cells containing three vector copies per cell (160). Other factors than the number of gene copies play an important role to achieve high production levels of antibodies. Therefore, industrial antibody expression platforms employ efficient screening systems in order to isolate the best of the high producers. However, there are also strategies to facilitate the isolation of high producer clones (161). To overcome negative effects of the integration site, protective cis-regulatory elements include insulators, boundary elements, scaffold/matrix attachment regions (S/MARs) (162), chromatin opening elements

(163), and antirepressor elements (164) were introduced into the vector which reduced the influence of heterochromatin and stabilize transgene expression (165, 166). Silencing can be blocked by inhibition of histone deacetylation using butyrate (167) which could enhance the protein expression levels of the cells (168) but can also induce apoptosis.

Recombination enzymes, like bacteriophage P1 Cre recombinase, lambda phage integrase, or yeast Flp recombinase can efficiently catalyze the site specific integration into defined chromosomal recombination exchange cassettes which have been introduced into producer cell lines (169, 170). The 2A/furin technology allows expression of both IgG chains as a single gene due to post-translational auto-cleavage of the viral protease 2A encoded by the linker and subsequent processing by the Golgi protease furin (171, 172).

Transient production of antibodies in mammalian cells

The generation of high producer cell lines has been dramatically improved and accelerated (161, 173), however it is still too expensive, time-consuming and laborious for research applications, or if large numbers of individual antibodies have to be produced. Here, transient and semi-stable mammalian antibody expression is much more suitable because it allows fast and parallelized production without any need to generate producer cell lines (174). Moreover, transient mammalian antibody production can be scaled up by employing batch or fed-batch bioreactor processes to more than 150 L production volumes (175). Therefore, transient antibody production is suitable for small-scale production in antibody screening (176), but also capable to generate grams of antibodies (177–179).

The human embryonic kidney (HEK) 293 cell lines have been widely used for transient protein expression because they can be very efficiently transfected with plasmid DNA. Some derivatives were further transformed either with the simian virus 40 (SV40) large T antigen, termed HEK293T, or with the Epstein Barr virus (EBV) nuclear antigen 1 (EBNA1), termed HEK293E, in order to mediate semi-stable episomal propagation of vectors containing an origin of replication (ori) of SV40 or EBV, respectively. Transient transfection of plasmid DNA in HEK293 cells can also be performed in large scale by calcium phosphate transfection (180), cationic liposomes, and polymers like polyethyleneimine (PEI) (181, 182).

Recently, transient production of IgG-like scFv-Fc antibodies in the HEK293-6E cell line, a genetically modified variant with a truncated version of EBNA1 growing in suspension and chemically defined serum-free medium (183, 184), achieved volumetric yields of up to 0.6 g/L by simple shake flask cultivation. Improved production media, fed-batch supplementation, and well-controlled bioreactor processes allow higher cell densities and prolonged production time, both enhancing the yield. Backliwal and colleagues (177) combined optimized PEI-based transfection at high-cell densities with the coexpression of cell cycle regulators p18 and p21, acidic fibroblast growth factor, valproic acid supplementation, consequent maintenance at high-cell densities of cells/milliliter and up-scaling to 2 L and achieved production levels of more than 1 g IgG within 2 weeks after transient transfection.

TRANSGENIC ORGANISMS
TRANSGENIC PLANTS

The development of transgenic plants for the expression of recombinant antibodies is becoming interesting, especially when high amounts are required. Up-scaling of this production system can be achieved more easily compared to other systems such as mammalian cell culture, where up-scaling of the fermentation process leads to increasing production costs. In theory, the costs of an IgA expressed in plants are only 1–10% compared to the expression in hybridoma cells (185).

The generation of genetically modified dicotyledonous plants is mainly done by the transfer of the expression cassette of the transgene with the help of *Agrobacterium tumefaciens*. In principle, the gene of interest is cloned into the T-DNA of a binary plasmid (186, 187) which is flanked by two 25 bp imperfect repeats. In most cases, the expression of the transgene is under the control of one or two (188) copies of the constitutive cauliflower mosaic virus (CaMV). In addition, a selection marker is located on the T-DNA and transferred into the host genome for effective screening of successfully transformed plants. After integration of the T-DNA into the host genome by non-homologous recombination complete plants can be regenerated from transformed pieces of the plant [RB (189)]. As this procedure requires several months of transformation and special regeneration protocols, transient expression systems have been developed which allow time saving production of recombinant proteins: McCormick and colleagues designed a tobacco mosaic virus (TMV) based vector for the secretory expression of different scFvs for the treatment of non-Hodgkin's lymphoma (190). Expression yields in *Nicotiana benthamiana* were up to 100–800 μg/mL in the crude secretory extract. Same technique has been applied for the expression of idiotype-scFvs for personalized vaccination of follicular B-cell lymphoma patients in a phase I clinical study (191). In this study, nearly half of the treated patients developed an antigen specific immune response despite differences in glycosylation pattern.

Differences in the glycosylation pattern between mammalia and plants are one of the main obstacles researchers have to overcome when developing therapeutic antibodies expressed in plants. Although plants are able to perform complex glycosylation, differences in glycosylation patterns, in particular β1,2-xylose and α1,3-fucose, can lead to immunogenicity of the therapeutic proteins (192–194). Therefore, different strategies have been developed to express recombinant proteins with a more mammalian-like glycosylation pattern. The first one is the retention of the protein in the endoplasmic reticulum (eR) as eR-associated N-glycosylation leads to the generation of oligomannose-type *N*-glycans which are identical in plants and mammalians (192, 195). One side effect of this localization is the accumulation to higher levels in the eR (196, 197). A second approach for the expression of proteins with mammalian-like glycosylation patterns is the usage of glyco-engineered plants. In most cases, RNA interference (RNAi) is used for the down-regulation of endogenous beta1,2-xylosyltransferase and alpha1,3-fucosyltransferase leading to a reduction of the xylosylated and core-fucosylated *N*-glycans (198–200). A second type of glyco-engineering in plants is the coexpression of genes which facilitates the expression of human-like *N*-glycans (201) or even

the *in planta* protein sialylation by the coexpression of six mammalian genes (202). An increasing effort has been put into the adaptation of N-glycosylation, but there are also some efforts in the engineering of sialylated mucin-type *O*-glycans to achieve the most human-like glycosylation patterns (203, 204). Alternatively, non-glycosylated antibodies which mediate protection against an inhalation anthrax spore challenge in non-human primates showed an improvement of the half-life in serum (205). Rodriguez and colleagues showed that the aglycosylated form of Nimotuzumab (currently in a phase II clinical study in the USA and Canada) produced in tobacco shares the *in vitro* and *in vivo* properties as well as the antitumor effect in nude mice with the glycosylated form (206).

Transient expression of an antibody in plants can be achieved using viral vectors. The main problem with this approach is the low infectivity with these vectors. Therefore, the more efficient transfer of *A. tumefaciens* was combined with the speed and high expression rate of plant RNA viruses (207). This system has been used for the expression of monoclonal antibodies in *Nicotiana benthamiana* with yields up to 0.5 g/kg fresh weight (208).

In principle, most plantibodies are expressed in tobacco (*N. tabacum* or *N. benthamiana*), but there are also production systems in *Lemna minor* (duckweed) (209–211), rice cell culture (212), *Arabidopsis thaliana* seeds (213, 214), *Medicago sativa* (alfalfa) (215), lettuce (216), and maize (217). HIV-1 neutralizing antibody 2G12 was expressed in the endosperm of maize and showed similar or even better neutralizing properties as its CHO-derived counterpart (218).

Besides the transfection or transformation of whole plants or at least organs, monoclonal BY-2 tobacco cell lines that grow in suspension have been developed (219). Flow cytometric analysis has been used to enrich cells expressing a fluorescent marker which was located on the same T-DNA with the antibody gene. Using this method for the enrichment of high expressing cells, production could be increased up to 13-fold and was shown to be stable for 10–12 months.

Much effort has been set into the establishment and development of plants producing antibodies for therapy, but so far none of these products has appeared on the market, despite of the estimated dramatic reduction of production costs (220). Nevertheless, at least two plant derived antibodies have been used in clinical trials: CaroRX was developed by Planet Biotechnology (Hayward, CA, USA) and is expressed in transgenic tobacco (221). This antibody binds to the streptococcal antigen I/II of *S. mutans*, the major causative agent of bacterial tooth decay and prevents the attachment of *S. mutans* to tooth enamel. CaroRX has entered clinical phase II (222, 223). A second plant-made anti-idiotype antibody against non-Hodgkin-lymphoma (NHL) which was successfully tested in clinical phase I study has been mentioned above (191, 224).

TRANSGENIC ANIMALS

In recent years the idea of expressing human antibodies in transgenic animals has increased. On the one hand the humanization of antibodies for therapeutics derived from hybridoma technology is still a laborious and time-consuming procedure which often requires the generation and characterization of a set of different humanized versions of the antibody. On the other hand the mouse or rat derived antibodies may elicit an immune response in patients (225, 226). One method for the generation of fully human binders is the antibody phage display technology (21).

Beside this, several researchers developed transgenic animals for the production and expression of human monoclonal and polyclonal antibodies: therefore, human antibodies have mostly been expressed in the milk of transgenic mice (227–230), goats (231), or even in eggs of transgenic chickens (232).

The first step toward the generation of human antibodies in animals by immunization was the transfer of a human minilocus containing unrearranged immunoglobulin variable, diversity, and joining elements linked to a human μ-chain into mice (233). In this study, approximately 4% of the extracted B-lymphocytes expressed human antibodies. The immunization of larger animals containing human chromosomal immunoglobulin loci would enable the production of even larger amounts of antibodies. Therefore, transgenic cattle were developed by the transfer of a human artificial chromosome vector containing the entire unrearranged sequences of the human immunoglobulin heavy and lambda light chain loci (234). For the improvement of the human antibody proportion and for safety reasons regarding the potential risk of BSE, the bovine immunoglobulin μ heavy chain locus and the bovine prion protein have been knocked out (235, 236). Finally, transgenic cattle carrying human immunoglobulin heavy and kappa-light chain loci have been used for immunization with anthrax protective antigen. The resulting polyclonal antibody mixture consisted of entirely human and chimeric immunoglobulins that showed high activity and were protective in an *in vivo* mouse challenge models (237). Rabbits and cattle were used for expression of a bispecific scFv targeting the melanoma-associated proteoglycan and the human CD28 molecule on T cells (238). The usage of different animals as a source for the generation of human polyclonal sera has already been initiated: the immunoglobulin gene loci have been knocked out in livestock such as pigs or rabbits (239–241). For a review of approaches for the generation of transgenic animals expressing polyclonal human antibodies see Houdebine (242).

Much of the energy in transgenic production has been set on the development of humanized mice or rats. The well established property of generating hybridoma cells from these species facilitates a streamlined approach for the generation of a cell line which stably expresses monoclonal antibodies (17, 243, 244). Using humanized mice, an anti-HIV-1 gp140 antibody was identified, but in contrast the low number of antigen specific hybridomas occurring during the generation of the clones has been observed (245). Therefore, the usage of humanized rats has been suggested to circumvent these problems and first antibodies have been developed with sub-nanomolar affinities using the so called OmniRat (246).

CONCLUDING REMARKS

Today, mammalian cell lines represent the most widely used expression system for the production of recombinant antibodies. Several other hosts are being developed which are even able to produce antibodies with human-like glycosylation patterns. In addition to this, there are several applications where the glycosylation

Table 1 | Production of recombinant antibodies by host.

Host	Antigen	Antibody format (clone)	Production system	Yield	Reference
GRAM-NEGATIVE BACTERIA					
Escherichia coli	Digoxin	Fab (26–10)	Shake flask	0.8 mg/L/OD$_{600}$	Levy et al. (49)
Escherichia coli	CD18	F (ab')$_2$	Fermentor	2.5 g/L	Chen et al. (247)
Escherichia coli	Lysozyme	scFv (D1.3)	250/400 mL shake flask	0.3–1.0 mg/L	Jordan et al. (84), Monsellier and Bedouelle (248), Thie et al. (70)
Escherichia coli	CRP	scFv (LA13-IIE3)	300 mL shake flask	0.55 mg/L	Jordan et al. (83)
Escherichia coli	Lysozyme	scFab (D1.3)	300 mL shake flask	9.5 µg/L	
Escherichia coli	MUC1	VHH	100 L shake flask	10 mg/L	Rahbarizadeh et al. (249)
Escherichia coli	*Clostridium difficile* toxin A	VHH (14 different)	Shake flask?	1.2–72.3 mg/L	Hussack et al. (250)
Escherichia coli	MUC1	scFv (2 different)	250 mL shake flask	0.46/1.3 mg/L	Thie et al. (70)
Escherichia coli	p815^{HER2}	Fab	10 L fermenter	1–2 g/L	Carter et al. (251)
Escherichia coli	Atrazine	Fab (K411B)	2 L fermenter	13.8 mg/L	Wiebe et al. (66)
Escherichia coli	PPL	VL dAb	1.5 L fermenter	35–65 mg/L	Cossins et al. (252)
Escherichia coli	phOx	scFv	50 mL shake flask	16.2 mg/L	Kipriyanov et al. (69)
Escherichia coli	phOx	scFv	3 L fermenter	1.2 g/L	Sletta et al. (68)
Escherichia coli	Scorpion toxin Cn2	scFv; Fab (BCF2)	n. d.	0.3 mg/L; 1.0 mg/L	Quintero-Hernández et al. (253)
Escherichia coli	TNF alpha	scFv	Shake flask?	45 mg/L	Yang et al. (254)
Escherichia coli	HSP70	Fab (cmHsp70.1)	8 L fermenter	>15 mg/L	Friedrich et al. (64)
Escherichia coli	Tissue factor	IgG	10 L fermenter	130–150 mg/L	Simmons et al. (73)
Escherichia coli	TAG-72	Fv (B72.3)	Shake flask; fermentor	40 mg/L; 450 mg/L	King et al. (255)
Escherichia coli	VEGF	scFv::SUMO	50 mL shake flask?	50.3 mg/L	Ye et al. (256)
Escherichia coli	HIV capsid	Fab, engineered	Shake flask	12 mg/L	Nadkarni et al. (257)
Escherichia coli	Ovarian carcinoma/CD3	scFv–scFv	250 mL shake flask	1.2 g/L	Zhao et al. (71)
Escherichia coli	Fibroblast growth factor receptor FGFR1	VHH	Shake flask	10–15 mg/L	Veggiani and de Marco (50)
Escherichia coli	Human prion	scFv	Shake flask	35 mg/L	Padiolleau-Lefevre et al. (258)
Escherichia coli	Lysozyme	scFv (D1.3)	LEX bioreactor (1.5 L)	~2 mg/L	Miethe et al. (67)
Escherichia coli	MUC1	scFv (HT186-D11)	LEX bioreactor (1.5 L)	~40 mg/L	
Escherichia coli	CD30	scFv (SH313-B5)	LEX bioreactor (1.5 L)	~38 mg/L	
Escherichia coli	Crf2	scFv (MS112-IIB1)	LEX bioreactor (1.5 L)	~4.5 mg/L	
Escherichia coli	Tubulin	scFv (different ones)	Shake flask (intracellular)	up to 50 mg/L	Philibert et al. (259)
Escherichia coli	n. d.	Fab	20 L; 75 L fed-batch bioreactor	0.7 g/L; 0.5 g/L	Nesbeth et al. (260)

(Continued)

Table 1 | Continued

Host	Antigen	Antibody format (clone)	Production system	Yield	Reference
Proteus mirabilis	FAP	scFv (OS4)	50 mL shake flask	∼12 mg/L	Rippmann et al. (74)
Proteus mirabilis	Phosphorylcholine	scFv-dHLX	n. d.	10–18 mg/L	Kujau et al. (30)
Pseudomonas putidas	Lysozyme	scFv (D1.3)	200 mL shake flask	1.5 mg/L	Dammeyer et al. (75)
Pseudomonas putidas	MUC1	scFv (HT186-D11)	200 mL shake flask	3.6 mg/L	
Pseudomonas putidas	CRP	scFv (TOB5-D4)	200 mL shake flask	2.9 mg/L	
GRAM-POSITIVE BACTERIA					
Bacillus brevis	uPA	Fab	2 L shake flask	100 mg/L	Inoue et al. (76)
Bacillus megaterium	Lysozyme	scFv (D1.3)	400 mL shake flask	0.41 mg/L	Jordan et al. (84)
Bacillus megaterium	CRP	scFv (LA13-IIE3)	300 mL shake flask	0.39 mg/L	Jordan et al. (83)
Bacillus megaterium	Lysozyme	scFab (D1.3)	300 mL shake flask	3.5 μg/L	Jordan et al. (83)
Bacillus subtilis	Digoxin	scFv	n. d.	12 mg/L	Wu et al. (78)
Lactobacillus paracasei	Rotavirus	VHH	n. d.	∼1 mg/L	Pant et al. (35)
Streptomyces lividans	Lysozyme	Fv	n. d.	∼1 mg/L	Ueda et al. (261)
EUKARYOTES					
Yeast					
Yarrowia lipolytica, Kluyveromyces lactis	Ras	scFv	Shake flasks	10–20 mg/L	Swennen et al. (262)
Pichia pastoris	Muc1	VHH	Baffled flasks	10–15 mg/L	Rahbarizadeh et al. (263)
Pichia pastoris	TNFα	VHH-Fc	Shake flasks	5 mg/L	Ji et al. (264)
Pichia pastoris	AaHI	VHH	Shake flasks	17 mg/L	Ezzine et al. (265)
Pichia pastoris	B-type natriuretic peptide	scFv	Shake flasks	150 mg/L	Maeng et al. (266)
Pichia pastoris	Atrazine	Fab-HRP	Shake flasks	3–10 mg/L	Koliasnikov et al. (267)
Pichia pastoris	Muc1	Bibody, tribody	Shake flasks	12–36 mg/L	Schoonooghe et al. (268)
Saccharomyces cerevisiae	71 Different	VHH	Shake flasks	<1 to >100 mg/L	Gorlani et al. (269, 270)
Pichia pastoris	HER2	scFv	Shake flasks	15–20 mg/L	Sommaruga et al. (271)
Pichia pastoris	n. d.	scFv	n. d.	300 mg/L	Khatri et al. (272)
Pichia pastoris	Keratin 8	sc (Fv)$_2$	Baffled shake flasks	4–5 mg/L	Jafari et al. (273)
Pichia pastoris	n. d.	IgG	0.5 L bioreactor	0.5–1 g/L	Barnard et al. (274)
Pichia pastoris	Rabies virus	scFv-Fc	80 L fermenter	60 mg/L	Wang et al. (275)
Pichia pastoris	HER2	IgG	3 L bioreactor	148–227 mg/L	Chen et al. (276)

(Continued)

Table 1 | Continued

Host	Antigen	Antibody format (clone)	Production system	Yield	Reference
Filamentous fungi					
Aspergillus niger var. *awamori*	ErbB2	IgG, Fab	Shake flasks	0.9 g/L; 0.2 g/L	Ward et al. (118)
Aspergillus niger var. *awamori*	Lysozyme	scFv	7 L fermenter	108.9 mg/L	Sotiriadis et al. (277)
Aspergillus oryzae	EGFR	VHH	Shake flasks	73.8 mg/L	Okazaki et al. (123)
Protozoa					
Leishmania tarentolae	16 Different scFv	scFv	Shake flasks	0.04–3.38 mg/L	Klatt and Konthur (128)
Insect cells					
Trichoplusia ni larvae	Rotavirus	VHH	Living larvae	257 mg/L	Gómez-Sebastián et al. (278)
Spodoptera frugiperda	Blood coagulation factor VIII	scFv	Shake flasks	3.2–10 mg/L	Kurasawa et al. (279)
Drosophila, S2	Glycoprotein H	Fab	Spinner flasks	16 mg/L	Backovic et al. (280)
Drosophila, S2	HIV	IgG	n. d.	5–35 mg/L	Johansson et al. (281)
Drosophila, S2	Bovine viral diarrhea virus, hepatitis C virus	scFv	n. d.	5–12 mg/L	Gilmartin et al. (282)
Spodoptera frugiperda, SF-9	gp41	IgG	T-flasks	3 mg/L	Palmberger et al. (283)
Trichoplusia ni	gp41	IgG	T-flasks	12 mg/L	Palmberger et al. (283)
*Sf*SWT-1 Mimic	gp41	IgG	T-flasks	3 mg/L	Palmberger et al. (283)
MAMMALIAN CELLS					
Transient					
HEK293T	CD200, SIRPγ	Fab	Genejuice, roller bottles	4 mg/L	Nettleship et al. (284)
HEK293T	n. d.	IgG	HEKfectin, tissue culture plates	1–14 mg/L	Li et al. (155)
CHO	n. d.	IgG1, IgG4	Lipofectamine	140 mg/L	Codamo et al. (285)
HEK293F	n. d.	IgG	293fectin	100–400 mg/L	Van Berkel et al. (286)
CHO DG44	RhD	IgG	PEI, square-shaped bottles	90 mg/L	Wulhfard et al. (287)
HEK293E	n. d.	IgG	PEI, square-shaped bottles	200 mg/L	Backliwal et al. (288)
CHO	n. d.	IgG	PEI, square-shaped bottles	60–80 mg/L	Wulhfard et al. (289)
HEK293E	RhD	IgG	PEI, square-shaped bottles	1.1 g/L	Backliwal et al. (177)
Stable					
CHO-K1	n. d.	IgG	Lipofectamine	0.05–0.45 mg/L	Li et al. (155)
CHO	HIV-1	scFv-Fc	PEI	5.78–45.49 mg/L	Mader et al. (290)

(Continued)

Table 1 | Continued

Host	Antigen	Antibody format (clone)	Production system	Yield	Reference
CHO	n. d.	IgG	Electroporation	4 g/L	Kober et al. (291)
NS0	n. d.	IgG	5 L bioreactor, fed-batch	800 mg/L	Spens and Häggström (292)
NS0	n. d.	IgG	2–100 L bioreactor	2.64 g/L	Burky et al. (293)
per.C6	n. d.	IgG	Hollow fiber bioreactor	1 g/L	Jones et al. (147)
per.C6	n. d.	IgG	Roller bottle culture	50–100 mg/L	Jones et al. (147)
per.C6	n. d.	IgM	Shake flasks	0.5–2 g/L	Tchoudakova et al. (294)
CHO	n. d.	VHH-Fc	Shake flasks	100 mg/L	Agrawal et al. (295)
CHO	HER2	IgG	Orbital shaking bioreactor	152 mg/L	Huang et al. (296)
Transgenic plants					
Chlamydomonas reinhardtii	CD22	Immunotoxin, exotoxin A	Particle bombardement	0.2–0.4% of chloroplasts	Tran et al. (297)
Nicotiana tabacum	BoNT/A	scFv	*Agrobacterium tumefaciens*	20–40 mg/kg	Almquist et al. (298)
Nicotiana benthamiana	HIV	IgG	CPMV	105.1 mg/kg	Sainsbury et al. (299)
Nicotiana tabacum	HCC	scFv-RNase	*Agrobacterium tumefaciens*	0.75–1.99 µg/g	Cui et al. (300)
Nicotiana benthamiana	Ebola virus GP1	IgG	*Agrobacterium tumefaciens*	0.4–0.5 mg/g	Huang et al. (301)
Transgenic animals					
Mouse	HBV	IgG	Milk	17.8 mg/mL	Zhang et al. (302)
Mouse	CD147	Chimeric IgG	Milk	1.1–7.4 mg/mL	Wei et al. (303)
Mouse	Hepatitis A virus	IgG	Milk	32 mg/mL	Zhang et al. (304)
Mouse	HER2	scFv-Fc	Milk	~120 ng/mL	Yuskevich et al. (305)
Chicken	CD2, prion peptide	Chimeric IgG	Egg white	<150 µg/mL	Kamihira et al. (306)

AaHI, toxin class I of Androctonus australis hector scorpion venom; BoNT/A, Bolutinum toxin serotype A; CPMV, cowpea mosaic virus; FAP, fibroblast activation protein alpha; HCC, hepatocellular carcinoma; phOx, 2-phenyl-oxazoline-5on; PEI, polyethyleneimine; PPL, peptostreptococcal protein L; RhD, rhesus factor D; uPA, urokinase type plasminogen activator.

pattern does not play a critical role, such as for *in vitro* diagnostics or in research. Therefore, bacteria, yeasts, filamentous fungi, and insect cells can be employed in order to lower the production costs of these products. In principle, transgenic plants and animals have the highest potential for up-scaling processes to theoretically unlimited production amounts. An overview of recombinant antibodies produced in different hosts is shown in **Table 1**. There, however, it must be discriminated between the yield of functional antibodies after purification and the total yield.

Antibody phage display is now a widespread method for the development of antibody fragments such as scFv or Fab. The expression host used in this technology is *E. coli* which is known

to be the best genetically examined organism providing a large set of molecular biological tools for genetical engineering. Consequently, both antibody generation and production can be performed without changing the production system. Using high-cell density fermentation, the yield can be up to 1–2 g/L depending on the individual antibody fragment. Antibody fragments expressed in *E. coli* are mainly secreted into the periplasm and have to be extracted from there. Gram-positive bacteria lack the outer membrane and are well suited for biotechnological processes due to their powerful secretion apparatus which allow easy purification directly from the cultivation supernatant. However, antibody production systems employing Gram-positive bacteria are still in the

developmental stage. However, larger antibody formats are very difficult to express in bacteria, if they can be expressed at all. Furthermore, the lack of a glycosylation apparatus limits their use, if effector functions are needed.

Yeasts, as an eukaryotic organism, has the capacity to perform post-translational modifications. In addition, they can be used even in high throughput processes and glyco-engineering enables the expression of recombinant proteins with human-like glycosylation. Nevertheless, the production of full-size immunoglobulins remains a challenge. Compared to yeasts, filamentous fungi are more difficult for the generation of transformed clones, but they have a long tradition for the usage in biotechnology and they have partially been used for the expression of IgGs. In contrast, the development of protozoa as an expression system for recombinant proteins and antibodies has just been started and is still in a developmental stage. However, the mammalian-like glycosylation pattern presents them a promising candidate for further exploitation.

Insect cells contain a better suited protein folding and secretion apparatus than prokaryotes. Their high robustness combined with less sophisticated requirements for fermentation provide some advantages compared to mammalian cells. However, the development of stable insect cell lines and process technology is not developed as far. Consequently, mammalian cell lines are most widely used for the production of therapeutic antibodies as they provide a sophisticated folding and secretion apparatus as well as human-like glycosylation. For the production of high levels of recombinant antibodies high technical efforts are needed leading to relatively high costs. The maximum reported yield of functional IgG was 5 g/L which cannot be achieved using other expression systems so far, but up-scaling of the production does not lead to a high reduction of the production costs.

For an efficient reduction of production costs, transgenic plants can be used as they represent a highly scalable expression system; cultivation can be easily expanded without a gross increase in costs. In contrast, the generation of transgenic plants remains very complex and difficult. The most important obstacle of transgenic plants is the downstream processing as tons of plant material may have to be processed. However, antibody production in milk or eggs of animals would also be highly scalable and permits easy downstream processing. Several livestock animal species have been developed for the expression of recombinant proteins, but generation of transgenic animals also is very laborious. An interesting approach is the combination of human transgenic animals with hybridoma technology for the development of human antibodies.

In principle, there is no "universal" production system which can guarantee high yields of recombinant antibody, particularly as every antibody-based molecule itself will cause its own issues in terms of expression.

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