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The production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi

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Abstract

In this review we will focus on the current status and views concerning the production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi. We will focus on single-chain antibody fragment production (scFv and V_{HH}) by these lower eukaryotes and the possible applications of these proteins. Also the coupling of fragments to relevant enzymes or other components will be discussed. As an example of the fusion protein strategy, the 'magic bullet' approach for industrial applications, will be highlighted.

Introduction

Antibodies (also called immunoglobulins) are glycoproteins, which specifically recognise foreign molecules. These recognised foreign molecules are called antigens. When antigens invade humans or animals, an immunological response is triggered which involves the production of antibodies by B-lymphocytes. By this immunological response, microorganisms, larger parasites, viruses and bacterial toxins can be rendered harmless. The unique ability of antibodies to specifically recognise and bind with high affinity to virtually any type of antigen, made them interesting molecules for medical and scientific research.

In 1975 Köhler and Milstein developed the monoclonal antibody technology [1] by immortalising mouse cell lines that secreted only one single type of antibody with unique antigen specificity, called monoclonal antibodies (mAbs). With this technology, isolation and production of mAbs against protein, carbohydrate, nucleic acids and hapten antigens was achieved. The technology resulted in a rapid development of the use of antibodies in diagnostics (*e.g.* pregnancy tests; [2]), human therapeutics and as fundamental research tools.

More applications outside research and medicine can be considered, such as consumer applications. Examples are the use of antibodies in shampoos to prevent the formation of dandruff [3] or in toothpaste to protect against tooth decay caused by caries [4]. For these purposes large quantities of antibodies are required. However, for these applications on a larger scale there were some major problems concerning the expensive production system based on mammalian expression, the difficulty of producing antibodies in bulk amounts and the low stability and solubility of some antibodies under specific (harsh) conditions.

In this review we will discuss the possibilities of large-scale production of antibodies and fragments thereof by relevant expression systems. Requirements are that the system used for production is cheap, accessible for genetic modifications, easily scaled up for greater demands and safe for use in consumer applications.

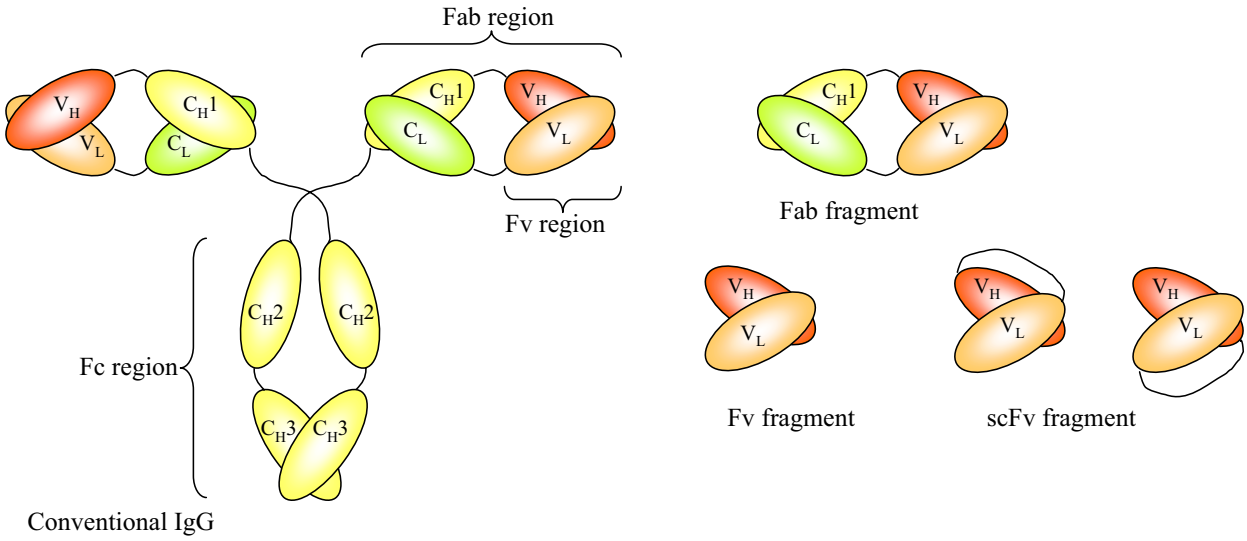

Figure 1
Schematical representation of the structure of a conventional IgG and fragments that can be generated thereof. The constant heavy-chain domains C_H1, C_H2 and C_H3 are shown in yellow, the constant light-chain domain (C_L) in green and the variable heavy-chain (V_H) or light-chain (V_L) domains in red and orange, respectively. The antigen binding domains of a conventional antibody are Fabs and Fv fragments. Fab fragments can be generated by papain digestion. Fvs are the smallest fragments with an intact antigen-binding domain. They can be generated by enzymatic approaches or expression of the relevant gene fragments (the recombinant version). In the recombinant single-chain Fv fragment, the variable domains are joined by a peptide linker. Both possible configurations of the variable domains are shown, i.e. the carboxyl terminus of V_H fused to the N-terminus of V_L and vice versa.

First, structure and characteristics of antibodies and antibody fragments generated thereof will be discussed, followed by the impact of recombinant DNA technology and antibody engineering techniques on the generation and modification of antibodies and antibody fragments. The modification of antibodies is of major interest since changes in their functionality and physico-chemical properties will broaden their application area. For most applications only the antigen-binding site of the native antibody molecule is required and even preferred. By the development of recombinant DNA technology and the increasing knowledge on the structure of antibody molecules created the opportunity to clone and engineer smaller fragments of antibody genes [5,6] and subsequent alter their functions, for example improve the affinity for their antigen. Besides that, recombinant DNA technology provides the possibility to generate fusion proteins or 'Magic bullets', consisting of an antibody fragment fused to an effector molecule.

In this review the various expression systems for these type of protein will be outlined. We will detail on using yeasts and filamentous fungi as suitable expression systems for antibody fragments and antibody fusion proteins.

Antibodies and their unique antigen binding domains

Whole antibodies

In vertebrates five immunoglobulin classes are described (IgG, IgM, IgA, IgD and IgE), which differ in their function in the immune system. IgGs are the most abundant immunoglobulins in the blood and these molecules have a molecular weight of approximately 160 kDa. They have a basic structure of two identical heavy (H) chain polypeptides and two identical light (L) chain polypeptides (Figure 1). The H and L chains, which are all β-barrels, are kept together by disulfide bridges and non-covalent bonds (for a review about antibody structure see [7]). The chains themselves can be divided in variable and constant domains. The variable domains of the heavy and light chain (V_H and V_L) which are extremely variable in amino acid sequences are located at the N-terminal part of the antibody molecule. V_H and V_L together form the unique antigen-recognition site. The amino acid sequences of the

remaining C-terminal domains are much less variable and are called C_H1, C_H2, C_H3 and C_L.

Fc fragment

The non-antigen binding part of an antibody molecule, the constant domain Fc mediates several immunological functions, such as binding to receptors on target cells and complement fixation (triggering effector functions that eliminate the antigen). The Fc domain is not essential for most biotechnical applications, relying on antigen binding. The Fc fragment, which is glycosylated, can have different effector functions in the different classes of immunoglobulins.

Antigen binding region

The unique antigen-binding site of an antibody consists of the heavy and light chain variable domains (V_H and V_L). Each domain contains four conserved framework regions (FR) and three regions called CDRs (complementarity determining regions) or hypervariable regions. The CDRs strongly vary in sequence and determine the specificity of the antibody. V_L and V_H domains together form a binding site, which binds a specific antigen.

Antibody fragments generated thereof

Several functional antigen-binding antibody fragments could be engineered by proteolysis of antibodies (papain digestion, pepsin digestions or other enzymatic approaches), yielding Fab, Fv or single domains (Figure 1).

Fab fragments

Fab fragments (fragment antigen binding) are the antigen-binding domains of an antibody molecule, containing V_H + C_H1 and C_L + V_L. Between C_L and C_H1 an interchain disulfide bond is present. The molecular weight of the heterodimer is usually around 50 kDa [8]. Fab fragments can be prepared by papain digestions of whole antibodies.

Fv fragments

The minimal fragment (~30 kDa) that still contains the whole antigen-binding site of a whole IgG antibody is composed of both the variable heavy chain (V_H) and variable light chain (V_L) domains. This heterodimer, called Fv fragment (for fragment variable) is still capable of binding the antigen [9]. Normally, native Fv fragments are unstable since the non-covalently associated V_L and V_H domains tend to dissociate from one another at low protein concentrations.

Single domains

Single domain antigen binding fragments (dAbs) or V_Hs were generated in the past [10,11]. They have good antigen-binding affinities, but exposure of the hydrophobic surface of the V_H to the solvent, which normally interacts with the V_L, causes a sticky behaviour of the isolated V_Hs. It turned out to be difficult to produce them in soluble form, although replacement of certain amino acids increased solubility of these single domains (see also **Llama Heavy-chain antibody fragments**) Besides that, their affinity for the antigen was much less compared with other antibody fragments [12].

***Heavy-chain antibodies in* Camelidae**

In 1993 Hamers-Casterman *et al.* [13] discovered a novel class of IgG antibodies in *Camelidae* (camels, dromedaries and llamas). These antibodies are devoid of light chains and therefore called 'heavy-chain' IgGs or HCAb (for heavy-chain antibody; Figure 2). HCAbs have a molecular weight of ~95 kDa instead of the ~160 kDa for conventional IgG antibodies. Their binding domains consist only of the heavy-chain variable domains, referred to as V_{HH}s [14] to distinguish it from conventional V_Hs. Since the first constant domain (C_H1) is absent (spliced out during mRNA processing due to loss of a splice consensus signal; [15,16]), the variable domain (V_{HH}) is immediately followed by the hinge region, the C_H2 and the C_H3 domains. Although the HCAbs are devoid of light chains, they have an authentic antigen-binding repertoire. The current knowledge about the genetic generation mechanism of HCAbs is reviewed by Nguyen *et al.* [17,18].

Recombinant antibodies, antibody fragments and antibody fusion proteins

The development and applications of recombinant DNA technology led to the design of several new antibodies and antibody fragments. Firstly, functionalities of these proteins may be altered resulting in novel and improved functions. One of the possible applications of recombinant whole antibodies is the use in human therapeutics (see also **Recombinant whole antibodies**). Secondly, smaller antibody fragments may be synthesised having the advantage over whole antibodies in applications requiring tissue penetration and rapid clearance from the blood or kidney. Moreover, the use of recombinant expression systems could also be the solution for large-scale production of antibody (fragments).

Recombinant whole antibodies

The development of human(ised) antibody molecules is mostly aimed at reduction of unwanted immunological properties in medical applications [19]. Repeated doses of foreign (murine) antibody molecules could lead to an immune response in patients recognising the mouse antibody as foreign. This so-called HAMA (human anti-mouse antibody) response can lead to severe health problems.

Two strategies are developed to reduce the antigenicity of therapeutic antibodies (see also [20]). One of these strategies is chimerisation. In this case the constant murine do-

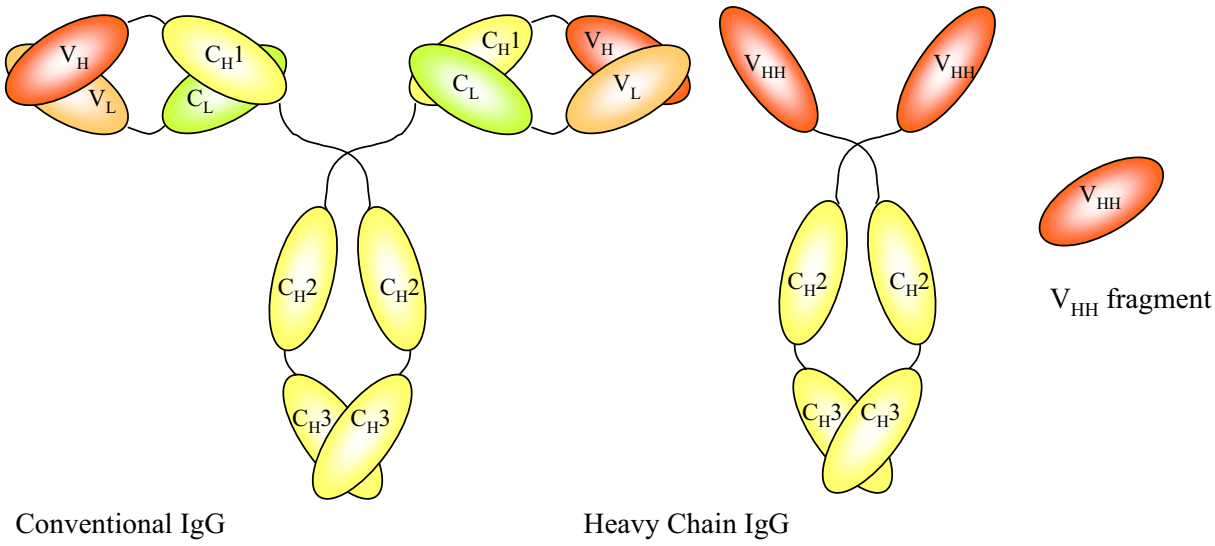

Figure 2
Schematical representation of the structure of a conventional IgG, a heavy-chain IgG antibody and the variable heavy-chain antibody fragment (V_{HH}) that can be generated of the latter. Heavy-chain antibodies found in llama and camel are only composed of heavy-chains and lack the light chain completely, as shown in this Figure. The antigen-binding domain consists of only the V_H domain, which is referred to as V_{HH} (variable heavy-chain antibody fragment), to distinguish it from a normal V_H. The constant heavy-chain domains C_H1, C_H2 and C_H3 are shown in yellow, the constant light-chain domain (C_L) in green and the variable heavy-chain (V_H or V_{HH}) or light-chain (V_L) domains in red and orange, respectively.

mains are replaced by human constant domains [21,22]. The second strategy is grafting of only the murine CDRs onto existing human antibody framework regions, which is called humanisation [22].

At present there are more than 10 recombinant antibodies approved by the US Food and Drug Administration (FDA) for use in medicine and many more are in a late stage of clinical trials. FDA approved recombinant mAbs are *e.g.* Herceptin™ (Genetech, San Francisco, CA), which targets and blocks the growth factor Her2 on the surface of breast cancer cells and Rituxan™ (IDEC Pharmaceuticals Inc., San Diego, CA) used against non-Hodgkin's lymphoma (see for more examples [23,24]). The use of recombinant antibodies for medical purposes does not require a cheap large-scale production process *per se*, since only a limited amount of pure preparations is needed.

Production of recombinant antibody fragments by Escherichia coli

Much work on antibody fragment production has been focussed on *Escherichia coli* as an expression system (reviewed in [25]). The advantage of this system is the ability to produce proteins in relative large amounts. Besides that, *E. coli* is easily accessible for genetic modifications, requires simple inexpensive media for rapid growth and they can easily be cultured in fermentors permitting large-scale production of proteins of interest. Several antibody fragments have been produced in functional form (*e.g.* [8,9,26,27]) and expression of relevant gene segments also permitted the production of the recombinant antibody fragments. The problem of stability has been tackled by generation of single-chain Fv (scFv) or disulfide stabilised Fv (dsFv) fragments.

Selection of antibody fragments with improved functionalities

In 1990 McCafferty *et al*. [28] showed that antibody fragments could be displayed on the surface of filamentous

phages, called phage-display. This technology is based on the fusion of the antibody variable genes to a phage coat protein gene (*e.g.* [20]). After displaying an antibody fragment on the protein surface of the phage, antigen specific phages can be selected and enriched by multiple rounds of affinity panning (*e.g.* reviewed in [29,30]). This technique makes it possible to select phages that bind almost any antigen, including those previously considered to be difficult, such as self-antigens or cell surface proteins.

Libraries can be prepared from variable genes isolated from immunised animals, non-immunised sources (naïve libraries, thus avoiding the need for immunisation) or even (semi-) synthetic libraries can be constructed. The V genes can be subjected to random mutagenesis, chain or DNA shuffling methods [31], mimicking the natural hypermutation mechanism.

Single-chain Fv fragments and multimers

An attractive recombinant antibody fragment is the single-chain Fv (scFv) fragment (reviewed in [32,33]). It has a high affinity for its antigen and can be expressed in a variety of hosts [34]. These and other properties make scFv fragments not only applicable in medicine (reviewed in [35]), but also of potential for biotechnological applications. In the scFv fragment the V_H and V_L domains are joined with a hydrophilic and flexible peptide linker, which improves expression and folding efficiency [36,37]. Usually linkers of about 15 amino acids are used, of which the $(Gly_4Ser)_3$ linker has been used most frequently [35]. Unfortunately, some scFv molecules have a reduced affinity compared to the parental whole antibody or Fab molecule [12,38,39]. Besides that, scFv molecules can be easily proteolytically degraded, depending on the linker used [40]. With the development of genetic engineering techniques these limitations could be practically overcome by research focussed on improvement of function and stability, as discussed in [32]. An example is the generation of disulfide-stabilised Fv fragments where the V_H-V_L dimer is stabilised by an interchain disulfide bond [38,41,42]. Cysteïnes are introduced at the interface between the V_L and V_H domains, forming a disulfide bridge, which holds the two domains together (reviewed in [43]).

Dissociation of scFvs results in monomeric scFvs, which can be complexed into dimers (diabodies), trimers (triabodies) or larger aggregates ([44], reviewed in [45]). The simplest designs are diabodies that have two functional antigen-binding domains that can be either similar (bivalent diabodies) or have specificity for distinct antigens (bispecific diabodies). These bispecific antibodies allow for example the recruitment of novel effector functions (such as cytotoxic T cells) to the target cells, which make them very useful for applications in medicine (reviewed in [46,47]).

Llama Heavy-chain antibody fragments (V_{HH}s)

The other type of interesting antibody fragments are V_{HH}s (see Figure 2) comprising the smallest available intact antigen-binding fragment (~15 kDa, 118–136 residues [48,49]). The affinities found for V_{HH}s were in the nanomolecular range and comparable with those of Fab and single chain Fv (scFv) fragments [50,51]. Besides that V_{HH}s are highly soluble and more stable than the corresponding derivatives of scFv and Fab fragments [50,52]. V_{HH}s carry amino acid substitutions that make them more hydrophilic and prevent the prolonged interaction with BiP (Immunoglobulin heavy-chain binding protein), which normally binds to the H-chain in the Endoplasmic Reticulum (ER) during folding and assembly, until it is displaced by the L-chain [53]. There are indications that this increased hydrophilicity improves secretion of the V_{HH}s from the ER. Hence, production of V_{HH}s in commercially attractive microorganisms may be favourable.

Several ways are described to obtain functional V_{HH}s: from proteolysed HCAb of an immunised camelid, direct cloning of V_{HH} genes from B-cells of an immunised camelid resulting in recombinant V_{HH}s or from naïve or synthetic libraries [49]. V_{HH}s with desired antigen specificity could be selected by phage display (see **Selection of antibody fragments with improved functionalities**). Using V_{HH}s in phage display is much simpler and more efficient as compared with Fabs or scFvs, since only one domain needs to be cloned and expressed to obtain a functional antigen-binding fragment [52,54].

As already noted before (see **Antibody fragments generated thereof**), classical V_Hs were difficult to produce in soluble form. To improve their solubility and prevent non-specific binding, residues located on the V_L side of V_Hs were replaced by 'V_{HH}-like' residues, mimicking the more soluble V_{HH} fragments. This process has been termed camelisation [55–57] and these camelised V_H fragments, particularly those based on the human framework, are expected to have significant advantages for therapeutical purposes in humans (reviewed in [58]).

Fusion proteins ('Magic bullets')

A completely new use of the binding capacity of antibody fragments is the design of a fusion approach, in which an effector protein is coupled to an antigen recognising antibody fragment. In human medicine this approach is referred to 'Magic bullet'. All kinds of molecules can be used as effector molecule only limited by the imagination. The gene encoding the effector may be directly fused to the gene of the antibody fragment of interest, resulting in novel bifunctional proteins [59]. Examples of the use of this approach will be given in the section **Antibody fragments and antibody fusion proteins for large-scale applications and consumer products**.

Table 1: Extracellular production of antibody fragments in prokaryotic expression systems.

Strains	Characteristics	Food grade	Production yields	References
Lactobacillus zeae	Gram$^+$	yes	scFv, ND* (secreted/cell-bound)	[91]
Bacillus subtilis	Gram$^+$	yes	10 to 15 mg/L scFv (secreted)	[143,144]
Streptomyces lividans	Gram$^+$	yes	1 mg/L Fv fragment (secreted)	[145,146]
Staphylococcus carnosus	Gram$^+$	yes	5–10 mg/L V_H domain (secreted)	[147]
Proteus mirabilis	Gram$^-$	no	40 to 200 mg/L scFv (secreted)	[148,149]
Escherichia coli	Gram$^-$	no	Several fragments (inclusion bodies/periplasmic space)	[25,34]

*ND = not determined

Applications of antibody fragments and antibody fusion proteins

Applications of antibody fragments in human medicine

The smaller the better

Most applications of recombinant antibody fragments are related to diagnosis and therapy in human medicine, which is especially focussed on the use of antibodies as the ideal cancer-targeting reagent (reviewed in [19,60–62]). For some clinical applications small antibody fragments have advantages over whole antibodies. The small size permits them to penetrate tissues and solid tumours more rapidly than whole antibodies [63] which recently also was shown for V_{HH}s [64]. Smaller antibody fragments have also a much faster clearance rate in the blood circulation, which leads to differences of selectivity [63]. Nowadays there are also promising pre-clinical and clinical trials with antibody fragments as diagnostic or therapeutical agents [61,65]. Another application of antibody fragments is to treat viral infections with so-called intrabodies, which are intracellular antibodies synthesised by the cell and targeted to inactivate specific proteins within the cell [66].

'Magic bullets' in medicine

The use of bi-functional molecules in medicine is aimed at delivery of a protein drug, which is only active where it is required. It thereby limits the dose of the drug, resulting in less side effects of the drug towards healthy tissue and/or less immunogenic response to the protein drug itself. Also the physical interaction between the target and the effector molecule increases the potency of the effector. Fusion proteins are ideal immuno agents for cancer diagnosis [67] and cancer therapeutics. An example is the use of cancer-specific bi-functional antibodies targeting potent cytotoxic molecules to tumour cells and subsequently eliminate these tumour cells without harming healthy cells [68].

Potential applications of V_{HH}s

Specific applications of V_{HH}s are foreseen in the following direction:

V_{HH}s as drug carriers

It is expected that V_{HH}s are also applicable in diagnosis and therapy in human medicine, especially when an economically feasible production, small size and stability are required (reviewed in [49]). Cortez-Retamozo et al. [64] recently showed that V_{HH}s specifically could be targeted to tumour cells, which together with the possibility of generation of bispecific V_{HH} constructs [69] is of major interest for cancer therapy.

V_{HH}s as delivery carriers in the brain

Antibodies and many other water soluble compounds are excluded from the brain by the blood-brain barrier (BBB), thus making treatment of brain-related disease very difficult. Recently, Muruganandam *et al*. [70] showed that V_{HH} were able to selectively bind to and transmigrate across the BBB in a human *in vitro* BBB model and partly *in vivo* in mice. This property can be exploited for the development of efficient antibody carriers suitable for delivery of macromolecules across the human BBB and subsequently for treatment of neurological diseases.

V_{HH}s as potent enzyme inhibitors

Hypervariable regions in V_{HH}s are on average longer than those of V_Hs [71,72]. The extended hypervariable regions of V_{HH}s are capable of penetrating deep into the cleft of active sites of enzymes, binding to novel epitopes that are not recognised by conventional antibodies [51,73,74]. Because of this property V_{HH}s may act as better potent enzyme inhibitors [51,75,76].

V_{HH}s in consumer products

Since llama V_{HH}s are very stable, even at high temperature, applications can be envisaged in which a high temperature step is involved (e.g. pasteurisation), without losing antigen-binding properties [50]. Recently it was shown that V_{HH}s could be used to prevent phage infection in cheese production processes [77], by recognising a structural protein of the phage, which is involved in recognition of the host Lactococcus lactis.

Table 2: Higher eukaryotic expression systems for heterologous protein production and possible advantages and disadvantages of the expression system.

Expression systems	Ease of molecular cloning	upscaling	Economic feasibility[1]	Pathogenic contaminants[2]	References[3]
Mammalian cells	+	+/-	+	+	[59,150–154]
Insect cells	++	+	+	+	[155–160]
Plants	++	+++	++	++	[92,93,161,162]
Transgenic animals*	+/-	+++	+/-	+/-	[163–166]
Yeasts	+++	+++	+++	+++	See references in section **Production of antibody fragments by lower eukaryotes**.
Filamentous fungi	+++	+++	+++	+++	[4,123,128]

+++ = excellent, ++ = good, + = sufficient, +/- = poor. * With transgenic animals in this context is mentioned the production of antibodies or antibody fragments in the milk of transgenic animals, for example rabbits, sheep, goats or cows [1] With economical feasibility is mentioned the time and cost of molecular cloning, upscaling and downstream processing (purification). [2] Pathogenic contaminants like viruses or pyrogens. [3] Articles dealing with production of antibodies, antibody fragments and antibody fusion proteins.

Antibody fragments and antibody fusion proteins for large-scale applications and consumer products

Many additional applications can be envisaged if an inexpensive and simple production system is available, yielding large amounts of antibody fragments that can be purified easily. The highly specific antigen-binding ability could be used for inactivating bacteria or specific enzymes that can cause spoilage of food. Other suggested applications are the use in biosensors, treatment of wastewater [78], industrial scale separation processes such as separation of chiral molecules [79], purification of specific components (proteins) from biological materials or the use as abzymes [80,81]. They have also been considered as components of novel consumer goods with new improved functionalities, in oral care and personal hygiene (*e.g.* in toothpaste or mouthwashes [82]). For dental applications antibody fragments can be coupled to enzymes to increase the concentration of antimicrobials like hypothiocyanate and hypohalites, for example glucose oxidase (GOX; [83]), galactose oxidase (GaOX; [84]) or lactate oxidase (LOX; [85]). Other examples are targeted bleach in laundry washing (*e.g.* detergents containing antibodies coupled to molecules that specifically remove difficult stains) or the use in shampoos where antibodies act to prevent dandruff by inhibiting growth of specific microorganisms causing this [3].

Suitable expression systems for the large-scale production of antibody fragments and antibody fusion proteins

To be able to use antibody fragments and antibody fusion proteins in these large scale applications, a suitable expression system has to be chosen. Several expression systems are available, both from prokaryotic (Table 1) and eukaryotic (Table 2) origin. Our main interest goes out to these systems that are able to economically produce large amount of proteins into the culture medium. Several of these systems can be considered as suitable (both from prokaryotic and eukaryotic origin). Hereafter several of these systems will be discussed, with an emphasis on yeast and fungal systems.

Drawbacks using E. coli as a host for antibody fragment production

As described in the section **Production of recombinant antibody fragments by *Escherichia coli***, this micro-organism has shown to be a potential expression host for antibody fragments and fusion proteins. Although the general production yields in shake-flask cultures are low (several mg/L), in fermentation processes several g/L could be obtained (reviewed in [86]). There are two possibilities of antibody fragment production in *E. coli*, either by secretion of the fragments into the culture medium and/or periplasmic space (the compartment between the inner and outer membrane) or preparation of inclusion bodies with subsequent *in vitro* folding. However, both strategies have disadvantages that make the use of this prokaryote not attractive for the large-scale production of antibody fragments and antibody fusion proteins. Firstly, the secretion of folded and fully assembled fragments in the medium or periplasmic space is often accompanied with cell lysis and subsequent product loss. Secondly, 'toxicity' of the antibody sequence and concomitant plasmid loss is frequently observed, which hamper high production levels (reviewed in [25]). Thirdly, expression of the fragments in inclusion bodies, which often results in insoluble protein aggregates [87], demands laborious and cost-intensive *in vitro* refolding (denaturation and renaturation) and purification steps. Hence, the final yield of fragments is only a small percentage of the protein that was initially present in the inclusion bodies even though purification steps are nowadays facilitated by affinity

chromatography using C-terminal polypeptide tails, like poly-His_6 or FLAG [88,89]. Recently, production of soluble and functional scFv by *E. coli* could be increased by improving disulfide bond formation activity in the cytoplasm, using mutants and overexpression of disulfide-bond isomerase [90]. Finally, *E. coli* is unable to carry out eukaryotic post-translational modifications and is therefore not suitable when glycosylation of antibody fragments or more importantly the fusion proteins is required.

Alternative prokaryotic expression systems

E. coli is not the only available prokaryotic expression system, although it is rather dominant in the field. Alternative prokaryotic expression systems are available for antibody fragment production (Table 1). However, these will encounter similar limitations as *E. coli*, even though most organisms described in Table 1 secrete the investigated antibody fragment into the culture medium. A field where production of antibody fragments in prokaryotic cells could still be interesting, is in food grade organisms used for delivery passive immunisation in humans, by means of functional foods. In a recent article, Kruger *et al*. [91] reported the production of scFv antibody fragments against *Streptococcus mutans* by the Gram positive food grade bacteria *Lactobacillus zeae*. In experimental animals a decrease of *S. mutans* and reduced development of caries was observed.

Eukaryotic expression systems

Also several eukaryotic systems can be envisaged for large-scale production of antibody fragments and antibody fusion proteins (see also [34]), like mammalian cells, insect cells, plants, transgenic animals and lower eukaryotes (see Table 2).

The production of therapeutical whole antibodies is well established in mammalian cells. However, large-scale production is expensive and time-consuming.

'Plantibodies' can be produced in several plant target organs (reviewed in [92]). Roots, storage organs (seeds and tubers) and fruiting bodies can be suitable for mass oral (edible) applications (see [93] and references therein). Expression of scFv in transgenic plants has been proposed as a way to produce and store pharmaceutical antibodies [94,95] and as means to block physiological processes in the plant itself [96] or establish plant pathogen resistance [97]. Plants show several advantages as large-scale antibody production systems, like the ease and low costs of growing plants, even in large quantities. However, the generation of transgenic plants that express antibodies is a time consuming process and the downstream processing to isolate the expressed antibodies from the plant parts is relatively expensive and laborious.

Production of antibody fragments by lower eukaryotes

An attractive possibility for the cost-effective large-scale production of antibody fragments and antibody fusion proteins are yeast or fungal fermentations. Large-scale fermentation of these organisms is an established technology already used for bulk production of several other recombinant proteins and extensive knowledge is available on downstream processes. Besides that, yeasts and filamentous fungi are accessible for genetic modifications and the protein of interest may be secreted into the culture medium. In addition, some of their products have the so-called GRAS (Generally Regarded As Safe) status and they do not harbour pyrogens, toxins or viral inclusions.

Methylotrophic and other yeasts

The methylotrophic and other yeasts like *Candida boidinii*, *Hansenula polymorpha*, *Pichia methanolica* and *Pichia pastoris* are well known systems for the production of heterologous proteins (reviewed in [98]). High levels of heterologous proteins (milligram-to-gram quantities) can be obtained and scaling up to fermentation for industrial applications is possible [99–101].

Especially the *P. pastoris* system is used in several industrial-scale production processes [102]. Ridder *et al.* [103] were the first to report the expression of a scFv fragment by *P. pastoris*. From then on several papers reported about the use of *P. pastoris* for the production of recombinant antibodies and fragments thereof [104,105]. In shake-flask cultures a level of 250 mg/L scFv was obtained [106] and Freyre *et al.* [107] were able to obtain even an expression level of 1.2 g/L scFv fragment under fermentation conditions. However, Cupit *et al*. [108] also showed that the production of antibody fragments by *P. pastoris* is not always a success story.

Based on the described results the commercial recombinant antibody production by *P. pastoris* is promising. However, products currently obtained from *P. pastoris* are not regarded as GRAS, which may limit its use.

Wood *et al.* [109] were the first to report the production of mouse IgM by the baker's yeast *S. cerevisiae*, although only unassembled chains were detected in the culture medium. However, the production of Fab fragments was possible as was first shown by Horwitz *et al.* [110]. Although the obtained levels were low, functional Fab fragments were secreted in the culture medium. Davis *et al.* [111] expressed scFv antibody fragments in *Schizosaccharomyces pombe.* Studies on the scFv production in the non conventional yeasts *Yarrowia lipolytica* and *Kluyveromyces lactis* resulted in 10–20 mg/L functional and soluble anti-Ras scFv [112].

Filamentous fungi: Trichoderma reesei *and* Aspergillus spp

Filamentous fungi, in particular species from the genera *Trichoderma* and *Aspergillus* have the capacity to secrete large amounts of proteins, metabolites and organic acids into their culture medium. This property has been widely exploited by the food and beverage industries where compounds secreted by these filamentous fungal species have been used for decades. This has led to the GRAS status for some of their products. Filamentous fungi like *A. awamori*, *A. niger* and *A. oryzae* are therefore suitable organisms for the production of commercially interesting homologous and heterologous proteins [113–115]. Strategies to improve protein secretion by filamentous fungi are extensively reviewed in [116–119].

Production strains of *Trichoderma reesei* (*Hypocrea jecorina*) have an exceptional secretion capacity up to 35 g protein/L, where half of the secreted protein consists of the cellulase cellobiohydrolase I (CBH1; [120]). Therefore, *Trichoderma* is considered as an excellent host for the production of heterologous proteins (reviewed in [121,122]). Nyyssönen *et al*. [123] reported a production of 1 mg/L in shake-flasks of Fab antibody fragments by *T. Reesei* Rut-C30. More strikingly, when the Fab antibody fragment chain was fused to the core-linker region of CBH1, a production level of 40 mg/L in shake-flasks and 150 mg/L in bioreactor cultivations was obtained [123,124].

The use of *S. cerevisiae* and *A. awamori* for the large-scale production of antibody fragments and fusion proteins

In our own laboratory at TNO Nutrition and Food Research in Zeist (The Netherlands) and in collaboration with Unilever Research Vlaardingen (The Netherlands) research on antibody fragment production in *S. cerevisiae* and *A. awamori* has been carried out [125,126]. The aim of this project was a detailed comparison of both expression systems, in relation to their possible large-scale production process of antibody fragments and fusion proteins. In the framework of this collaboration also a new *A. awamori* expression system, based on xylose induction was developed [127].

The use of S. cerevisiae *and* A. awamori *for the large-scale production of scFv*

To investigate the feasibility of a large-scale cost-effective process for the extracellular production of (functionalised) scFv fragments initially *S. cerevisiae* was used. However, it was shown that *S. cerevisiae* was a poor host for the production of scFv, since the secretion of scFv was hampered by improper folding of the fragments, because large aggregates were formed in the ER and vacuolar-like organelles. It was hypothesised that the exposure of the hydrophobic surfaces on the V_L and V_H chains of scFv plays an important role in the accumulation of scFv in the cell [128]. Shusta *et al.* [129] reported the increase of scFv production up to 20 mg/L in *S. cerevisiae* by optimising the expression system by overexpression of two ER resident chaperones and reduction of growth temperature. Kauffman *et al.* [130] showed that overexpression of scFv in *S. cerevisiae* resulted in cellular stress, displayed by decreased growth rates and induction of the Unfolded Protein Response (UPR). It was hypothesised that a functional UPR was required to decrease the malfolded scFv in the ER, leading to a recovery from cell stress.

As further improved levels were desired also a fungal expression system was considered [4]. In shake-flask cultures a production level of 10 mg/L was achieved by using *A. awamori* as production host. As secretion of a heterologous protein can be greatly enhanced by fusing it to a "carrier" protein such as glucoamylase (GLA; [117,118]), also this fusion-approach was employed. Analysis of the culture medium of transformants carrying the fusion construct revealed a production of approximately 50 mg/L scFv in the culture medium [4]. Several commercially interesting scFv fragments were investigated for their ability to be produced by *A. awamori* using the GLA-fusion strategy. The results showed that the production levels differed significantly between the different scFv transformants. Interestingly, in some cases increased levels of scFv detected in the culture medium corresponded to an increase of transcription level of the ER chaperone BiPA [131], indicating that the antibody fragments, like in *S. cerevisiae*, may have problems with correct folding and aggregate in the fungal cell.

To increase production levels, successful 10 L and 1,5 × 10^4 L scale fermentations were carried out resulting in 200 mg/L scFv under optimal conditions. However, variable amounts of scFv dimers and other multimers were observed. Recent fermentation experiments performed by Sotiriadis *et al.* [132] showed that the highest scFv level was observed when induction was started in the late exponential phase. An increase of the carbon and nitrogen source concentrations and a decreased of the concentration of the inducer, resulted in increased product yields.

Production of Llama V_{HH} antibody fragments by S. cerevisiae *and* A. awamori

Although the production of scFv fragments by *S. cerevisiae* and *A. awamori* was successful, levels up to several g/L were not achieved. Possibly the hydrophobic regions of the scFv, responsible for keeping the variable regions of the heavy and light chains together, could also interact with other molecules in the cell. Aggregation of scFv in *S. cerevisiae* may result in accumulation and subsequent degradation (of a part) of the antibody fragment molecules [128] as frequently observed when expressing heterolo-

gous proteins that exhibit hydrophobic surfaces [133]. Interestingly, antibody fragments devoid of these hydrophobic surfaces could be obtained from camels, dromedaries and llamas (V_{HH}s, see **Llama Heavy-chain antibody fragments (V_{HH}s)** and [13]), providing an option to improve production levels in relevant microorganisms [126].

V_{HH}s could be produced in *E. coli* up to levels of 6 mg/L, were found to be extremely stable, highly soluble and reacted specifically and with high affinity with antigens [52]. V_{HH}s were produced in *S. cerevisiae* at levels over 100 mg/L in shake-flask cultures [134], although considerable amounts of V_{HH}s were detected intracellularly. From a 1,5 × 10^4 L fed-batch fermentation, 1.3 kg of V_{HH}s was obtained, which clearly showed that these fragments could be produced in this host more efficiently than scFv fragments [135]. For a cost-effective large-scale process for the production of V_{HH}s in *S. cerevisiae* further improvement is required. Van der Linden *et al.* [54] showed that production of V_{HH}s by *S. cerevisiae* could be improved by DNA shuffling techniques, in which three homologous V_{HH} genes were randomly fragmentated and reassembled subsequently.

Based on the fact that *A. awamori* performed superior for scFv also the possibility of V_{HH} production by *A. awamori* was investigated. As a model V_{HH}s against the hapten RR6 were chosen [134]. Gene fragments coding for anti-RR6 V_{HH}s were cloned in an expression vector containing the highly inducible endoxylanase promoter. Recent experiments (Joosten *et al*. submitted) showed that functional V_{HH}s could be produced in the culture medium in shake-flask cultures, albeit at relatively low levels. For further optimisation a carrier strategy and controlled fermentations will be carried out.

Production of 'Magic bullets' by A. awamori

A major research interest is the production of fusion proteins or 'Magic bullets', consisting of an antibody fragment (scFv or V_{HH} fragment) fused to an enzyme of interest. In our laboratory research has been carried out with a few examples of scFv fragments coupled to glucose oxidase (GOX). GOX is already for many years an interesting enzyme for coupling to antibodies for killing cells [136]. A scFv, which recognises for example oral *Streptomycetes*, when fused to GOX, which is an antimicrobial enzyme, may kill bacteria by generation of the bactericidal hydrogen peroxide. In activity assays it was shown that the fusion protein produced by *A. awamori* was functional, both in binding to the antigen and GOX activity [4].

In the detergent industry enzymatic bleaching may be a good alternative to the current chemical bleaching used. To make these laundry-cleaning products more effective, the production of Magic bullets by filamentous fungi or yeasts is of interest. An enzyme coupled to an antibody fragment recognising persistent stains from e.g. azo-dyes [134] results in a more directed bleaching process, resulting in lower amounts of required detergent, reduction of harmful effects of the enzyme to the textile and lower environmental burden (see Figure 3).

Currently we are investigating the feasibility of production of V_{HH}-enzyme fusions by *A. awamori*. One of the V_{HH}s used is a model llama V_{HH}, recognising the azo-dye Reactive Red 6 (RR6 [134]). As a bleaching enzyme, the *Arthromyces ramosus* peroxidase (ARP) [137,138] was genetically linked to the V_{HH} fragment. This peroxidase utilises hydrogen peroxide to catalyse the oxidation of a wide range of organic and inorganic compounds, which makes the enzyme suitable for use in bleaching processes [139]. ARP alone could be produced in high amounts by *A. awamori* (800 mg/L; Lokman *et al.* submitted). Preliminary results showed the feasibility of fusion protein production by *A. awamori*, yielding high levels of ARP-V_{HH} fusion protein in controlled fermentation experiments (Joosten *et al.* manuscript in preparation). The fusion protein showed both ARP activity and azo-dye binding activity.

In future experiments V_{HH}s fragments can be replaced by other more relevant antibody fragments, for example those binding tomato or blood spots. Also the peroxidase part of the fusion can be further optimised.

Conclusions and future prospects

Recent developments in the fields of antibody engineering and expression systems have enabled the engineering and production of antibodies and antibody fragments for a wide variety of applications. A lot of examples are already mentioned, but presumably more applications can be envisaged. The development of the 'Magic bullet' approach will even increase the interest in antibodies and their related products, also for applications in human medicine. A recently envisioned application that is of much interest, is the use of antibody fragments in micro-arrays. Antibody arrays can be used for proteomic analysis by comparing the differences in presence of proteins in healthy and diseased cells. For this purpose antibody fragments derived from large phage-antibody libraries can be used as probes to capture proteins on chips in a high-throughput system (reviewed in [140,141]). In this respect, V_{HH}s fragments are of great interest, due to their simple and stable structure.

In this review we evaluated whether the yeast *S. cerevisiae* and the filamentous fungus *A. awamori* are suitable expression systems for the large-scale production of antibody fragments and antibody fusion proteins. Although

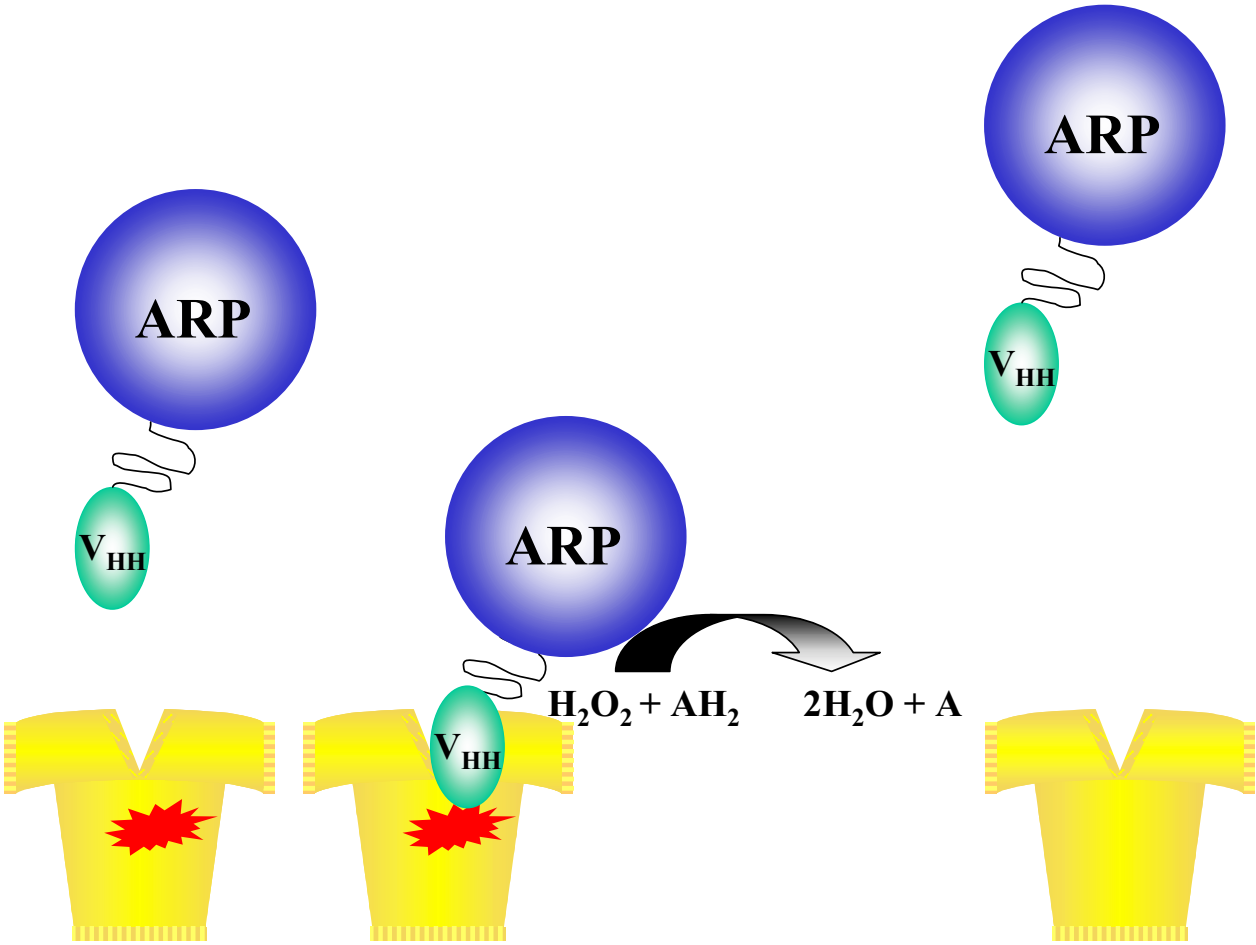

Figure 3
Schematical example of the 'Magic bullet' approach in consumer applications, where an antibody fragment (in this case a llama variable heavy-chain antibody fragment; V_{HH}) recognising a spot on textile, is coupled to an effector molecule (in this case *A. ramosus* peroxidase; ARP). ARP is a peroxidase, which utilises hydrogen peroxide to catalyse the oxidation of a wide range of organic and inorganic compounds, which makes the enzyme suitable for use in bleaching processes [139].

A. awamori is not the best expression system for the production single antibody domains (scFv and V_{HH} fragments), in particular for the production of antibody fusion proteins filamentous fungi offer significant potential. In particular in those cases where specific post-translational modification (*e.g.* N-glycosylation) is required for functional expression of the effector protein (also in relation to pharmaceutical applications). In contrast to *S. cerevisiae*, filamentous fungi do not show extensive hyperglycosylation [142].

Both the scFv-GOX as well as results from ARP-V_{HH} fusion proteins showed that the filamentous fungal system is a promising candidate for the production of antibody fusion proteins. In the future production of other fusion proteins can be investigated in this or other fungal expression systems, allowing a potential breakthrough for antibody technology in producing large amounts of specific recognition units coupled to effector molecules for consumer applications.

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