Research Article

**Pichia pastoris** secretes recombinant proteins less efficiently than Chinese hamster ovary cells but allows higher space-time yields for less complex proteins

Andreas Maccani1,2*, Nils Landes1,2*, Gerhard Stadlmayr3,4, Daniel Maresch4, Christian Leitner2, Michael Maurer1,5, Brigitte Gasser1,2, Wolfgang Ernst1,2, Renate Kunert1,2 and Diethard Mattanovich1,2

1 Austrian Centre of Industrial Biotechnology (ACIB GmbH), Vienna, Austria
2 Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria
3 Christian Doppler Laboratory for Antibody Engineering, University of Natural Resources and Life Sciences, Vienna, Austria
4 Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria
5 School of Bioengineering, University of Applied Sciences FH-Campus Vienna, Vienna, Austria

Chinese hamster ovary (CHO) cells are currently the workhorse of the biopharmaceutical industry. However, yeasts such as *Pichia pastoris* are about to enter this field. To compare their capability for recombinant protein secretion, *P. pastoris* strains and CHO cell lines producing human serum albumin (HSA) and the 3D6 single chain Fv-Fc anti-HIV-1 antibody (3D6scFv-Fc) were cultivated in comparable fed batch processes. In *P. pastoris*, the mean biomass-specific secretion rate ($q_p$) was 40-fold lower for 3D6scFv-Fc compared to HSA. On the contrary, $q_p$ was similar for both proteins in CHO cells. When comparing both organisms, the mean $q_p$ of the CHO cell lines was 1011-fold higher for 3D6scFv-Fc and 26-fold higher for HSA. Due to the low $q_p$ of the 3D6scFv-Fc producing strain, the space-time yield (STY) was 9.6-fold lower for *P. pastoris*. In contrast, the STY of the HSA producer was 9.2-fold higher compared to CHO cells because of the shorter process time and higher biomass density. The results indicate that the protein secretion machinery of *P. pastoris* is much less efficient and the secretion rate strongly depends on the complexity of the recombinant protein. However, process efficiency of the yeast system allows higher STYs for less complex proteins.

**Keywords:** CHO cells · Fed batch · Protein secretion · Recombinant protein production · Volumetric productivity

1 Introduction

Human protein therapeutics became more and more important for the treatment of various diseases over the last decades. Today, many different production systems are in use for the expression of heterologous proteins ranging from bacterial hosts to transgenic animals. To date, approved biopharmaceutical products are produced in a limited number of expression systems (in particular *Escherichia coli*, *Saccharomyces cerevisiae*, and Chinese hamster ovary (CHO) cells) [1], but non-conventional systems are catching up [2]. The choice of the most suitable expression system strongly depends on the complexity of the product as well as the need for correct post-translational modifications (PTMs) such as glycosylation, disulfide bond formation, phosphorylation, and proteolytic processing which might be required for biological efficacy.

* These authors contributed equally to this work.

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Mammalian cells are currently the expression system of choice for the production of complex therapeutic proteins which require proper folding, human-like PTMs or multimeric assembly. Various mammalian cell lines such as CHO, mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293), and human embryonic retinoblast (PER.C6) cells have been established. However, almost all approved mammalian cell-derived biopharmaceutical proteins have been produced in CHO cells [1]. Mammalian cells have been used for more than three decades for the production of recombinant proteins and initially they were considered as the least effective production system reaching product titers of only 50 mg L\(^{-1}\) in the mid-1980s. But mainly due to media and bioprocess optimization, product titers ranging from 1 to 5 g L\(^{-1}\) are typically reached in industry today [3]. Compared to microbial expression systems, mammalian cell cultures grow very slowly and reach only a low biomass density. Moreover, media costs are generally higher, although chemically defined, serum- and protein-free media have been developed for CHO cells. Additionally, cell line development is a very time-consuming process which takes at least 6 months [4]. In spite of these drawbacks, mammalian cells and especially CHO cells are currently the workhorse in biopharmaceutical industry, mainly because they are the only established expression system which is able to produce complex recombinant proteins with human-like glycoforms which are bioactive in humans. But also yeast expression systems have been used for the production of therapeutic proteins since the early 1980s. They can grow on inexpensive, chemically defined media to very high cell densities. Until 2009, all approved yeast-derived biopharmaceutical proteins were expressed in S. cerevisiae. However, expression systems based on non-conventional yeasts have been developed during the last two decades. Especially the methyloctrophic yeasts Pichia pastoris and Hansenula polymorpha are frequently applied for recombinant protein production. In 2009, Ecallantide (trade name Kalbitor) was the first therapeutic derived from P. pastoris that gained FDA approval [5]. The main advantages of yeasts over bacterial expression systems such as E. coli are their ability to secrete recombinant proteins into the culture broth as well as the absence of endotoxins. Moreover, yeasts do not contain oncogenic or viral DNA [6]. Furthermore, yeasts are eukaryotes and so they have the capability of protein processing such as protein folding and PTM-like glycosylation. N-linked glycosylation patterns however differ significantly from human and can impact the serum half-life and immunogenicity of therapeutic proteins [7, 8]. The high mannose content and the absence of sugar residues typical for human glycoproteins such as sialic acid are a major drawback of yeasts compared to mammalian expression systems. Because of this, much effort has been put into the engineering of strains that are able to form human-like glycans. The synthesis of fully humanized N-glycans in P. pastoris has been achieved [9], as well as the production of recombinant human proteins with humanized N-glycans [10].

Although a huge amount of information about the individual expression systems as well as comparative reviews can be found in literature, a quantitative comparison of heterologous protein production data is very difficult. The productivities of the individual systems strongly depend on the expressed recombinant protein. However, the proteins produced in microbial processes are generally different ones than those expressed in mammalian cells. Smaller proteins are supposed to be produced more economically in microbial cells, whereas mammalian expression systems are exclusively used for large glycosylated proteins. However, due to the recent advances in glycoengineering, yeasts become a more and more attractive alternative for the recombinant production of complex proteins.

In this study, process relevant parameters of high producing recombinant P. pastoris strains and CHO cell lines secreting the same model proteins were compared. For downstream processing, the product concentration as well as the relative purity of the culture supernatant is of high importance. Beside media costs, the achievable space-time yield (STY) is the crucial criterion to assess the economic efficiency of the fermentation process. The STY depends on the one hand on the specific growth rate (\(\mu\)) and the achievable biomass density and on the other hand on the ability for product formation and secretion which is described by the specific product secretion rate (\(q_p\)). Two model proteins with different complexity were selected in order to challenge the expression systems in different ways. One of them, human serum albumin (HSA) is a monomeric and non-glycosylated protein that can be produced at very high levels in P. pastoris [11]. As a second more complex model protein, a single chain Fv-Fc fusion antibody (3D6scFv-Fc) derived from the monoclonal anti-HIV-1 antibody 3D6 [12] was designed. This protein is homodimeric and contains the Fc-specific glycosylation site. For both host systems, transgene copy number was increased by gene amplification in order to establish high producing strains and cell lines which then were cultivated in standard fed batch processes using the same bioreactor system. Comparing the process relevant parameters highlighted the strengths and limitations of P. pastoris and CHO cells for the production of recombinant proteins.

2 Materials and methods
2.1 Model protein construction

The 3D6scFv-Fc antibody was designed by combining the variable heavy chain (\(V_H\)) and the variable light chain (\(V_L\)) domain of the monoclonal antibody 3D6 [12] via a
(GGGGS)₆ linker and fusing this single-chain fragment variable (scFv) construct to the human IgG1 fragment crystallizable (Fc) region. The cDNAs of 3D6scFv-Fc and human serum albumin (HSA) were codon optimized for CHO cells and \( P. \) \( \text{pastoris} \) respectively and synthesized (Geneart, Germany).

### 2.1 \( P. \) \( \text{pastoris} \) expression vector

For both proteins, codon optimized genes were cloned into the multiple cloning site (ShiI, ShiII) of the in-house vector pPUZZLE containing the Zeocin resistance cassette for selection and the NTS region of the ribosomal DNA locus as genome integration sequence [13]. The expression of both model proteins was controlled by the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter of \( P. \) \( \text{pastoris} \). HSA was secreted by means of its native secretion leader. For secretion of the 3D6scFv-Fc antibody the prepro leader sequence of the \( S. \) \( \text{cerevisiae} \) alpha mating factor was used.

### 2.2 CHO cells expression vectors

Both target genes were cloned into the pCI-neo mammalian expression vector (Promega, WI, USA) which carries the human cytomegalovirus (CMV) immediate-early enhancer/promoter to drive the constitutive expression of the inserted gene as well as the neomycin phosphotransferase gene for selection (pCI-neo_\( \text{HSA}_{-} \text{CHO} \), pCI-neo_\( \text{3D6scFv}_{-} \text{CHO} \)). For secretion of HSA the native leader was used. The 3D6scFv-Fc antibody was secreted using the human Ig heavy chain leader. Additionally, a second plasmid (p2-dhfr) which contains the dihydrofolate reductase gene under the control of the SV40 early promoter was used for gene amplification.

### 2.2.1 \( P. \) \( \text{pastoris} \) strains and CHO cell lines

The establishment of a high producing \( P. \) \( \text{pastoris} \) strain for each model protein was based on the procedure of post-transformational vector amplification via repeated selection on stepwise increased antibiotic concentrations as described previously [14].

Plasmids linearized with SpeI were transformed into \( P. \) \( \text{pastoris} \) SMD1168H (Life Technologies, CA, USA) using electroporation (2 kV, 4 ms, GenePulser, Bio-Rad, CA, USA). After regeneration, the cell suspension was plated on YPD agar plates (10 g L⁻¹ yeast extract, 10 g L⁻¹ peptone, 20 g L⁻¹ glucose, and 20 g L⁻¹ agar) containing 25 μg mL⁻¹ Zeocin. Initially, 24 clones for each model protein were picked from the 25 μg mL⁻¹ Zeocin containing YPD agar plates, screened in shake flask and analyzed by SDS-PAGE, western blot, and ELISA. Out of those, the best 12 clones were stepwise transferred to YPD agar plates with increasing Zeocin concentrations (100, 500, 1000, 2500, and 5000 μg mL⁻¹). Thus, 12 clone families were generated, each one containing six clones which were descended from different Zeocin levels. Thereby, the clone selected on the lower Zeocin level represents the parental strain of the clone selected on the next higher level. Screening of the corresponding clones was carried out in shake flask cultures on a Multitron II shaker (Infors, Switzerland). Therefore, a single colony of the desired clones was cultivated in 5 mL of YPD (10 g L⁻¹ yeast extract, 10 g L⁻¹ peptone, and 20 g L⁻¹ glucose) medium supplemented with the respective amount of Zeocin. Such pre-cultures were shaken at 180 rpm for 24–48 h at 28°C. After measuring the optical density (OD₆₀₀) of the pre-cultures, main cultures (10 mL YPD medium in a 100 mL shake flask) were inoculated to an OD₆₀₀ of 0.1 and grown for 48 h at 28°C and 180 rpm. Additional glucose (100 μL of 50% w/v glucose) was added to the cultures after 12, 24, and 36 h. The cultures were harvested after 48 h of cultivation. Wet cell mass concentrations were determined by centrifugation of 1 mL culture broth for 1 min at 17000g and 4°C. Aliquots of the supernatant as well as cell pellets were stored at −20°C until further analysis.

### 2.2.2 CHO cell line development

Two stable recombinant CHO cell lines producing 3D6scFv-Fc and HSA, respectively were established. Protein-free cultivated dihydrofolate reductase deficient (dhfr) CHO cells DUXX-B11, ATCC CRL-9096 [15] were used as host cell line. These cells were co-transfected with the plasmids pCI-neo_\( \text{HSA}_{-} \text{CHO} \) or pCI-neo_\( \text{3D6scFv}_{-} \text{CHO} \) and p2-dhfr using polyethyleneimine (PEI) as previously described [16]. Selection of recombinant cell lines was performed in the presence of G418 and the absence of hypoxanthine and thymidine (HT). For this purpose the protein-free CHO medium ProCHO5 (Lonza, Switzerland) supplemented with 4 mM l-glutamine and 0.5 mg mL⁻¹ G418 was used. To select single clones, limiting dilution was conducted by seeding the cells into 96-well plates for 24 h after transfection. For each cell line, transfections were done in four independent experiments using 5 × 10⁶ cells respectively. Cells were cultivated in a 37°C, 5% carbon dioxide environment. Additionally, 0.05 μM methotrexate (MTX) was added to the medium for gene amplification and stepwise increased to 0.1 μM in the subsequent passages. Best producing clones were identified by screening the supernatants using product specific ELISA assays. The number of clones was stepwise reduced and the culture volume increased to 10 mL using 48-well plates and T25 cell culture flasks. Finally, the four best producing clones were transferred into 125 mL spinner flasks and propagated in 50 mL suspension cultures at 50 rpm and 37°C. These clones were evaluated regarding specific growth rate and specific productivity for at least ten passages. The best performing clone was then subcloned by limiting dilution in 96-well plates. The cultures were treated as before and MTX concentration was stepwise increased to 0.4 μM in the subsequent pas-

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sages. The four best producing clones were again transferred into 125 mL spinner flasks and evaluated regarding specific growth rate and specific productivity for at least ten passages. For the two best performing clones, the MTX-pressure was stepwise increased to 0.8 μM and finally to 1.6 μM. The best performing clone at 0.4 μM MTX was subcloned by limiting dilution in 96-well plates for a third time. During culture volume expansion, the clones were adapted to DMEM/Ham’s F12 (1:1) supplemented with 4 mM L-glutamine, 0.25% soy peptone (Quest International, The Netherlands), 0.1% Pluronic F68, protein-free supplement (Polymun Scientific, Austria), and 0.4 μM MTX. The four best producing clones were again transferred into 125 mL spinner flasks and evaluated regarding specific growth rate and specific productivity for at least ten passages. Working cell banks were generated at the different stages of gene amplification and subcloning.

2.3 Fed batch cultivation

2.3.1 P. pastoris cultivation

Glucose limited fed batch cultivations of the selected P. pastoris high producing strains were carried out in duplicate in 1.0 L bioreactors (SR0700ODLS, DASGIP, Germany) with a fed batch starting volume of 350 mL as described previously [17]. Pre-cultures for fed batch experiments were inoculated from a cryo-stock and grown at 25°C in 1000 mL shake flasks containing 100 mL of YPD medium with corresponding amounts of Zeocin. Cultures were shaken at 180 rpm for 24–48 h. Pre-culture cells were harvested and used to inoculate the bioreactor to the desired starting optical density (OD$_{600}$) of 3.0. Therefore, a defined culture broth volume was withdrawn, transferred into sterile 50 mL centrifuge tubes and centrifuged (1504 g, 4 min, 25°C). After washing, the cells were re-suspended in 40 mL of sterile batch medium and used to inoculate the bioreactor, which was prefilled with 410 mL of sterile batch medium. After complete consumption of glycerol in the batch phase, fed batch cultivation was initiated by starting the balance controlled feed pumps. A constant feed of 2.38 g h$^{-1}$ fed batch medium was applied for 113 h. The air flow rate was 13.5 L h$^{-1}$. Foam formation was antagonized by controlled addition of 5% w/w antifoam solution (Glanapon 2000, Bussetti, Austria). Samples for determination of cell dry mass were taken once a day. Optical density (OD) was measured at a wavelength of 600 nm after dilution in ddH$_2$O. Yeast dry mass concentrations were determined with a bioprofile analyzer (BioProfile 100 Plus, Nova Biomedical, MA, USA).

2.4 Biomass concentration determination

2.4.1 P. pastoris

Optical density (OD) of P. pastoris cultures was measured at a wavelength of 600 nm after dilution in ddH$_2$O. Yeast dry mass concentrations were determined in duplicate for each sample. Three milliliters of culture broth were centrifuged at 4300 g for 5 min, pellets were washed with 5 mL ddH$_2$O, centrifuged and re-suspended in ddH$_2$O. This suspension was transferred to a pre-weighed beaker and dried at 105°C for 24 h.

2.4.2 CHO cells

Cell concentration was determined by counting the nuclei of lysed cells with a Z2 Coulter Counter (Beckman...
and the determined average dry mass of one CHO cell.

For CDM determination, more than $9 \times 10^7$ cells were collected by centrifugation, washed with Dulbecco’s PBS and dried as described above. The CDM was determined once per fermentation. The time course of the CDM concentration was calculated based on the cell concentration and the determined average dry mass of one CHO cell.

2.5 Analytical methods

2.5.1 ELISA

The concentrations of the secreted products were determined from the culture supernatants using sandwich ELISA assays. For 3D6scFv-Fc, 96-well immunosorbent plates (Nunc MaxiSorp, Thermo Fisher Scientific, MA, USA) were coated with 0.33 μg mL$^{-1}$ goat anti-human IgG (γ-chain specific) antibody (I3382, Sigma-Aldrich) diluted in coating buffer (0.1 M Na$_2$CO$_3$/NaHCO$_3$, pH 9.6) at 4°C overnight. After each incubation step, the plates were washed three times using washing buffer (phosphate-buffered saline (PBS)) containing 0.1% Tween 20, pH 7.4. Affinity purified 3D6scFv-Fc was used as a standard protein at a starting concentration of 100 ng mL$^{-1}$. Standard and samples were serially diluted in washing buffer containing 1% bovine serum albumin (BSA) and applied onto the pre-coated plates. After 1 h, captured 3D6scFv-Fc was incubated with 0.5 μg mL$^{-1}$ horseradish peroxidase (HRP) conjugated goat anti-human albumin antibody (A80-129P, Bethyl) detected using a horseradish peroxidase (HRP) conjugated goat anti-human albumin antibody (A80-129P, Bethyl) diluted in 0.15 M citric acid buffer, pH 5.0 containing 0.02% NaN$_3$. The mean specific product secretion rate $q_{P,\text{mean}}$ (mg g$^{-1}$ h$^{-1}$) between two consecutive sampling points was calculated according to Equation (1), where $t_i$ (h) is the time at the end of cultivation. In this study $t_i$ (h) represents the time of the feed start because $q_{P,\text{mean}}$ was determined for the feed phase.

$$ q_{P,\text{mean}} = \frac{1}{t_i - t_0} \sum_{i=1}^{k} \frac{2(P_i - P_{i-1})}{CDM_{i-1} + CDM_i} $$

2.5.2 SDS–PAGE and western blot

Sample supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE Novex 4–12% Bis-Tris Gels (Life Technologies) and MOPS buffer according to the manufacturer’s instructions. Proteins were visualized by silver staining [19] or transferred to a polyvinylidene difluoride (PVDF) membrane for western blot analysis. Semi wet blotting was applied using the XCell II Blot Module (Life Technologies) according to the supplier’s manual. The membrane was blocked with washing buffer (PBS, 0.1% Tween 20) containing 3% skim milk powder for 1 h. To detect 3D6scFv-Fc, the membrane was incubated with alkaline phosphatase (AP) conjugated goat anti-human IgG (γ-chain specific) antibody (A3187, Sigma-Aldrich) 1:5000 diluted in washing buffer containing 3% skim milk powder. 5-Bromo-4-chloro-3-indolyl phosphate combined with nitro blue tetrazolium (BCIP/NTB) staining was used for the colorimetric detection of AP activity. HSA was detected using a horseradish peroxidase (HRP) conjugated goat anti-human albumin antibody (A80-129P, Bethyl) in a 1:30 000 dilution. For protein visualization the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was used.

2.6 Parameter calculations

Smoothing of experimental data for CDM and secreted product concentration was done using the smoothing spline algorithm of the Matlab Curve Fitting Toolbox (The MathWorks, MA, USA). A smoothing parameter of $p = 0.2$ was used for smoothing the CDM data, whereas a smoothing parameter of $p = 0.00001$ was applied for smoothing of the product concentration data. Specific secretion rates and STYs were calculated based on smoothed product concentrations as described in the following. The specific product secretion rate $q_p$ (mg g$^{-1}$ h$^{-1}$) between two consecutive sampling points was calculated according to Equation (2), where $t_i$ (h) is the later point in time. $P_i$ (mg) is the total amount of secreted product at the time $t_i$ and CDM$_i$ (g) represents the total CDM at the time $t_i$.

$$ q_p = \frac{1}{t_i - t_{i-1}} \frac{2(P_i - P_{i-1})}{CDM_{i-1} + CDM_i} \quad (1) $$

The mean specific product secretion rate $q_{P,\text{mean}}$ (mg g$^{-1}$ h$^{-1}$) was calculated with Equation (2), where $t_0$ (h) is the time of the feed start because $q_{P,\text{mean}}$ was determined for the feed phase.

$$ q_{P,\text{mean}} = \frac{1}{t_i - t_0} \sum_{i=1}^{k} \frac{2(P_i - P_{i-1})}{CDM_{i-1} + CDM_i} \quad (2) $$

The STY (mg L$^{-1}$ h$^{-1}$) at a given point $t_i$ (h) was calculated according to Equation (3), where $t_0$ (h) represents the time of fermentation start. $P_i$ (mg) is the total amount of product at the time $t_i$, $V_i$ is the total culture volume at the time $t_i$.

$$ \text{STY} = \frac{1}{V_i} \frac{P_i - P_0}{t_i - t_0} \quad (3) $$

The specific growth rate $\mu$ (h$^{-1}$) between two consecutive sampling points was calculated according to Equation (4).

$$ \mu = \frac{1}{t_i - t_{i-1}} \ln \frac{CDM_i}{CDM_{i-1}} \quad (4) $$
3 Results

3.1 Strain and cell line development

High-producing P. pastoris strains and CHO cell lines expressing 3D6scFv-Fc and HSA were developed. The key data of strain and cell line development are shown in Table 1. To achieve high-level expression, amplification of the transgenes was conducted by stepwise increasing of the Zeocin concentration in case of P. pastoris and the MTX concentration for CHO cells according to established standard protocols.

The selection of high-producing P. pastoris strains was based on the determination of biomass-specific product yields in correlation to gene copy number. For 3D6scFv-Fc, the maximal product yield in shake flask screening cultures was obtained from a strain which was selected on 500 μg mL⁻¹ Zeocin (Supporting information, Fig. S1A). Further increment of selection pressure resulted in decreased product yields and gene copy numbers. In contrast, maximal product yields for HSA producing strains were already reached at a Zeocin concentration of 100 μg mL⁻¹ (Supporting information, Fig. S1B). Increasing antibiotic concentrations led to higher copy numbers, whereas product yields remained unchanged. Based on the desired strain properties (high product yields and high gene copy numbers), the 3D6scFv-Fc producing strain derived from 500 μg mL⁻¹ Zeocin and the HSA producing strain derived from 1000 μg mL⁻¹ Zeocin were chosen for bioreactor cultivation.

The established CHO clones were evaluated by monitoring the specific productivity $q_p$ and cell growth over several passages. Increasing the MTX concentration from 0.1 to 0.4 μM improved the productivity considerably (Supporting information, Fig. S1C and D). However, a further increase of MTX concentration could not raise the specific productivity significantly. The determined gene copy numbers clearly correlated with the specific productivities. For this reason the clones at 0.4 μM MTX were selected for the final subcloning step to establish clonal high producing CHO cell lines.

3.2 Fed batch cultivation

The established high producing P. pastoris strains and CHO cell lines were cultivated in a fed batch process using comparable bioreactor systems (Table 1).

For P. pastoris cultures, the substrate feed was started after around 30 h of batch phase and was maintained for 113 h in order to reach a yeast dry mass concentration of 100 g L⁻¹. The time courses of CDM and product concentration of the 3D6scFv-Fc and HSA producing P. pastoris strains are shown in Fig. 1A and B.

Product concentrations were measured in the cell free culture supernatant, corrected for the volume of the biomass and recalculated to total culture volume as previously described [20]. After biomass correction, the P. pastoris 3D6scFv-Fc cultivation yielded 8.8 ± 0.1 mg L⁻¹. In contrast, a product concentration of 380.2 ± 32.7 mg L⁻¹ could be achieved in the P. pastoris HSA process. The cell viability remained above 98% during the cultivations.

In case of the CHO cell lines, feeding was started after an initial batch phase of 50 h. The process was stopped after the viability dropped to 70%. CDM, cell viability and product concentration of the CHO 3D6scFv-Fc and CHO HSA cultivations are shown in Fig. 1C and D. The dura-

| Table 1. Key data of P. pastoris and CHO system comparison |
|-----------------------------------------------------------|
| **P. pastoris**                                          | **CHO cells**                                   |
| Host           | DUKX-B11                                      |
| Promoter       | CMV promoter                                  |
| Leader HSA     | Native                                       |
| Leader 3D6scFv-Fc | α-factor                                  |
| Integration    | rDNA locus                                   |
| Selection      | Zeocin resistance                             |
| Amplification  | Zeocin                                       |
| Bioreactor system | SR0700ODLS, DASGIP                           |
| Cultivation mode | Fed batch                                  |
| Temperature    | 37°C                                        |
| Dissolved Oxygen | 30% air saturation                           |
| Stirrer speed  | 80 rpm                                       |
| pH             | 7.00                                         |
| Batch medium   | DMEM/Ham’s F12 based                          |
| Feed medium    | DME/Ham’s F12 based, enriched with glucose and amino acids |
| Feeding strategy | Glucose-limited, constant feed rate (2.38 g h⁻¹) |
|                | Constant residual glucose concentration of 1–2.5 g L⁻¹, feed rate adjusted accordingly |
Figure 1. Time courses of fed batch cultivations. Mean cell dry mass concentration, cell viability and product concentration of the (A) *P. pastoris* 3D6scFv-Fc, (B) *P. pastoris* HSA, (C) CHO 3D6scFv-Fc, and (D) CHO HSA cultivations. Arrows mark the feed start. Data represent mean values ± standard deviation of two independent cultivations (CHO HSA: data of one cultivation after 163 h). Curves for cell dry mass and product concentration were calculated using the smoothing spline algorithm of the Matlab Curve Fitting Toolbox. Biomass-specific secretion rates of (E) 3D6scFv-Fc producers and (F) HSA producers and space-time yields of (G) 3D6scFv-Fc producers and (H) HSA producers during the feed phase. Data were calculated using smoothed product concentrations of two independent cultivations. *P. pastoris* and CHO cells were cultivated in comparable DASGIP bioreactor systems. *P. pastoris* cultures were inoculated with an optical density (OD600) of 3.0 and grown at 25°C, pH 5.85, and 20% dissolved oxygen. A constant feed rate of 2.38 g h⁻¹ fed batch medium (500 g L⁻¹ glucose) was applied for 113 h. The CHO cell concentrations at cultivation start were adjusted to 2.5 × 10⁵ cells mL⁻¹ and the cultures were grown at 37°C, pH 7.0, 30% dissolved oxygen, and an agitation speed of 80 rpm. The feed rate was adjusted daily to maintain a constant residual glucose concentration of 1.0–2.5 g L⁻¹. Product concentrations were determined using ELISA and are expressed as milligram product per liter of culture broth.
tion of the CHO 3D6scFv-Fc fermentations was 234 h leading to a final product concentration of 158.9 ± 0.6 mg L⁻¹. The CHO HSA fermentation could be maintained for 379 h and yielded 129.3 mg L⁻¹.

Nearly 50-fold higher product levels were obtained for HSA in comparison to 3D6scFv-Fc with P. pastoris, whereas product concentrations were in a similar range for both proteins in the CHO cultivations. Notably, three- to four-fold higher final HSA concentrations were obtained in P. pastoris fermentations, while for 3D6scFv-Fc more than 12-fold higher product levels were reached with CHO cells.

The quality of the expressed proteins and the purity of the culture supernatant were evaluated by SDS-PAGE and western blot. The results at two points in time (after start of the feed and at the end of the process) of one representative fermentation run are shown in Fig. 2. The recombinant protein constituted the major product in the supernatant in all cultivations.

The binding affinity of the 3D6scFv-Fc antibody to the HIV-1 envelope protein UG37 gp140 was measured using bio-layer interferometry (Supporting information, Fig. S2). Very similar binding properties (k_on, k_off, and K_D values) could be observed for 3D6scFv-Fv derived from P. pastoris and CHO cells (Supporting information, Table S2). The determined affinity constant K_D was 3.7 nM in both cases.

Figure 2. Fed batch culture supernatants after feed start and at the end of the process. Silver stained SDS–PAGE was used to analyze the total protein content in the culture supernatant. Western blot analysis was used to identify the recombinant proteins HSA (67 kDa) and 3D6scFv-Fc (homodimer: approx. 110 kDa), respectively. (A) Silver stained SDS–PAGE and (B) western blot under reducing conditions of the HSA producing CHO and P. pastoris clones. (C) Silver stained SDS–PAGE under non-reducing conditions, (D) Western blot under non-reducing, and (E) reducing conditions of the 3D6scFv-Fc expressing CHO and P. pastoris clones. Equal volumes of supernatant were loaded onto the gel. Samples of two independent cultivations were analyzed. The results of one representative replicate per clone are shown. Compared samples were always analyzed on the same gel.

3D6scFv-Fc glycosylation pattern was analyzed by mass spectrometry. CHO cell-derived 3D6scFv-Fc antibodies contained complex-type N-glycans which were mainly fucosylated, whereas P. pastoris-derived 3D6scFv-Fc antibodies had exclusively high-mannose-type N-glycans ranging from Man9 to Man16 (Supporting information, Fig. S3).

The quality of secreted HSA was further analyzed on the level of secondary structure. Therefore, far-UV circular dichroism spectra were recorded. The comparison to a reference albumin derived from human serum revealed no significant differences, showing that the HSA produced in P. pastoris and CHO cells was properly folded (Supporting information, Fig. S4).

The biomass-specific product secretion rate q_P is an essential parameter to assess the secretion performance of an expression system. Figure 1E and F show the corresponding time courses of q_P for both organisms and model proteins. In P. pastoris, q_P mean was significantly lower (40-fold) for 3D6scFv-Fc than for HSA, whereas the specific product secretion rates were very similar for both model proteins in the CHO cell lines. Furthermore, q_P clearly declined during the process in the P. pastoris strains. Large differences in q_P could be observed comparing both organisms. The mean specific secretion rates in the CHO cell lines were 1011-fold higher for 3D6scFv-
The STY is an important parameter to evaluate the performance of the whole process. The time courses of the STY during the feed phase for both organisms and model proteins are shown in Fig. 1G and H. In P. pastoris the STY at the end of the process was significantly higher for the HSA compared to the CHO cell line due to the 

4.1 Cell line and strain development

A major advantage of the P. pastoris system is that stable clones can be developed within a short time. A single round of screening after transformation may be sufficient to achieve a production clone, requiring about 2 weeks. Additional rounds of amplification as described here may extend this to 2 months.

Although site-specific integration of transgenes is possible in CHO cells using recombinase-mediated cassette exchange [21, 22], random integration is still commonly used. However this necessitates extensive screening, because the chances of isolating high-producing clones depend on the number of clones that have been screened. Furthermore, the establishment of a high-producing system is that stable clones can be developed within a short time. A single round of screening after transformation may be sufficient to achieve a production clone, requiring about 2 weeks. Additional rounds of amplification as described here may extend this to 2 months.

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ic byproducts such as ammonium [23], lactate [24] as well as elevated pCO₂ and hyperosmolality [25]. In *P. pastoris* cell density is mainly limited by the technical feasibility of biomass removal from the culture broth. Therefore one major aim of CHO process development is to achieve higher cell densities by optimizing the fed batch strategy [26], or by removing spent medium using perfusion [27]. While the low $\mu_{\text{max}}$ of CHO cells extends process duration markedly, microbial processes are commonly not limited by the cell’s ability to achieve high growth rates but by technical limits of heat and oxygen transfer. It should be noted that the standard fed batch performed here for *P. pastoris* is rather long at low feed rates, leaving room for optimization.

### 4.3 Productivity

One means to optimize feed rates is to adapt the feed profile to the optimal trajectory of STY. We have shown before [20] that specific secretion rate and specific growth rate correlate strictly in *P. pastoris* which is also observed here. In CHO cells this correlation is rather weak (Fig. 3). Therefore different optimization strategies need to be applied to the two production platforms. In *P. pastoris*, optimization leads to initial high feed rates for rapid accumulation of biomass, followed by a phase of decreasing $\mu$, thus allowing time for product accumulation [20]. In CHO cells, the fed batch strategy rather aims at maintaining reasonably high viable cell concentrations. This difference in feed strategy leads to large differences in process duration spanning from 50 to 150 h for *P. pastoris* up to 21 days for CHO cells [28]. The STY is a measure for the product output per bioreactor volume and time, and thus illustrates the respective capital costs per unit of product. STY is reverse proportional to process duration, so that in other words the capital costs per unit product increase proportionally with the time needed to achieve a defined amount of product. Especially for the biopharmaceutical industry capital costs for production plants are a major factor of total production costs [29], so that maximizing STY is a valid optimization strategy.

Our results illustrate clearly that specific productivity (mean values during feed phase) in *P. pastoris* depends strongly on the product, being 40-fold lower for the antibody fragment compared to HSA. Low $q_p$ of the antibody fragment cannot be explained by protein size and number of disulfide bonds as HSA is a large protein of 67 kDa composed of three domains with a total of 17 intramolecular disulfide bonds and one free cysteine, compared to about 110 kDa and 10 disulfide bonds of the dimeric antibody fragment. It is more likely that folding efficiency and thermodynamic stability account for differences in $q_p$ as described for different variants of scFv fragments [30]. Notably this does not account for CHO cells as $q_p$ is the same for both proteins in this expression system. It may be speculated that CHO cells are specifically better suited to produce antibodies or their fragments while other proteins like HSA are well produced also in lower eukaryotic expression systems.

### 4.4 Product quality

Downstream processing is the major cost factor in a biopharmaceutical production process accounting for 50–80% of the total manufacturing costs [31]. Hence, it is very important that an expression system produces the desired protein at high relative purity to facilitate purification. Both production systems in this study are able to deliver very pure supernatants that contain low amounts of host cell proteins. Certainly, the produced proteins should be correctly folded and fully assembled to ensure biological activity. In the CHO processes we did not observe any product degradation. In case of *P. pastoris*...
very little amounts of degraded HSA could be detected. However, most of the HSA had the correct size and was properly folded. The 3D6scFv-Fc antibody was predominantly assembled as a dimer of the correct size in both expression systems. Not surprisingly, differences could be observed in the N-glycosylation pattern of the 3D6scFv-Fc antibody. The P. pastoris-derived antibodies were partly unglycosylated or contained glycans of the high-mannose-type. In contrast, the Fc glycans present in antibodies produced in CHO cells had the complex-type biantennary structures which are typically found in human IgGs [32]. As expected, the differences in IgG-Fc glycosylation did not impair binding to the epitope. However, it is well known that Fcγ receptor and complement mediated effector functions (antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity) are strongly affected by the IgG glycoform [33]. Hence, glycoengineered P. pastoris strains [34] would be required to produce fully functional IgGs or scFv-Fc antibodies.

### 4.5 Concluding remarks

In this study, we could demonstrate that in contrast to CHO cells the secretion capacity of P. pastoris is highly dependent on the complexity of the produced recombinant protein. In P. pastoris the specific product secretion rates are considerably lower compared to CHO cells. However, the shorter process times and the higher cell densities of P. pastoris processes can compensate for the lower secretion potential. Whether P. pastoris or CHO cells are the more adequate expression system strongly depends on the particular protein. Considering the two model proteins used in this study, we concluded that antibodies and antibody fragments may be produced more economically in CHO cells. On the contrary, P. pastoris seems to be the better choice for the production of less complex proteins such as HSA.

The results of this study clearly indicate that the protein secretion machinery is much more efficient in mammalian cells than in yeast. The mechanisms that control and limit recombinant protein expression and secretion in yeast as well as in mammalian cells are still poorly understood. Hence, a comprehensive comparison of these expression systems on a physiological level using various omics technologies might be very useful to identify the bottlenecks of heterologous protein secretion in lower eukaryotes.

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