

Research Article

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

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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.

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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.

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Abbreviations: 3D6scFv-Fc, 3D6 single chain Fv-Fc anti-HIV-1 antibody; CDM, cell dry mass; CHO, Chinese hamster ovary; HSA, human serum albumin; MTX, methotrexate; PTM, post-translational modification; STY, space-time yield

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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 multi-meric 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 methylotrophic 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 human-

ized *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 (μ) 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

(GGGS)₃ 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. pastoris* respectively and synthesized (Genearth, Germany).

2.1.1 *P. pastoris* expression vector

For both proteins, codon optimized genes were cloned into the multiple cloning site (SbfI, SfiI) 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. 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. cerevisiae* alpha mating factor was used.

2.1.2 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_HSA_CHO, pCI-neo_3D6scFv_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 *P. pastoris* strains and CHO cell lines

2.2.1 *P. pastoris* strain development

The establishment of a high producing *P. 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. 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 flasks 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 DUKX-B11, ATCC CRL-9096 [15] were used as host cell line. These cells were co-transfected with the plasmids pCI-neo_HSA_CHO or pCI-neo_3D6scFv_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-

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 (SR0700DLS, 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 (1504g, 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 (CDM), product concentrations and cell viability were taken every day. Viability of cells was analyzed with a cell viability kit (BD Biosciences, CA, USA) on a flow cytometer as described previously [18].

Batch medium contained per liter: 40 g glycerol, 12.6 g $(\text{NH}_4)_2\text{HPO}_4$, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.9 g KCl, 0.022 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2 g citric acid monohydrate, 4.6 mL PTM1 trace salts stock solution, and 2 mL of a 0.2 g L⁻¹ biotin (Sigma–Aldrich, MO, USA) solution. The pH was set to 5.85 with 25% w/w HCl. Fed batch medium contained per

liter: 550 g glucose-H₂O, 10 g KCl, 6.45 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.35 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 12 mL PTM1 trace salts stock solution, and 2 mL of a 0.2 g L⁻¹ biotin solution. PTM1 trace salts stock solution contained per liter: 6.0 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.08 g NaI, 3.36 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.2 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.02 g H_3BO_3 , 0.82 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 20.0 g ZnCl_2 , 65.0 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 5.0 mL H_2SO_4 (95–98% w/w).

2.3.2 *CHO* cell cultivation

Fed batch cultivation was conducted in 800 mL cell culture bioreactors (DS0700TPSS, DASGIP, Germany) with a starting working volume of 500 mL. The inocula were expanded in spinner flasks starting from the working cell bank. Exponentially growing cells from passage six were used for inoculation. The initial cell concentration was 2.5 × 10⁵ cells mL⁻¹. The cultures were maintained at 37°C, pH 7.0, 30% dissolved oxygen, and an agitation speed of 80 rpm. The initial batch medium was DMEM/Ham's F12 (1:1) supplemented with 4 mM L-glutamine, 0.25% soy peptone (Quest International), 0.1% Pluronic F68, and protein-free supplement (Polymun Scientific). The feed medium for the 3D6scFv-Fc expressing cell line contained 20 g L⁻¹ D-glucose, 28 mM L-glutamine, 5× MEM amino acids (PAA, Austria), and 10× MEM non essential amino acids (PAA). The feed medium for the HSA producing cell line was composed of 10 g L⁻¹ D-glucose, 14 mM L-glutamine, 2.5× MEM amino acids, and 5× MEM nonessential amino acids. Both feed media were based on DMEM/Ham's F12 (1:1) supplemented with 0.25% soy peptone, 0.1% Pluronic F68, and protein-free supplement.

After 50 h batch cultivation, the cultures were continuously fed at a constant feed rate. The feed rate was adjusted daily to maintain the glucose concentration of the culture broth within the target range of 1.0–2.5 g L⁻¹. Samples for off-line analyses were taken once a day. D-Glucose, L-glutamine, L-glutamate, and ammonium concentrations were measured 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 ddH₂O. Yeast dry mass concentrations were determined in duplicate for each sample. Three milliliters of culture broth were centrifuged at 4307g for 5 min, pellets were washed with 5 mL ddH₂O, centrifuged and re-suspended in ddH₂O. 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

Coulter, CA, USA). Cell viability was determined by trypan blue exclusion using a hemocytometer.

For CDM determination, more than 9×10^7 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 $0.33 \mu\text{g mL}^{-1}$ goat anti-human IgG (γ -chain specific) antibody (I3382, Sigma-Aldrich) diluted in coating buffer ($0.1 \text{ M Na}_2\text{CO}_3/\text{NaHCO}_3$, 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 $0.5 \mu\text{g mL}^{-1}$ horseradish peroxidase (HRP) conjugated goat anti-human IgG (γ -chain specific) antibody (62-8420, Life Technologies) for 1 h. Staining was conducted using 100 mg mL^{-1} o-phenylenediamine diluted in 0.15 M citric acid buffer, pH 5.0 containing 0.02% H_2O_2 . After stopping the reaction with 25% H_2SO_4 , absorption was measured at 492 nm (620 nm reference wavelength) using the infinite M1000 microplate reader (Tecan, Switzerland). HSA concentrations were determined using the Human Albumin ELISA Quantification Set (E80-129, Bethyl, TX, USA) according to the manufacturer's instructions.

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 Super-Signal 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 ($\text{mg g}^{-1} \text{ h}^{-1}$) between two consecutive sampling points was calculated according to Equation (1), 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

$$q_p = \frac{1}{t_i - t_{i-1}} \frac{2(P_i - P_{i-1})}{\text{CDM}_{i-1} + \text{CDM}_i} \quad (1)$$

The mean specific product secretion rate $q_{p,\text{mean}}$ ($\text{mg g}^{-1} \text{ h}^{-1}$) was calculated with Equation (2), where t_E (h) is the time at the end of cultivation. In this study t_0 (h) represents the time of the feed start because $q_{p,\text{mean}}$ was determined for the feed phase

$$q_{p,\text{mean}} = \frac{1}{t_E - t_0} \sum_{i=1}^E \frac{2(P_i - P_{i-1})}{\text{CDM}_{i-1} + \text{CDM}_i} \quad (2)$$

The STY ($\text{mg L}^{-1} \text{ h}^{-1}$) at a given point t_i (h) was calculated according to Equation (3), where t_0 (h) represents the time of fermentation start. P_0 (mg) is the total amount of product at the time t_0 . 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 μ (h^{-1}) between two consecutive sampling points was calculated according to Equation (4)

$$\mu = \frac{1}{t_i - t_{i-1}} \ln \frac{\text{CDM}_i}{\text{CDM}_{i-1}} \quad (4)$$

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 $\mu\text{g mL}^{-1}$ 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 $\mu\text{g mL}^{-1}$ (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 $\mu\text{g mL}^{-1}$ Zeocin and the HSA producing strain derived from 1000 $\mu\text{g mL}^{-1}$ 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 $8.8 \pm 0.1 \text{ mg L}^{-1}$. In contrast, a product concentration of $380.2 \pm 32.7 \text{ mg L}^{-1}$ 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

	<i>P. pastoris</i>	CHO cells
Host	SMD1168H	DUKX-B11
Promoter	GAP promoter	CMV promoter
Leader HSA	Native	Native
Leader 3D6scFv-Fc	α -factor	Human Ig heavy chain
Integration	rDNA locus	Random
Selection	Zeocin resistance	DHFR deficiency, G418 resistance
Amplification	Zeocin	Methotrexate
Bioreactor system	SR0700ODLS, DASGIP	DS0700TPSS, DASGIP
Cultivation mode	Fed batch	Fed batch
Temperature	25°C	37°C
Dissolved Oxygen	20% air saturation	30% air saturation
Stirrer speed	400–1250 rpm	80 rpm
pH	5.85	7.00
Batch medium	Synthetic, 40 g L^{-1} glycerol	DMEM/Ham's F12 based
Feed medium	Synthetic, 500 g L^{-1} glucose	DMEM/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

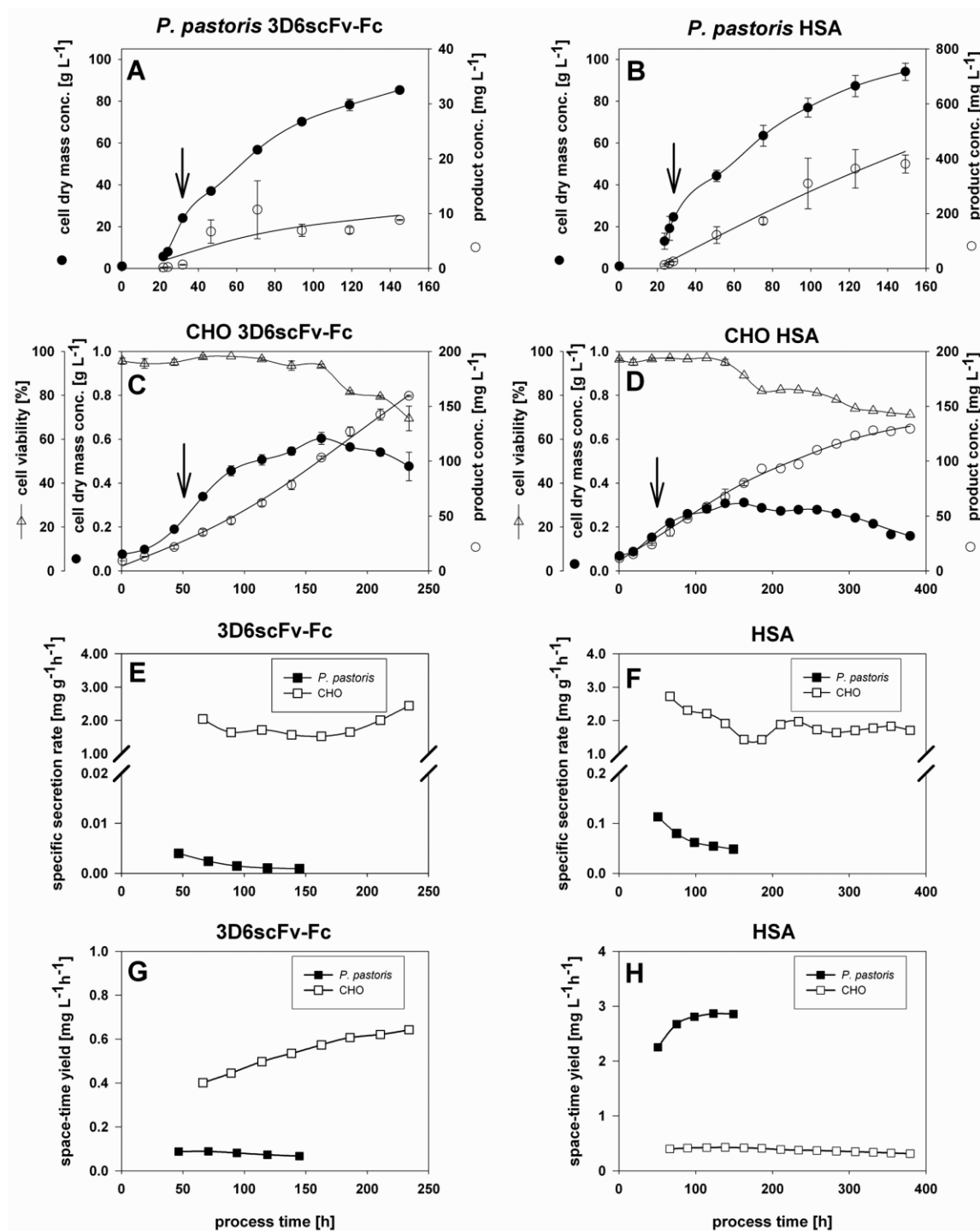


Figure 1. Time courses of fed batch cultivations. Mean cell dry mass concentration, cell viability and product concentration of the (A) *P. pastoris* 3D6scFv-Fc, (B) *P. pastoris* HSA, (C) CHO 3D6scFv-Fc, and (D) CHO HSA cultivations. Arrows mark the feed start. Data represent mean values \pm standard 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 (OD_{600}) 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 2.5×10^5 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.

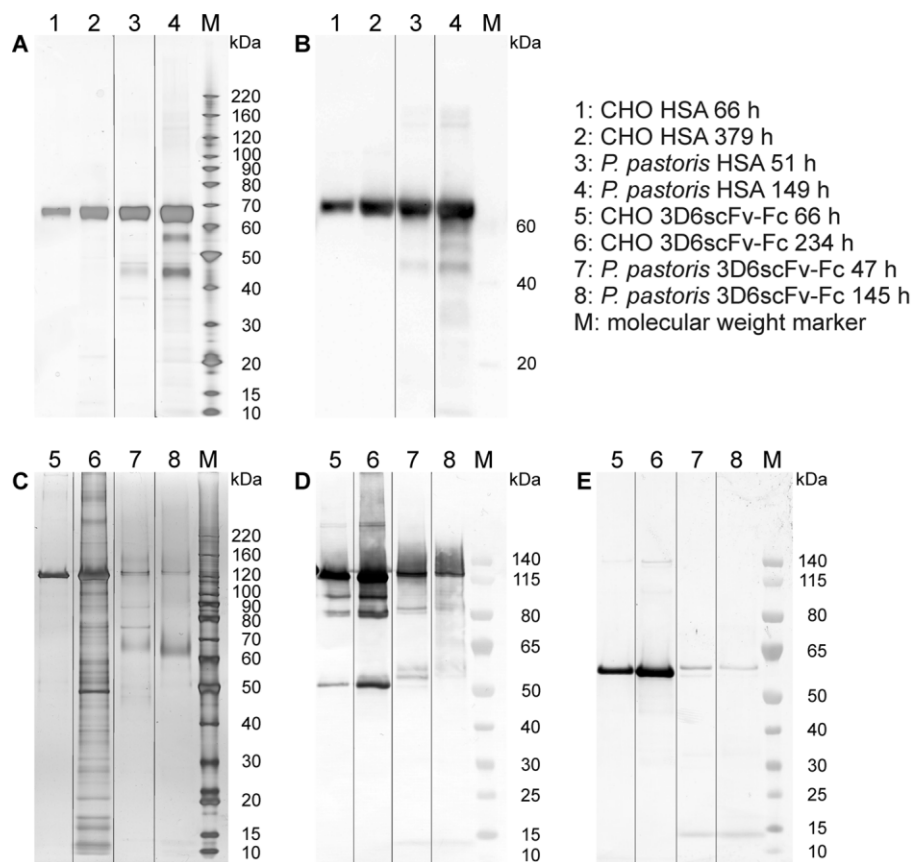


Figure 2. Fed batch culture supernatants after feed start and at the end of the process. Silver stained SDS–PAGE was used to analyze the total protein content in the culture supernatant. Western blot analysis was used to identify the recombinant proteins HSA (67 kDa) and 3D6scFv-Fc (homodimer: approx. 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.

tion of the CHO 3D6scFv-Fc fermentations was 234 h leading to a final product concentration of $158.9 \pm 0.6 \text{ mg L}^{-1}$. 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-Fc derived from *P. pastoris* and CHO cells (Supporting information, Table S2). The determined affinity constant K_{D} was 3.7 nM in both cases.

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*, $q_{\text{p,mean}}$ 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-

Table 2. Overview of the results from the fed batch cultivations

	<i>P. pastoris</i> 3D6scFv-Fc	CHO 3D6scFv-Fc	<i>P. pastoris</i> HSA	CHO HSA ^{e)}
Duration (h)	145	234	149	379
Maximal cell dry mass concentration CDM_{max} (g L ⁻¹) ^{a)}	85.3 ± 1.0	0.604 ± 0.027	94.1 ± 4.2	0.311 ± 0.009
Maximal specific growth rate μ_{max} (h ⁻¹) ^{a)}	0.147 ± 0.008	0.028 ± 0.002	0.148 ± 0.003	0.023 ± 0.002
Gene copy number	10 ± 1 ^{c)}	18 ± 2 ^{d)}	11 ± 2 ^{c)}	130 ± 16 ^{d)}
Product concentration (supernatant) (mg L ⁻¹) ^{a)}	12.3 ± 0.1	159.4 ± 0.7	551.0 ± 36.4	129.4
Product concentration (broth) (mg L ⁻¹) ^{a)}	8.8 ± 0.1	158.9 ± 0.6	380.2 ± 32.7	129.3
Mean specific secretion rate $q_{P,mean}$ (feed phase) (mg g ⁻¹ h ⁻¹) ^{b)}	0.0018	1.82	0.071	1.86
Maximal specific secretion rate $q_{P,max}$ (feed phase) (mg g ⁻¹ h ⁻¹) ^{b)}	0.0040	2.36	0.113	2.77
Space-time yield (process end) STY_{end} (mg L ⁻¹ h ⁻¹) ^{b)}	0.067	0.642	2.856	0.312
Maximal space-time yield STY_{max} (feed phase) (mg L ⁻¹ h ⁻¹) ^{b)}	0.088	0.642	2.866	0.427

a) Mean ± standard deviation ($n = 2$), two independent fed batch cultivations.

b) Calculated with smoothed product concentrations, two independent fed batch cultivations.

c) Mean ± standard deviation ($n = 4$), data from strain selection.

d) Mean ± standard deviation ($n = 2$), data from cell line selection.

e) Data of one cultivation after 163 h.

Fc and 26-fold for HSA compared to the *P. pastoris* strains (Table 2).

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 3D6scFv-Fc expressing strain (more than 40-fold difference), whereas it was in the same range for both model proteins in the CHO cell lines (less than two-fold difference). Due to the low q_P of the 3D6scFv-Fc producing *P. pastoris* strain, the STY at the end of the process was 9.6-fold lower in *P. pastoris* in comparison with the corresponding CHO cell line. In contrast, the STY of the HSA producers was 9.2-fold higher in *P. pastoris* compared to the CHO cell line due to the shorter process time and the higher biomass density (Table 2).

All process-relevant parameters of the fed batch cultivations are summarized and compared in Table 2.

4 Discussion

In this study, we compared the ability of the two frequently applied expression systems *P. pastoris* and CHO cells for recombinant protein production. To conduct a thorough comparison, we established *P. pastoris* strains and CHO cell lines which express the same model proteins and cultivated them in fed batch processes using comparable bioreactor systems (Table 1). Both expression systems have their advantages and disadvantages considering clone development, cell growth, cell density, productivity, and product quality.

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 stable CHO cell line requires steps of amplification and single cell cloning and so the whole procedure is very time-consuming and labor-intensive. Our standard procedure includes initial single-cell cloning after transfection followed by amplification, screening, and selection of the best clone. This clone will then be subjected to two additional rounds of single-cell cloning, amplification, and selection. Hence the whole clone development process from transfection to cell banking takes at least 6 months. Although high-throughput cell screening systems such as FACS-based screening have been developed, the clone development time of CHO cells cannot keep up with the very short development times of *P. pastoris* and microbial systems in general.

4.2 Cell growth and cell density

One major limitation of the CHO cell system is low specific growth rate, leading to long process times and limited cell densities. Cell growth and viable cell concentrations are additionally limited by the accumulation of tox-

ic byproducts such as ammonium [23], lactate [24] as well as elevated $p\text{CO}_2$ 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 μ_{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 μ , 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 suit-

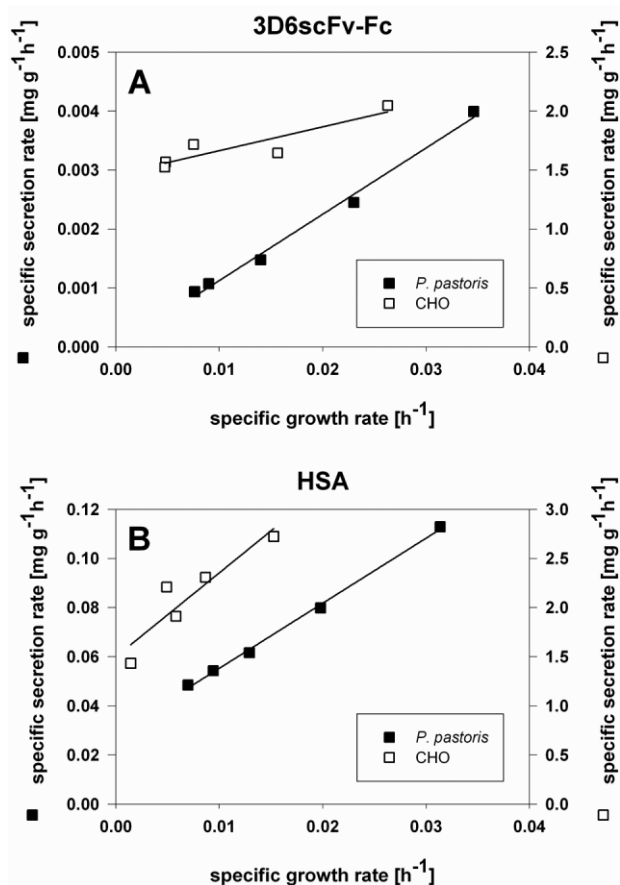


Figure 3. Relationship between biomass-specific secretion rate q_p and specific growth rate μ during the feed phase of fed batch cultivations. (A) 3D6scFv-Fc and (B) HSA producing *P. pastoris* and CHO clones. *P. pastoris* and CHO cells were cultivated as described for Fig. 1. Product concentrations were determined using ELISA. Biomass-specific secretion rates were calculated using smoothed product concentrations (smoothing spline algorithm of the Matlab Curve Fitting Toolbox) of two independent cultivations. Specific growth rates represent mean values of two independent cultivations.

ed 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 Fc γ 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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The authors declare no financial or commercial conflict of interest.

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This Special Issue on “Biomolecular Technology of Proteins – BioToP” compiles selected peer-reviewed publications of students from the BioToP PhD program at the Vienna Institute of BioTechnology (VIBT) of the University of Natural Resources and Life Sciences, Vienna, Austria (BOKU) and is edited by Co-Editor-in-Chief Prof. Alois Jungbauer. The cover represents the interdisciplinary and international character of BioToP. Idea: Andreas Maccani, Dagmar Brugger, Stefan Hofbauer, Vaibhav Jadhav, Gerald Klanert, Daniel Kracher, Iris Krondorfer, Irene Schaffner. Image: Dagmar Brugger.

Biotechnology Journal – list of articles published in the April 2014 issue.

Editorial: Biomolecular Technology of Proteins – BioToP

Christian Obinger

<http://dx.doi.org/10.1002/biot.201400106>

Review

Chlorite dismutases – a heme enzyme family for use in bioremediation and generation of molecular oxygen
Stefan Hofbauer, Irene Schaffner, Paul G. Furtmüller and Christian Obinger

<http://dx.doi.org/10.1002/biot.201300210>

Research Article

Convenient microtiter plate-based, oxygen-independent activity assays for flavin-dependent oxidoreductases based on different redox dyes

Dagmar Brugger, Iris Krondorfer, Kawah Zahma, Thomas Stoisser, Juan M. Bolivar, Bernd Nidetzky, Clemens K. Peterbauer and Dietmar Haltrich

<http://dx.doi.org/10.1002/biot.201300336>

Research Article

Fungal secretomes enhance sugar beet pulp hydrolysis

Daniel Kracher, Damir Oros, Wanying Yao, Marita Preims, Iva Rezic, Dietmar Haltrich, Tonci Rezic and Roland Ludwig

<http://dx.doi.org/10.1002/biot.201300214>

Research Article

The human anti-HIV antibodies 2F5, 2G12, and PG9 differ in their susceptibility to proteolytic degradation: Down-regulation of endogenous serine and cysteine proteinase activities could improve antibody production in plant-based expression platforms

Melanie Niemer, Ulrich Mehofer, Juan Antonio Torres Acosta, Maria Verdianz, Theresa Henkel, Andreas Loos, Richard Strasser, Daniel Maresch, Thomas Rademacher, Herta Steinkellner and Lukas Mach

<http://dx.doi.org/10.1002/biot.201300207>

Research Article

Expression of human butyrylcholinesterase with an engineered glycosylation profile resembling the plasma-derived orthologue

Jeannine D. Schneider, Alexandra Castilho, Laura Neumann, Friedrich Altmann, Andreas Loos, Latha Kannan, Tsafir S. Mor and Herta Steinkellner

<http://dx.doi.org/10.1002/biot.201300229>

Research Article

In *Pichia pastoris*, growth rate regulates protein synthesis and secretion, mating and stress response

Corinna Rebnegger, Alexandra B. Graf, Minoska Valli, Matthias G. Steiger, Brigitte Gasser, Michael Maurer and Diethard Mattanovich

<http://dx.doi.org/10.1002/biot.201300334>

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 Maccani, Nils Landes, Gerhard Stadlmayr, Daniel Maresch, Christian Leitner, Michael Maurer, Brigitte Gasser, Wolfgang Ernst, Renate Kunert and Diethard Mattanovich

<http://dx.doi.org/10.1002/biot.201300305>

Technical Report

Endogenous microRNA clusters outperform chimeric sequence clusters in Chinese hamster ovary cells

Gerald Klanert, Vaibhav Jadhav, Konstantina Chanoumidou, Johannes Grillari, Nicole Borth and Matthias Hackl

<http://dx.doi.org/10.1002/biot.201300216>

Regular Articles

Research Article

Peptide microarrays enable rapid mimotope optimization for pharmacokinetic analysis of the novel therapeutic antibody IMAB362

Karsten Schnatbaum, Hans-Ulrich Schmoltd, Martin Daneschdar, Laura M. Plum, Janina Jansong, Johannes Zerweck, Yvonne Kühne, Antonia Masch, Holger Wenschuh, Markus Fiedler, Özlem Türeci, Ugur Sahin and Ulf Reimer

<http://dx.doi.org/10.1002/biot.201300456>

Research Article

Dual salt mixtures in mixed mode chromatography with an immobilized tryptophan ligand influence the removal of aggregated monoclonal antibodies

Judith Vajda, Egbert Mueller and Eva Bahret

<http://dx.doi.org/10.1002/biot.201300230>

Research Article

Generic chromatography-based purification strategies accelerate the development of downstream processes for biopharmaceutical proteins produced in plants

Johannes F. Buyel and Rainer Fischer

<http://dx.doi.org/10.1002/biot.201300548>

Research Article

Reduced graphene oxide hydrogels and xerogels provide efficient platforms for immobilization and laccase production by *Trametes pubescens*

Susana Rodriguez-Couto, Alejandro Arzac, Gracia Patricia Leal and Radmila Tomovska

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