Evaluating the impact of two different serum-free media and bioreactor systems on growth and productivity of three recombinant IgM-expressing CHO cell lines

Master thesis

Vienna, April 2015

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Acknowledgements

I’d like to thank Prof. Renate Kunert for the invaluable academic advice and support during the creation of my master thesis in her working group.

I’d like to thank Alexander Mader and David Reinhart for the extensive support throughout the practical work of my master thesis.

I further want to thank Willibald Steinfellner, Lukas Damjanovic, Patrick Mayrhofer and Wolfgang Sommeregger for technical support.

At last, I’d like to thank everybody in the working group for the friendly atmosphere and the encouragement to critically analyse my own work.

Abstract

Three immunoglobulin M (IgM) secreting Chinese hamster ovary (CHO) cell lines (HB617, 2G12-GL, 2G12) originating from the DG44 CHO host cell line were used in lab-scale fermentation trials aimed at comparing two different fermentation vessels (shaking flasks vs. DASGIP bioreactor) as well as two different serum-free nutrient media (simple DMEM/Ham's vs. rich ProCHO5). All methods were performed in batch mode and their performance was evaluated with respect to their specific productivity (q_P), specific growth rate (μ) and other important parameters such as viability, antibody titre and peak cell concentration. Fermentation in shaking flasks turned out to be superior to the DASGIP bioreactor as the viability of the cultures could be extended for up to 2 days (+ 22 %) and end titres increased from 365 to 502 $\mu g \times mL^{-1}$ (HB617), 46 to 96 $\mu g \times mL^{-1}$ (2G12-Gl) and 32 to 41 $\mu g \times mL^{-1}$ (2G12). The higher titres, however, were mainly caused by longer process durations. Further analysis revealed no improvement of q_P and μ. In respect of the two investigated media, results show that using the nutrient-poor DMEM/Ham's (D/H) medium had led to shorter process durations of up to 5 days less (- 45 %) as it is assumed that the culture becomes limited by nutrient(s). Furthermore, antibody titres decreased drastically from 502 to 62 $\mu g \times mL^{-1}$ (HB617), 96 to 6 $\mu g \times mL^{-1}$ (2G12-Gl) and 42 to 11 $\mu g \times mL^{-1}$ (2G12), which could not be explained by the shorter process duration. q_P decreased by 57 % and 75 % for HB617 and 2G12-Gl, respectively but remained the same for 2G12. At last, no difference in μ could be observed. In addition to the batch trials, a medium adaptation was conducted to adapt the investigated cell lines from the nutrient-rich ProCHO5 to the nutrient-poor DMEM/HAM's F12 (plus supplements) medium. This adaptation was deemed successful in shaking flasks and a subsequent subcloning procedure yielded an increase in q_P of 24% (HB617), 337% (2G12-Gl) and 617% (2G12). Lastly, a vessel adaptation from 50 mL spinner flasks into 125 mL shaking flasks in ProCHO5 medium was performed. This adaptation succeeded but we observed no change in process parameters such as q_P and μ. It has further been shown that the three cell lines under investigation showed different gene copy numbers (GCN) of the target genes coding for the single IgM subunits (light chain, LC; heavy chain, HC; and joining chain, JC). A real-time PCR (qPCR) analysis was performed and the GCN, relative to the β-actin gene of a non-producer CHO cell line, for each gene was obtained. The analysis

revealed that the relative GCN correlates directly with the overall specific productivities of the respective cell lines.

1 Introduction

The challenges for the production of safe recombinant proteins for therapeutic use are predominantly the correct folding of the protein and correct post-translational modifications (PTM's) such as the accurate addition of sugar residues. These premises can be met using mammalian cell culture, such as CHO cells. Since the glycosylation patterns of most CHO cell lines have been found to be sufficiently similar to that of the human species (Hossler et al., 2009) and also profitable titres of up to 10 $g \times L^{-1}$ (Kim et al., 2012) have been reported, CHO cell culture can be performed in suspension or as an adherent culture and is nowadays widely used in the production of therapeutic recombinant proteins, thus providing broad knowledge about cost-efficient cultivation of CHO cells on an industrial scale. It is estimated by Kim et al. (2012) that about 70% of all recombinant therapeutic proteins are today produced in CHO cell culture, though being not the only mammalian cell line available for commercial production. Approvals have been issued for therapeutics produced in baby hamster kidney (BHK), mouse myeloma-derived (NS0), human embryonic kidney (HEK-293) and the human adenovirus-transformed retina-derived PerC6 cell line (Havenga et al, 2008). Since CHO cells, however, are among the best known cell lines, authorities such as the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA), specify the safety issues related to mammalian cell culture, like absence of most human pathogenic viruses (e.g. HIV, hepatitis B virus and herpes simplex virus) and lack of prions to be within acceptable limits (Wurm and Hacker, 2011; Xu et al., 2011) in order to assure adequately safe products. These arguments and the broad availability of well-established transfection protocols mainly contribute to the popularity of CHO cell culture among the pharmaceutical industry.

First described by Urlaub and Chasin (1980), the CHO cell line consists of fibroblasts originating from an ovarian explant of the Chinese hamster, which had once undergone spontaneous immortalization during continuous *ex vivo* cultivation. By then, the CHO genome has undergone several alterations and mutations, which has led to a number of different cell lines showing different properties (Wurm and Hacker, 2011). Recently, the CHO-K1 genome has been sequenced by Xu et al. (2011) and is assumed to have a size about 2.45 Gb

harbouring 25,000 genes in 21 chromosomes. In general, genomic diversity between CHO cell lines is very high due to their genomic instability. The number of chromosomes of a specific cell line can vary significantly and is estimated to range from 18-22. Only few of those chromosomes, however, are present in a diploid state since many sister chromosomes have been altered due to extensive genomic re-organisation in the course of long-time cultivation. This has lead to a very heterogeneous CHO genome with mostly haploid chromosomes.

Many CHO cell lines used today in pharmaceutical production of recombinant proteins share one common feature: The lack of the dihydrofolate reductase (DHFR) gene, which encodes the enzyme tetrahydrofolate dehydrogenase (DHFR) (7,8-dihydrofolate:$NADP^+$ oxidoreductase; EC 1.5.1.3). The extent of this deletion varies between different CHO cell lines. While DG44 has both DHFR alleles deleted, other cell lines, such as DUKX-B11, show deletions in one allele and mutations in the other, thus switching it off. CHO cell lines that lack the DHFR gene are often subject to auxotrophic selection based on DHFR (in-)activity. Therefore, $DHFR^-$ mutants cannot survive on hypoxanthine and thymidine (HT) free medium. DHFR activity can be restored by transfecting CHO cells with the DHFR gene and subsequently selecting them on HT free medium. Co-transfection of the gene of interest (GOI) is then performed in a way that ensures an insertion spatially close to the DHFR gene. Furthermore, DHFR is also used as an amplification marker since it converts dihydrofolate into tetrahydrofolate and thus is responsible for the *de novo* synthesis of purines. This gene amplification is commonly induced via adding gradually increasing amounts of methotrexate (MTX) to the medium, which inhibits DHFR activity. The inhibition of DHFR is known to induce gene amplification of the DHFR gene (Kaufman et. al, 1983), which then compensates for this inhibition of DHFR activity via elevated expression of that protein. If the GOI is located near the (introduced) DHFR gene, it is assumed to get co-amplified along with the DHFR gene, which then is expected to augment the mRNA content and subsequently the cell's overall productivity. Other gene amplification systems, such as that based on the glutamine synthetase (GS) system, are also available and work in the same fashion. GS is responsible for the synthesis of glutamine (Gln) from glutamate and ammonia in most mammalian cells and thus is crucial for the cells supply with Gln. Cells which lack GS or in which GS is inhibited (e.g. by chemicals such as methionine sulphoximine) must be supplied

with Gln via the medium, however, cells tend to amplify this gene in order to survive on Gln depleted medium.

The major downside of using CHO cell lines is their genomic instability, which has been described before (Beckmann et al., 2012; Cao et al., 2012). Long time cultivation (> 420 days) leads to the occurrence of several subpopulations due to genomic re-organization. This problem can also occur despite constant MTX pressure, although current research results are ambiguous (Barnes et al., 2003). This represents a problem since these subpopulations tend to have different specific productivities in respect to the target protein. If this occurs, subcloning procedures are necessary to re-establish the initial q_P value.

In this thesis, CHO cells originating from the DG44 host cell line were used to produce three different IgM antibodies called HB617, 2G12 and 2G12-Gl. All antibodies were produced as fully functional pentamers and their epitopes target two different antigens. The cell lines 2G12-Gl and 2G12 produced an IgM targeted against HIV-1 (Kunert et al., 1998), while HB617 produces an IgM targeted against a tumour (Jursik et al., 2009). The IgM HB617 was originally expressed in an Epstein Barr virus transformed B cell line called HB617, which was known for activating a certain population of CD4+ cells responsible for tumour elimination (Jursik et al., 2009). The IgM 2G12 originates from the HIV-1 recognizing IgG 2G12, which then was subjected to class switch to IgM 2G12. 2G12 recognizes a complex N-linked carbohydrate motif on gpl20 of HIV-1. Both HC, LC and JC of 2G12 and HB617 were transfected via DNA:polyethylenimine polyplexes into CHO DG44 DHFR deficient cells (Life Technologies, US-CA) adapted to DMEM/Ham's medium. All cell lines also contain a neomycin resistance gene as a selectable marker (Chromikova et. al, 2014).

In this thesis, different approaches to enhance specific productivity have been investigated by adjusting different process parameters: Two different fermentation environments (shaking flask or DASGIP bioreactor), two different serum-free media (supplemented DMEM-Ham's or ProCHO5) and several other minor adjustments in process parameters (pre-warming the medium, glutamine feeding and using different inoculum cell concentrations) have been tried out. Adjusting the process parameters to the needs of the cell culture is critical, since non-optimized process conditions have been shown to lead to a collapse of production due to e.g. rapid accumulation of toxic side products such as lactate and ammonia. Furthermore it has been previously observed that low concentrations of glutamine (Gln) (< 0.1 mM) and glucose

(Glc) (< 0.7 mM) can further lead to error-prone glycosylations (Butler and Meneses-Acosta, 2012). Control and permanent monitoring of these process parameters thus was assumed to yield an improved performance of the cell lines. Consequently, the DASGIP bioreactor was believed to perform better compared to the shaking flasks.

In addition to the above, a medium adaptation from the nutrient-rich ProCHO5 medium to DMEM/Ham's F12 was conducted. The goal was to establish a stable continuous culture, which could later be used as a Master Cell Bank for batch fermentations in D/H medium. Since the adaptation of the CHO cells to a new environment would supposedly lead to alterations on the genetic level, the emergence of several sub-populations was assumed, which would have led to poor reproducibility and an unstable culture in general. To tackle these problems a standard subcloning procedure was performed after medium adaptation to identify the best-producing cells, which would then have been used for further cultivation.

2 Materials & Methods

2.1 Cell lines

Tab. 1: Description of all cell lines used in this thesis. The cell lines were cultivated at the given methotrexate concentration and were passaged for the given number prior to freezing.

Cell line	MTX concentration	No. of passages
CHO HB617 3E11/5G7	95 nM	67
CHO 2G12 IgM Gl 1F11/6D8	95 nM	10
CHO 2G12 IgM 9D8/4D10	95 nM	78

Tab. 1 gives the detailed name of each IgM-secreting CHO clone used as well as the number of passages they were cultivated prior to this thesis' experiments. All cell lines originated from the DG44 CHO cell line and were transfected as described above. The cell lines were cultivated in ProCHO5 medium supplemented with 0.1% Pluronic F-68, 4 mM glutamine and the given MTX concentration prior to freezing. Thawing was conducted at room temperature until the content of the vial turned liquid. Then, the sample was quickly transferred into freshly prepared ProCHO5 medium in a T-flask and incubated at 37°C and 7% CO_2

atmosphere. After the culture reached a sufficient viability, it was transferred into 50 ml TECHNE spinner flasks containing the same medium. All inoculums for the conducted cultivations and adaptations were taken from these spinner flasks.

At the end of this thesis' experiments, all cell lines were passaged altogether 75 times until the newly obtained subclones were stored in liquid N_2 at -192°C in D/H medium.

2.1.1 Adaptation processes

For the conducted adaptations three different adaptations were investigated

a) ProCHO5 (Spinner) ==> D/H (T25) ==> **D/H (Spinner) (failed)**

b) ProCHO5 (Spinner) ==> D/H (T25) ==> **D/H (shaking flasks)**

c) ProCHO5 (Spinner) ==> **ProCHO5 (shaking flasks)**

2.1.1.1 Medium adaptation

The cell lines were adapted from their original ProCHO5 medium to D/H medium to test its influence on the culture performance. To achieve this, the strains were first passaged 4 times in the spinner in ProCHO5 medium (as described in chapter 2.2.1) until sufficient viability (>80%) was achieved. After that, an aliquot was taken from the spinner and put into a T25 flask, which already contained some fresh D/H medium plus supplements. The broth was not centrifuged prior to inoculation in order to provide some growth factors to the subsequent passage, which was believed to improve the viability of the culture. Thus, the final medium contained ProCHO5 and D/H medium in a 1:1 ratio. This procedure was continued for 35 passages with passage ratios varying from 1:1 to 1:4, hence consistently diluting the portion of ProCHO5 from the first passage. After the viability had risen again to >90%, an adaptation to the spinner was attempted. As this failed, efforts had been made to adapt the culture currently cultured in T25-flasks to shaking flasks, which eventually succeeded. This culture was then kept for 12 passages after which they were used to inoculate the D/H batch trials described in section 2.2.2.1. Before those trials, after 7 passages, an aliquot from the continuous culture was used for subcloning.

2.1.1.2 Vessel adaptation

In addition to the spinner culture, the establishment of a continuous maintenance culture in the shaking flask was attempted using ProCHO5. For that, an aliquot was taken from the

maintenance spinner after 23 passages in ProCHO5. Aliquots were again not centrifuged but taken as such and diluted with fresh ProCHO5 medium in a 125 mL shaking flask. This culture was kept in the CO_2 incubator at 37°C, 7% CO_2 and 140 rpm and observed for 14 passages.

2.2 Fermentations

All cell lines used had previously been established in ProCHO5 medium in spinner flasks and were received from a working cell bank stored in liquid N_2 at -192 °C. For the maintenance culture, cell lines were routinely propagated in ProCHO5 medium in 50 mL TECHNE spinner flasks starting with a cell concentration of 2.5×10^5 cells×mL^{-1} and passaged every three to four days. For all fermentations in 125ml shaking flasks the cultures were incubated at 37°C and under 7% CO_2 atmosphere. The incubator used was a CO_2 incubator from Kühner.

The two different media used for fermentation, termed “ProCHO5” and “D/H“, were serum-free and had the following composition:

- **“ProCHO5”:**
  ProCHO5 (Lonza) serum-free medium contained 0.1% Pluronic F-68 and was additionally supplemented with 4 mM L-alanyl-glutamin from Biochrom (Cat.No. K0302) and phenol red.
- “**D/H”:**
  DMEM/Ham's F-12 (BIOCHROM) was supplemented with 4 mM L-alanyl-glutamin from Biochrom (Cat.No. K0302), a protein-free additive, Pluronic F-68 and soy peptone prior to use.

For further information about the medium composition and supplements, see Tab. 2 and Tab. 3

For batch cultivation, three different combinations of media and vessel type were investigated (for detailed description see 2.2.2).

a) ProCHO5 medium in shaking flasks

b) ProCHO5 medium in DASGIP bioreactor

c) D/H medium in shaking flasks

Tab. 2: Composition of the respective medium additives used.

Name	Ingredients
PF-Additive 100xstock solution	6.12 g×L^{-1} Fe-(III)-citrate 2.5 µM Na-selenite 352 mg×L^{-1} L-ascorbic acid 153 mg×L^{-1} ethanolamine
Soypeptone stock	125 g×kg^{-1}
Pluronic F68	10 % Pluronic F68 from Sigma-Aldrich

Tab. 3: Composition of DMEM/Ham's F-12 from BIOCHROM according to the manufacturer.

Substance	Concentration [mg×L^{-1}]	Substance	Concentration [mg×L^{-1}]
NaCl	6999.5	L-leucine	59
KCl	311.8	L-lysine×HCl	91.25
Na_2HPO_4	71	L-methionine	17.24
$NaH_2PO_4×H_2O$	62.5	L-phenylalanine	35.5
$MgSO_4×7H_2O$	100	L-proline	17.25
$MgCl×6H_2O$	61	L-serine	26.25
$CaCl_2$	116.61	L-threonine	53.5
$Fe(NO_3)_3×9H_2O$	0.05	L-tryptophane	9
$FeSO_4×7H_2O$	0.417	L-tyrosine	38.7
$CuSO_4×5H_2O$	0.00125	L-valine	52.85
$ZnSO_4×7H_2O$	0.432	Cholin chloride	9
D-Glucose	3151	a-Biotin	0.00365
$NaHCO_3$	2438	Folic acid	2.65
Na-Pyruvate	55	D-Ca-panthothenate	2.24
Phenol red	12.5	Myo-inositol	12.6
L-alanine	4.5	Nicotinamide	2.02
L-arginine×HCl	147.5	Pyridoxal×HCl	2
L-asparagineH_2O	7.5	Pyridoxin×HCl	0.031
L-aspartic acid	6.65	Riboflavin	0.22

L-cysteine×HCl	15.75	Thiamine×HCl	2.17
L-cystine	24	Vitamine B12	0.68
Putrescine×2HCl	0.081	Hypoxanthine	2.05
L-glutaminic acid	7.35	Thymidine	0.37
Glycine	18.75	Lipoic acid	0.11
L-histidine×HCl×H_2O	31.5	Linoleic acid	0.042
L-isoleucine	54.5		

2.2.1 Maintenance cultivation

Maintenance culture was performed continuously over the course of about 6 months to obtain an inoculum for the batch trials performed in shaking flask or the DASGIP bioreactor. All maintenance cultures for ProCHO5 trials were kept in a 125 mL TECHNE spinner flask using a working volume of approximately 50 mL per spinner. Cells were passaged every 3-4 days to keep the culture constantly in the exponential growth phase. During each passage step, cells were diluted to a cell density of approximately 2.5×10^5 viable cells×mL^{-1} with fresh medium. The slightly acidic broth was not removed but just diluted with fresh medium to provide growth factors and to prevent the pH to drift into the basic region. As a medium, ProCHO5 supplemented with 4 mM alanyl-glutamine, 0.095 µM methotrexate and 0.5 g×L^{-1} G418 was used. Spinner flasks were kept in the 37°C incubation room in a closed spinner under gentle stirring. During initial inoculation of each spinner, the headspace was saturated with pure gaseous CO_2 to provide a sufficient buffer capacity for the initial growth phase. During all later passages this was omitted, since the broth was assumed to contain enough buffer capacity due to the CO_2 and lactate already produced by the culture itself, to prevent pH to shift above pH 7.

As all attempts to adapt the D/H cultures to the spinner flask failed (see section 2.1.1.1), all maintenance cultures for D/H medium were kept in 125 mL shaking flasks in DMEM Ham's F12 medium from BIOCHROM supplemented with 4 mM alanyl-glutamine, protein free (PF)-Additive, soy peptone, Pluronic F-68, 0.095 µM MTX and 0.5 g×l^{-1} G418. Passage was again performed every 3-4 days by diluting the broth to approximately 2.5×10^5 viable cells×mL^{-1}. Again, the cell broth was not discarded but used for the subsequent passage. The shaking flasks were kept in the CO_2 shaking incubator at 37°C 7% CO_2 and 140 rpm.

Tab. 4: Culture parameters for the maintenance cultures used as inoculums for the batch trials.

Reactor	125 mL Spinner (TECHNE) **OR** 125 mL shaking flask
Medium and supplements	ProCHO5 plus 4 mM glutamine, phenol red, 0.095 µM methotrexate and 0.5 $g \times l^{-1}$ G418 **OR** D/H medium plus 4 mM glutamine, protein free additive, soy peptone, Pluronic F-68, 0.095 µM methotrexate and 0.5 $g \times l^{-1}$ G418
Working volume	50 mL
Inoculum cell concentration	2.5×10^5 viable cells$\times mL^{-1}$
Culture parameters	37°C, pH 7.0, 50 rpm (spinner) **OR** 37°C, pH 7.0, 140 rpm, 7% CO_2 (shaking flask)
Passage	every 3 to 4 days

2.2.2 Batch cultivation

2.2.2.1 Shaking flask trials

All shaking flask trials were done in 125 mL Corning shaking flasks, with a maximum working volume of 70 mL and a starting inoculum cell concentration of $2x10^5$ $c \times mL^{-1}$. All trials were performed at 37°C, 7% CO_2 atmosphere and vigorous shaking at 140 rpm with loose screw caps to allow gas exchange.

Inoculation was performed with an initial cell concentration of $2x10^5$ viable cells$\times mL^{-1}$. To accomplish that, the respective amount of cell broth was taken either directly from the maintenance culture in a TECHNE spinner (for all fermentations performed in ProCHO5) or from a D/H-adapted culture in a shaking flask (for all fermentations in D/H medium). Medium adaptation was performed as described in section 2.1.1.1. The inoculum for each trial performed in ProCHO5 was taken from the maintenance cultures adapted to ProCHO5 in

spinners for 6 and 15 passages, respectively. Each inoculum for the D/H batches was taken from continuous cultures which were cultivated in 100% D/H medium in shaking flasks for 12 passages. It has to be noted, however, that to that point, no subcloning had been performed yet with those cultures used for inoculating these D/H batches.

After drawing off some of the broth it was centrifuged to remove the supernatant and the pellet was taken up in a few mL of fresh fermentation medium. The supernatant was removed to avoid influences caused by cell-secreted factors present in the culture's supernatant. Furthermore, all maintenance cultures (in ProCHO5 as well as in D/H medium) contained MTX and G418, which were omitted in the subsequent batches.

Batch fermentation was carried out in shaking flasks at several different conditions (as stated in Tab. 5). Sampling was done daily according to protocol (see section 2.4 "Analysis") under sterile conditions under a laminar flow hood (Thermo Fisher). Each shaking flask was shaken rigorously immediately prior to sampling to ensure an equal distribution of cells.

Tab. 5: List of all different conditions tested during batch trials performed in shaking flask. The number of replicates is included. Trials which did not yield improved performances were conducted less than three times.

MEDIUM	CONDITIONS		REPLICA
ProCHO5	Standard	37°C 7% CO_2 140 rpm $2x10^5$ $c \times mL^{-1}$ inoculum	3
	Double inoculum	Standard with 4×10^5 $c \times mL^{-1}$ inoculum	2
	Glutamine Feeding	Standard with Gln replenishment on day 5	2
	Pre-incubation	Standard with pre-incubation of the sterile medium under process conditions	1
D/H	Standard	Standard with adaptation of the culture to the shaking flask for 12 passages	3

Tab. 6: Culture parameters for all batch cultivations in shaking flasks.

Reactor	125 mL shaking flask
Medium and supplements	ProCHO5 plus 4 mM glutamine and phenol red **OR** D/H medium plus 4 mM glutamine, protein-free additive, soy peptone and Pluronic F-68
Working volume	70 mL
Inoculum cell concentration	2×10^5 viable cells×mL^{-1} **OR** 4×10^5 viable cells×mL^{-1}
Culture parameters	37°C, pH 7.0, 7% CO_2, 140 rpm

2.2.2.2 DASGIP Bioreactor Fermentation

In the DASGIP bioreactor trials, the only medium used was ProCHO5. Batch trials were performed in duplicates using a DASGIP bioreactor (Eppendorf). To assure the absence of pyrogens in the whole system, all plastic parts were de-pyrogenized by soaking them in 1 M NaOH o/n, while all glass parts were de-pyrogenized by applying dry heat at 220°C o/n. Then, the bioreactor was assembled (including all probes) and all open ends and tubings (e.g. gas outlets and inlets) were plugged with a 0.2 µm hydrophobic filter to avoid contamination after sterilization. The ready-built bioreactor was then sterilized by autoclaving it at 121°C for 30 min. Before and after autoclaving, a pressure-drop test was done to detect any leaks in the construction, which would pose a threat to the sterility. To assess the sterility of the DASGIP bioreactor and the functionality of the whole system, ~200 mL of sterile medium was added and incubated for 2-3 days under standard process conditions (37°C, stirring at 80 rpm). If a contamination would have occurred, a visible turbidity of the medium in addition to a decrease in pH and O_2-saturation would have been observed due to microbial growth. If none of the above had occurred, sterility was assumed and the subsequent fermentation was started.

To obtain a sufficient cell number for each inoculum, a pre-cultivation was performed in shaking flask for 4-5 days under standard conditions (37°C, 7% CO_2, 140 rpm in a shaking flask) until the exponential growth phase was reached. The inoculum for each pre-cultivation

was in turn taken from the maintenance ProCHO5 culture in the spinner flask, which have been cultivated in the spinner for 17 and 22 passages. Prior to the inoculation of the DASGIP bioreactor the pre-culture was centrifuged and the pellet was taken up in an equal amount of fresh fermentation medium to prevent any growth factors, MTX and G418 in the supernatant from entering the bioreactor system. Inoculation was then performed by transferring the broth into a flask with a sterile tubing system attached, which allowed an aseptic transfer from the flaks into the DASGIP bioreactor.

The inoculation was properly designed to achieve an initial working volume of 600 mL and a starting inoculum cell concentration of 2×10^5 cells×mL^{-1}. During fermentation, pH was measured on-line using a built-in pH meter (Mettler-Toledo). If required, the pH was adjusted automatically by the inflow of CO_2 or by automatically adding a bolus feed of 0.5 M NaOH. Temperature was controlled automatically using the built-in heating plate and was measured on-line using a temperature probe (Eppendorf). Dissolved oxygen (DO) was also monitored on-line using a pO_2 probe (Broadly James) and controlled via the inflow of O_2. Fermentation was performed under constant stirring at 80 rpm using a blade stirrer. When excessive foaming had occurred, anti-foam agent (Antifoam C Emulsion; Sigma-Aldrich) was added manually.

Prior to sampling, 5 mL of sample was drawn and discarded to clear the sampling tubing from old broth. Subsequently, sample was drawn for analysis of the broth as described under section “2.4 Analysis”.

T**ab. 7: Culture parameters for the batch cultivation in the DASGIP bioreactor.**

Reactor	1 L DASGIP bioreactor
Medium and supplements	ProCHO5 plus 4 mM glutamine plus phenol red
Working volume	600 mL
Inoculum cell concentration	2×10^5 viable cells×mL^{-1}
Culture parameters	37°C, pH 7.0, 80 rpm

2.3 Subcloning in D/H medium

Since each adaptation to a different condition (medium or vessel) is assumed to produce a heterogeneous culture, subcloning should be performed after such a step to ensure the establishment of a well-producing culture. Heterogeneity is presumably caused by single clones being able to cope better with the changing environment than others. In addition to the general instability of the CHO genome, heterogeneous cultures are very likely to arise especially over long-term continuous cultivation and even if culture conditions are kept constant. Thus, subcloning provides a very valuable tool for either maintaining efficiency of a well-established continuous culture or for recovering/improving efficiency of a recently adapted cell culture, as performed in this thesis. After the medium conversion to D/H medium was deemed successful (see section 2.1.1.1), subcloning was performed. For this purpose a sterile 384-well plate (Nunc) was used. Each well was filled with 50 µl of cell suspension, which was diluted in beforehand to finally contain in average one cell per well. As nutrient medium, a special "subclone"-mix was created, which contained fresh D/H medium supplemented with 4 mM glutamine, protein-free additive, soy peptone, Pluronic F-68, 0.5 $g \times l^{-1}$ G418 and 0.095 µM MTX as well as the sterile-filtered supernatant of a previous cultivation in a 1:1 ratio. This procedure should ensure that the cells growing on the 384-well plate were supplemented with 1) sufficient nutrients from the fresh medium and 2) various growth factors released from the growing CHO cells of the previous cultivation.

Cells harvested from cultures adapted to D/H-medium in shaking flasks were then taken up in the subclone mix. The 384-well plate was then filled aseptically under the laminar flow hood using a multipette. Microscopy immediately performed afterwards revealed that each well contained up to 5 cells instead of the desired 1 cell per well. Despite this unintended event, subcloning was continued without any further actions taken.

The 384-well plates were then incubated at 37°C and 7% CO_2 for 12 days until visible growth and acidification of the medium (indicated by the phenol red added) was observed in the wells. Now, those 80 wells showing the best growth were chosen, transferred aseptically onto a new 96-well plate and filled up to 300 µl with fresh D/H medium with supplements. After a 7 day incubation period, a qualitative IgM ELISA was performed to identify eight out of eighty wells containing the highest IgM concentration, which were then expanded on a new 96-well plate with fresh D/H medium as described before and finally put into a T25 flask for

further cultivation. The cells were then cultivated for another 3 passages to obtain the specific productivities of each clone. Of each cell line, the three best producing clones were then selected for further cultivation.

2.4 Analysis

2.4.1 Cell Counts

Cell counts were routinely performed with a Beckman Coulter Counter (Z2 series). Channel width was 50 µm and the analysed volume per sample was 100 µl. In this method, the cell nuclei were directly measured via impedance. Samples were prepared as followed:

Two millilitres of sampled broth was centrifuged for 10 min at 230×g and the supernatant discarded. The pellet was then re-suspended by vigorous shaking, taken up in 1 mL of "CC-Buffer" (containing 0.1 M citric acid 2 % (w/w) Triton X-100) and incubated at RT for at least 1 hour to up to one day. This was supposed to lyse the cell membrane but leave the nucleus membrane intact. Immediately prior to the measurement, the sample was diluted with the commercial diluent "ISOTON II" purchased from Beckman to obtain counts between 10,000 and 20,000 which was considered the linear range for the measurement. The measured value was then corrected for the dilution and the aliquot taken from the broth to obtain the cell concentration in cells×mL^{-1}.

2.4.2 Viability

Cell viability was routinely assessed visually using a Neubauer improved haemocytometer. 500 µl of cell broth were mixed with 100 µl of a 0.4% trypan blue dye (Sigma Aldrich) which stains dead cells only and allowed to distinguishing live from dead cells. Viability was calculated as

$$\text{Viability } [\%] = \frac{\text{counted live Cells}}{\text{counted total Cells}} * 100$$

2.4.3 IgM ELISA

IgM ELISA was routinely performed using a polyclonal IgM standard from human serum purchased from Sigma Aldrich (Cat. No. I8260). ELISA was performed using a 96 well-plate F96 Maxisorp (Nunc). Plates were evaluated on a TECAN Infinite M1000 plate reader.

On each plate, nine samples were applied, one for each column. In addition, one blank and two standard rows were applied to each plate. The samples were then diluted 1:2 with dilution buffer until the end of the column.

Tab. 8: Ingredients of all buffers and antibody solutions used for the IgM- ELISA.

Name	Ingredients
Coating buffer	0.1 M $NaHCO_3$ pH 9.6 – 9.8
Wash buffer	phosphate buffered saline pH 7.2 – 7.4 + 0.1 % Tween 20
Dilution buffer	Wash buffer + 1 % BSA (4°C)
Staining buffer	Na_2HPO_4 / citric acid buffer pH 5 (4°C)
Coating antibody solution	Goat anti human Ig (µ-chain specific), 1 mg×mL^{-1}, from Sigma (I1636), working solution was diluted 1:1000 in coating buffer
Detection antibody solution	Goat anti human kappa light chains-peroxidase, 1 mg×mL^{-1}, from Sigma (A7164), working solution was diluted 1:1000 in dilution buffer
Standard IgM	IgM from human serum, 200 ng×L^{-1}, from Sigma (I8260)

The ELISA was performed according to the following protocol:

1. Coating of MAXISORP plates from Nunc by pipetting 100 µL of coating antibody solution (1 µg×mL^{-1}) in coating buffer in each well. Incubation was done for 2 h at RT or overnight at 4 °C.

2. Dilute samples to the concentration of the standard used, which ranged from 200 ng×mL^{-1} to about 6 ng×mL^{-1} depending on the dilution. All dilutions were made in dilution buffer.

3. Wash coated MAXISORP plates three times with washing buffer.

4. Transfer 50 µL of the diluted sample into each well. Incubate for 1 h at RT at constant shaking. After incubation, wash each plate three times with wash buffer.

5. Add 50 µL of the detection antibody solution (1 µg×mL^{-1}) in dilution buffer to each well. Incubate for 1 h at RT at constant shaking. After incubation, wash each plate three times with wash buffer.

6. Add 100 µL of a solution containing 1 µL of ortho-phenylendiamin-dihydrochloride, 0.06 µL 30% H_2O_2 and 99 µL staining buffer. Incubate until the samples are sufficiently coloured (yellow) and then stop the reaction by adding 100 µL of 2.5 M H_2SO_4.

7. Measure the fluorescence of the samples with a TECAN reader at 492/620 nm. Data processing was performed with the provided Magellan calculation software from TECAN.

From these eight measurements per sample, a mean was calculated and used for assessing the IgM concentration. Outliers were manually deleted in order to obtain a relative standard deviation of <10%. For each mean value, a minimum of three values were used.

2.4.4 Metabolite analysis

Analysis of the metabolites glucose, glutamine, lactate, ammonium and glutamate were routinely performed potentiometrically using a Bioprofile Analyzer 100plus device (Nova Biomedical). One mL of supernatant or broth was used for analysis.

2.4.5 FACS analysis

To investigate whether the HC and the LC of the IgM were differently expressed during the course of the fermentation, the cell population of one DASGIP trial was sampled on day 2, 4, 6, 8 and 10 of the fermentation to perform FACS analysis. To do so, the µ HC and the kappa LC of the IgM molecules present within the cell were labelled with the fluorescent dye fluorescein isothiocyanate (FITC) using FITC-labelled antibodies specific against one of the two chains. Since the same reporter dye was used for both chains, two separate assays had to be performed. This resulted in fluorescent cell populations which were then detected in the

FACS device at an excitation/emission wavelength of 495/519 nm, respectively. As a negative control, a non-producing K1-host cell line was chosen.

The FACS protocol went as followed:

a) Fixation step

- Take an appropriate aliquot from the broth to obtain at no more than 1×10^6 cells.
- Centrifuge for 10 min at 230×g. Discard the supernatant.
- Take up the pellet in 1 mL ice cold 70% EtOH (non-methylated). Add the EtOH drop wise under constant shaking to avoid cell aggregates.
- Store the samples at 4°C until further use

b) Labelling step

- Spin down sample from the fixation step described above for 10 min at 230×g and discard the supernatant.
- Add 1 mL of PBS + 0.1% Triton X-100 drop wise to each pellet and dissolve it by gentle mixing.
- Split up sample into 2 × 0.5 mL and spin it down for 10 min at 230 g. Discard the supernatant.
- Add 1 mL of PBS + 0.1% Triton X-100 + 10% FBS drop wise to each pellet and dissolve it by gentle mixing.
- Spin down at 230 g for 10 min and discard the supernatant.
- Take up each pellet in 100 µL PBS + 0.1% Triton X-100 + 10% FBS
- Incubate the samples 30 min at 37°C
- Add 100 µl of a 1:100 diluted (in PBS + 0.1% Triton X-100 + 10% FBS) anti-human-µ-chain-FITC (in goat) or anti-human-kappa light chain-FITC (in goat) to each

sample, depending on which chain shall be stained. Since the fluorescent dye is light-sensitive, the samples were protected from light from now on until analysis.

- Incubate the samples for 1 h at 37°C
- Add 1 mL PBS + 0.1% Triton X-100
- Spin down at 230×g for 10 min and discard supernatant
- Take up pellet it 200 µl PBS and transfer sample to tubes for flow cytometry from Sarstedt (Cat. No. 55.1579)
- The samples were stored in the dark at RT until analysis.

c) FACS analysis and evaluation

The Forward Scatter (FS) to Sideward Scatter (SS) ratio of the negative sample was used in order to distinguish the live cells from the dead cells and agglomerates. For this, a spheroid gate was drawn around the most densely populated area in the FS:SS plot and then applied to the other samples. To further distinguish the IgM-secreting ("positive") from the non-secreting ("negative") counts, another gate was set along the axis showing the signal intensity of the negative control. This axis showed the frequency of each occurring signal. In this plot, a linear gate was drawn which excluded 99% (arbitrarily chosen) of all counts obtained by the negative control. This gate was then assumed to contain exclusively IgM-secreting live cells and exclude all non-secreting CHO cells. This population of cells was then compared between the single days (2, 4, 6, 8 and 10) of the DASGIP fermentation.

2.4.6 DNA isolation

For DNA extraction, 2×10^6 viable cells were harvested from one DASGIP trial on day 3, 4 and 5 in order to obtain healthy cells currently in the exponential growth phase. Each sample was washed with phosphate buffered saline (PBS) and the cell pellet was stored at -20°C until isolation. DNA isolation was performed on ice using a QIAGEN QIAamp DNA Blood Mini kit (Cat. No. 51104) according to the following procedure:

- Thaw frozen cell pellet
- Mix 200 µl PBS + 20 µl Proteinase K (from kit) per pellet
- Add the mix to each pellet
- Add 4 µl RNAse A (from kit) per pellet
- Add 200 µl Buffer AL (from kit). Mix.
- Incubate at 56°C for 10 min
- Spin down
- Add 200 µl EtOH absolute. Mix.
- Apply sample to the spin column provided
- Centrifuge for 1 min at full speed
- Discard eluate
- Add 500 µl Buffer AW1 (from kit) to the column
- Centrifuge for 1 min at full speed
- Discard eluate
- Add 500 µl Buffer AW2 (from kit) to the column
- Centrifuge for 3 min at full speed
- Place column in a fresh collection tube
- centrifuge for 1 min at full speed
- Place column in a 1.5 mL microcentrifuge tube
- Add 200 µl dH_2O
- Incubate for 1 min at RT
- Centrifuge for 1 min at full speed
- Use 2 µl of eluate to measure the DNA content at the Implen NanoPhotometer P-300. Peaks at 230 nm were considered solvent, peaks at 260 nm were considered to be DNA/RNA and peaks at 280 nm indicated a protein contamination.
- Store DNA at 4°C for further handling

2.4.7 qPCR

To determine the relative gene copy number of the target genes for the kappa light chain, µ-heavy chain and the joining chain, the copy number of the respective gene locus was measured relative to a housekeeping gene. For this purpose β-actin a crucial component of the cytoskeleton was chosen. β-actin belongs to those housekeeping genes which are more commonly used for internal standardization of RT-qPCR results (Bahr et al., 2009). By assuming that β-actin is present in an equal amount (= gene copy number) in every single cell and each cell line, we could determine the amount of target GOI relative to the β-actin gene. The aim was to investigate whether a higher GCN also coincided with a higher specific productivity q_P. Since the three cell lines under investigation showed substantial differences in their q_P, we expected also differences in their GCN. With this analysis we also could assess to which extent the GCN could explain the different q_P's, since the specific productivity of a cell can be determined by a number of factors other than the GCN alone, such as the efficiency of transcription, translation, PTM, folding, secretion, etc. There was, however, the possibility of a false-positive signal if only a (non-functional) part of the gene would have been integrated into the genome. All samples for qPCR-analysis originated from a single DASGIP fermentation in ProCHO5 medium. Three biological samples were analyzed for each target gene (β-actin, HC, LC, JC). These samples were aliquots taken from the DASGIP fermentation on Days 3, 4 and 5, at which the culture was currently in the exponential phase of growth and was thus considered to be in a similar and thus comparable metabolic state. Further, two technical replicates were carried out and at each technical replicate each sample was again applied in triplicate. In final, the total value consisted of:

(No. of technical replicates) × (No. of biological samples) × (No. of repeats) = 18 values

All qPCR runs were performed on a BIORAD MJ Mini Personal Thermal Cycler with a Mini Opticon RT-PCR System and was performed as followed:

100 µl of genomic DNA ($c = 1\ ng \times \mu l^{-1}$) from the previous DNA isolation were prepared with dH_2O and incubated at 99°C for 10 min.

A so-called “MasterMIX” was prepared as followed:

- 370 µL Supermix
- 22.2 µL sense primer
- 22.2 µL antisense primer
- 14.8 µL probe (labelled with FAM)
- 200 µL ultrapure water

The “Supermix” was purchased from Peqlab and was a KAPA Probe Fast QPCR Mastermix. All primers and probes were purchased from Sigma Aldrich and contained the sequences described in Tab. 9. After preparation of the “MasterMIX” 56 µl of it was added to 9.9 µl of the template (the genomic DNA) and from that, 3 µl were pipette in three wells on a “Low 48-well White” plate from BIORAD. The plate was then sealed with a heat-resistant plastic film to avoid evaporation during the qPCR and put into the Thermocycler. The following Cycle program was used:

First cycle:	95 °C for 5 min
Subsequent cycles (40×):	95 °C for 15 sec
	55 °C for 1 min

Tab. 9: Primer and probe sequences. All probes were labelled with FAM and were homologue to a stretch of the amplicon DNA.

Name	Sequence 5' => 3'	Amplicon size
JC sense	CTCTGAACAACCGGGAGAAC	194 bp
JC antisense	GTTCCGGTCGTAGGTGTAGC	
JC probe	[6FAM]CACCTGTCCGACCTGTGCAAGAA[TAM]	
LC sense	TGTGCCTGCTGAACAACTTC	183 bp
LC antisense	AGGCGTACACCTTGTGCTTC	
LC probe	[6FAM]AGCAGCACCCTGACCCTGTCCAA[TAM]	
β-actin sense	TTGCTGATCCACATCTCCTG	97 bp
β-actin antisense	TGAGCGCAAGTACTCTGTG	
β-actin Probe	[FAM]CCATCCTGGCCTCACTGTCCACCT[TAM]	
HC sense	GAGGAGGAGTGGAACACAGG	118 bp
HC antisense	ACGTTGTACAGGGTAGGTTTGC	
HC probe	[6FAM]CACGAGGCCCTGCCTAACCG[TAM]	

Data processing was performed with the BioRad CFX Manager 3.0. As baseline correction, the "linear regression" option was chosen. The C_q-values (Takeoff points) obtained from this software were subsequently analysed using a simple EXCEL sheet using a method described by Heid et al. (1996). In our method we further took potential inefficiencies of the polymerase into account and thus calculated the efficiency of each PCR step by ourselves using a mathematical approach following the model LinRegPCR described in Ruijter et al. (2013). We calculated those efficiencies for the polymerase for each target gene with the software LinRegPCR. The Efficiency E describes the fold-increase of target DNA per PCR-cycle and should ideally be exactly 2. However, due to enzyme inactivation, inhibition or other effects the polymerase might have a lower efficiency, which was estimated using the model mentioned above.

Tab. 10: Calculated efficiency for each PCR round using the software LinRegPCR. The ideal efficiency describes the fold-increase of target DNA per PCR-cycle and should be exactly 2.

Gene	Efficiency E
Beta-actin (Reference)	1.977
HC	1.955
LC	1.954
JC	1.929

The gene copy numbers for each cell line were calculated as followed:

$$\frac{\text{Mean}(E_{ref}^{Cq_{ref}})}{\text{Mean}(E_{target}^{Cq_{target}})} = \text{relative gene copy number (rgcn)}$$

The value obtained basically mirrors how much higher the C_q-value of the reference gene β-actin is compared to that of a target gene. Since the C_q-value correlates indirectly with the amount of DNA present in the sample, a high value indicates that there was less $DNA_{Reference}$ present compared to DNA_{Target}. So a high value indicated a high relative gene copy number of the target gene compared to the β-actin gene.

2.4.8 Agarose gel electrophoresis

Agarose gels were run to determine the quality of the DNA purification and the qPCR. The genomic DNA was applied to a 0.8% agarose gel to determine impurities caused by proteins or single nucleotides by comparison with the spectroscopic measurement. Proteins could have been present due to poor purification efficiencies, while single nucleotides would indicate degradation processes of the DNA. Since the latter could not be detected by the photometric method applied, the quality had to be checked on an agarose gel. Therefore, another 2% agarose gel was run with the amplicons obtained from the qPCR run. For this purpose, the amplicons after the qPCR were subject to gel electrophoresis. The amplicons would have been checked for primer dimers which could have influenced the previous qPCR efficiency. As a marker, the Gene Ruler DNA Ladder mix from Thermo Scientific was used.

Genomic DNA of each cell line cultivated in the DASGIP on ProCHO5 medium was separated on a 0.8% agarose gel created with TAE buffer (containing 40 mM Tris, 20 mM acetic acid and 1 mM EDTA) with 0.002% ethidium bromide (EtBr). The gel was run in TAE buffer containing EtBr. A constant voltage of 100 V was applied during the course of the run, which took approximately 30 min to finish. Samples were taken from Day 4, 5 and 6 from each cell line. As a positive control, DNA of a DG44 non-secreting cell line was purified and applied on one line. 200 ng DNA were applied per pocket.

Amplicon DNA of each cell line cultivated in the DASGIP on ProCHO5 medium was separated on a 2.0% agarose gel created with TBE (containing 89 mM Tris, 89 mM boric acid and 2,1 mM Na_2-EDTA.2xH_2O (Titriplex III)) buffer with 0.002% EtBr. The gel was run in TBE buffer containing EtBr. Samples were extracted from the "Low 48 well White" plates after qPCR had been conducted. All samples were analysed from Day 4 and the amplicons of HC, LC, JC as well as of the housekeeping gene, β-actin were applied on the gel.

All gels were evaluated with a Gel-Doc XR from Biorad.

3 Results

3.1 Fermentations

3.1.1 Influence of fermentation vessel (in ProCHO5)

In this section, the behaviour of the cultivated cell lines is compared with respect to the cultivation vessel used. During the fermentation in the shaking flasks and the DASGIP bioreactor the temperature, stirrer/shaking speed and the starting inoculum cell concentration were kept constant. Only in the DASGIP bioreactor the pH and pO_2 were additionally controlled. It is evaluated, which of the three CHO cell lines (HB617, 2G12-Gl, 2G12) performs best in the given fermentation vessel. Trials in shaking flask were done in triplicates, while trials in the DASGIP bioreactor were done in duplicates.

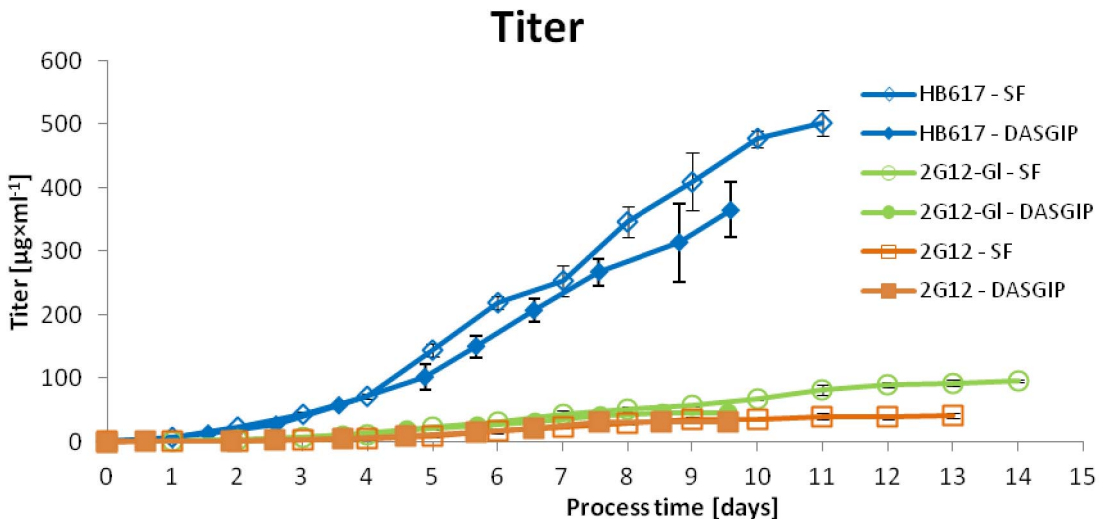

Fig. 1: Product titre of the respective cell lines during fermentation in shaking flasks (empty boxes) and in the DASGIP bioreactor (filled boxes).

Fig. 1 shows the titres of the respective IgM produced in the different cell lines. Cultivation in shaking flasks and in the DASGIP bioreactor was compared. The batch cultures in shaking flasks reached maximal product concentrations of 502 µg×mL^{-1} (HB617), 96 µg×mL^{-1} (2G12-Gl) and 41 µg×mL^{-1} (2G12). During fermentation in the DASGIP system, 27 % (HB617), 52 % (2G12-Gl) and 22 % (2G12) lower final titres were obtained.

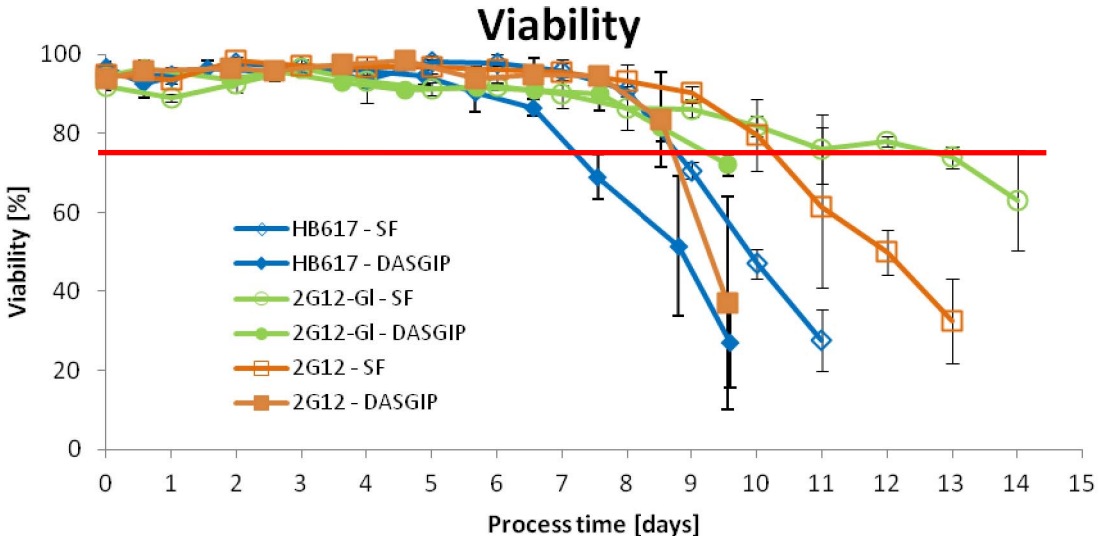

Fig. 2: Viability of the respective cell lines during fermentation in shaking flasks (empty boxes) and in the DASGIP bioreactor (filled boxes). Fermentation was considered finished after viability dropped below an arbitrary threshold (<80%), which is indicated by the horizontal red line.

As shown in Fig. 2, cultivation in the shaking flask led to a considerably longer (≤ 2 days) viability of the culture compared to cultivation in the DASGIP. The viability of the cell line HB617 always dropped below 80% at first, while 2G12-Gl dropped below 80% at last.

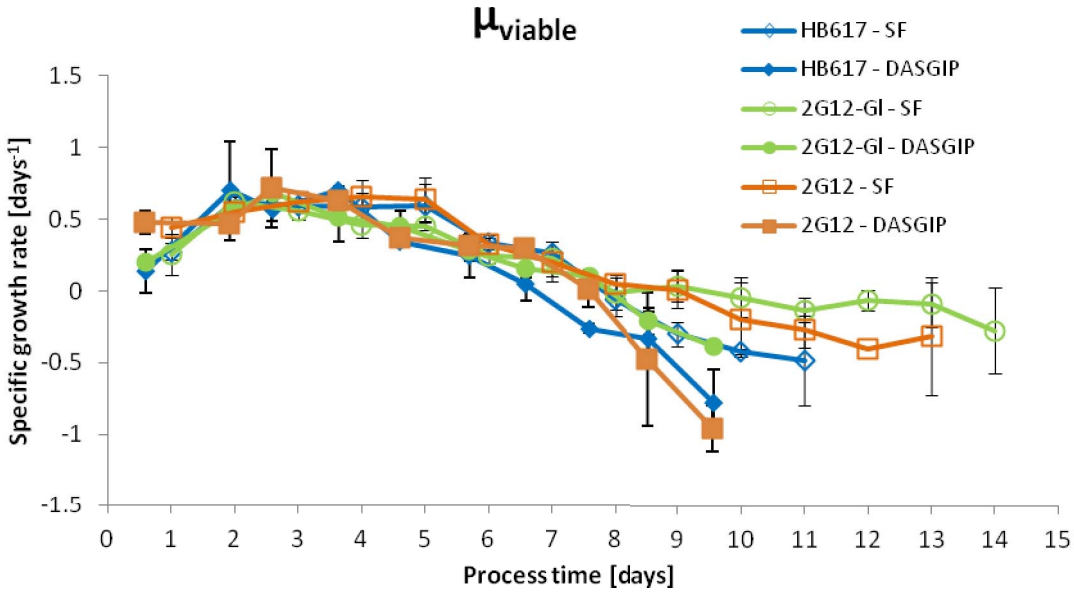

Fig. 3: Growth rate of the respective cell lines during fermentation in shaking flasks (empty boxes) and in the DASGIP bioreactor (filled boxes). Maximum growth rates achieved were 0.7 day^{-1} for both culture methods.

As shown in Fig. 3, μ of all investigated cell lines were quite similar to each other independent from whether they were grown in shaking flasks or the DASGIP bioreactor. During the exponential growth phase (day 1 to 7) in the shaking flask, μ of 0.47 day^{-1} (HB617), 0.40 day^{-1} (2G12-Gl) and 0.49 day^{-1} (2G12) were observed. In the DASGIP bioreactor, respective μ of 0.39 day^{-1} (HB617), 0.41 day^{-1} (2G12-Gl) and 0.47 day^{-1} (2G12) were obtained.

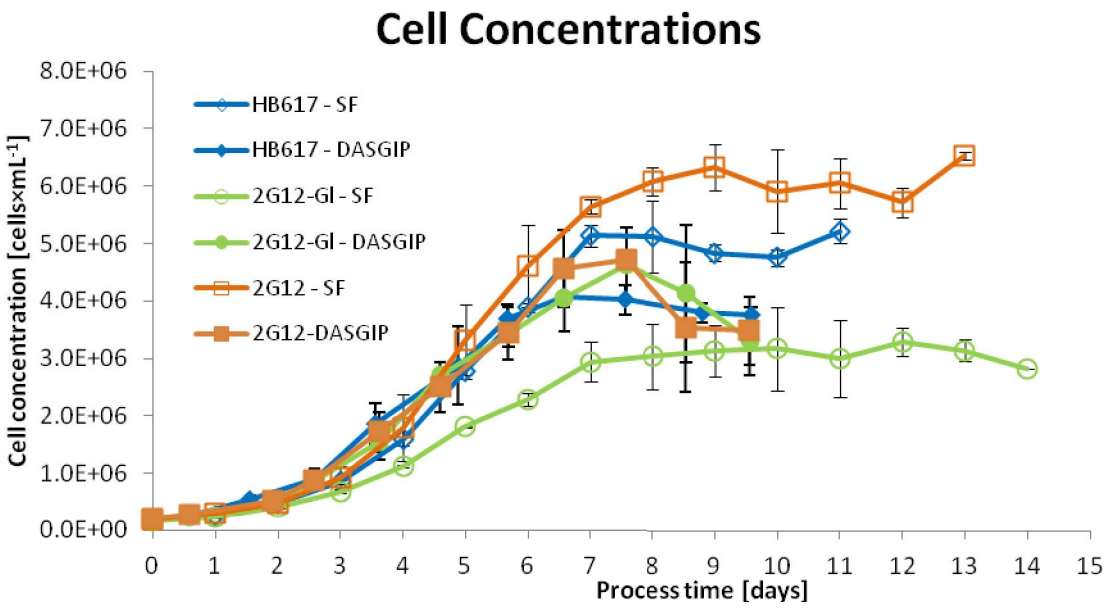

Fig. 4: Cell counts of the respective cell line during fermentation in shaking flasks (empty boxes) and in the DASGIP bioreactor (filled boxes).

Fig. 4 shows that the peak cell concentrations were similar for all cell lines in the DASGIP (4.1 - 4.7×10^6 cells×mL^{-1}). However, in the shaking flask, the maximum cell counts were quite different with 4.9×10^6 cells×mL^{-1} (HB617), 2.7×10^6 cells×mL^{-1} (2G12-Gl) and 5.7×10^6 cells×mL^{-1} (2G12).

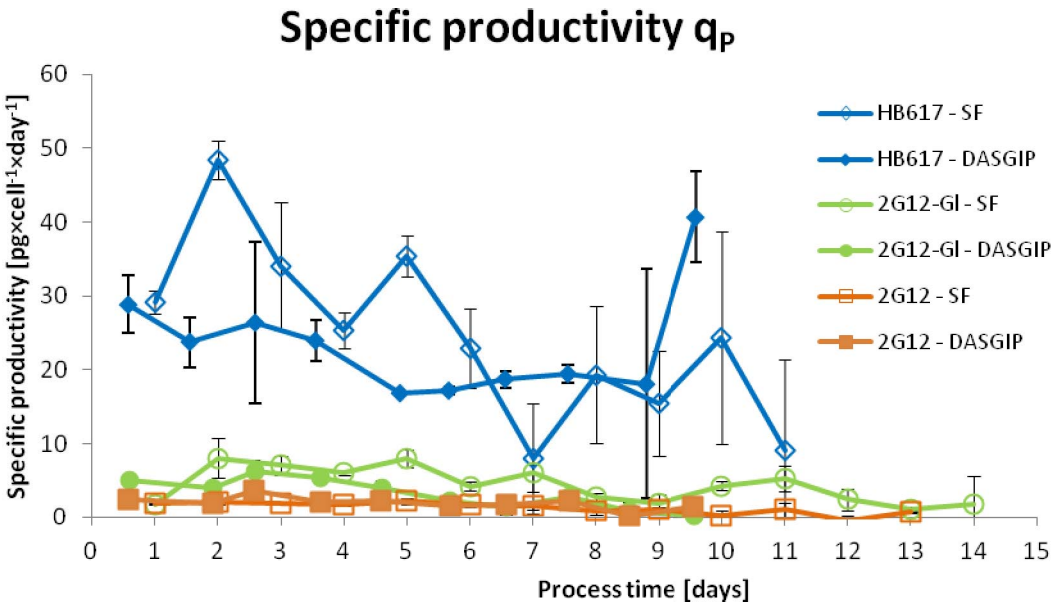

Fig. 5: Cell-specific productivity of the respective cell line during fermentation in shaking flasks (empty boxes) and in the DASGIP bioreactor (filled boxes).

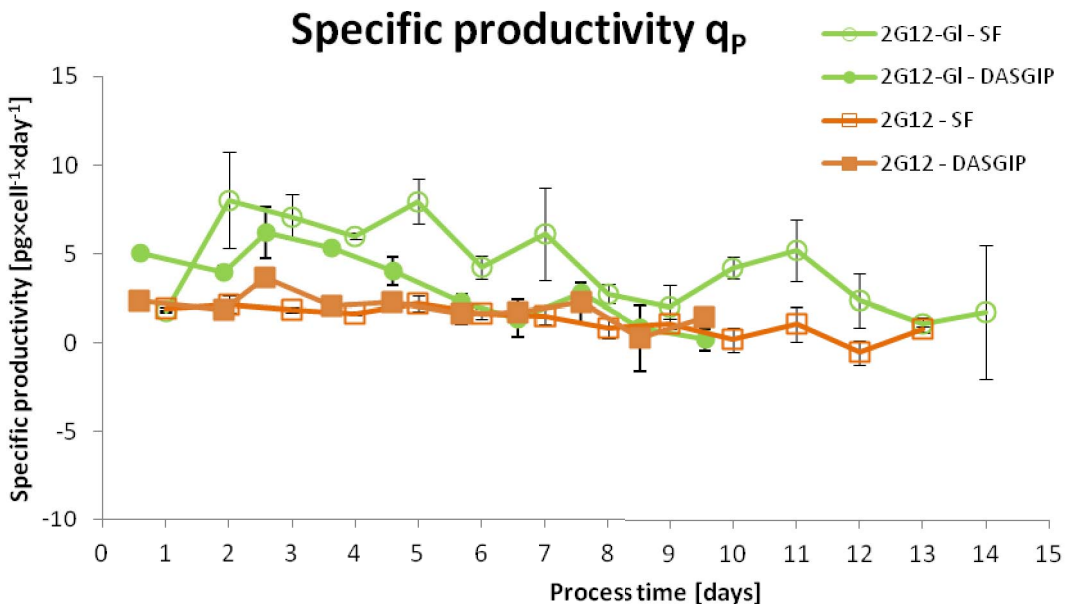

Fig. 6: Close-up of the cell-specific productivity of the respective cell line during fermentation in shaking flask (empty boxes) and in the DASGIP bioreactor (filled boxes).

The specific productivity of each cell line is shown in Fig. 5 and Fig. 6. Over the whole course of the fermentation (for the DASGIP and the shaking flasks, respectively) q_P-values of 20.46 and 19.79 pg×cell^{-1}×day^{-1} (pcd) for HB617, 2.28 and 4.13 pcd for 2G12-Gl and lastly 1.56 and 1.05 pcd for 2G12. For direct comparison, see **Tab. 11**.

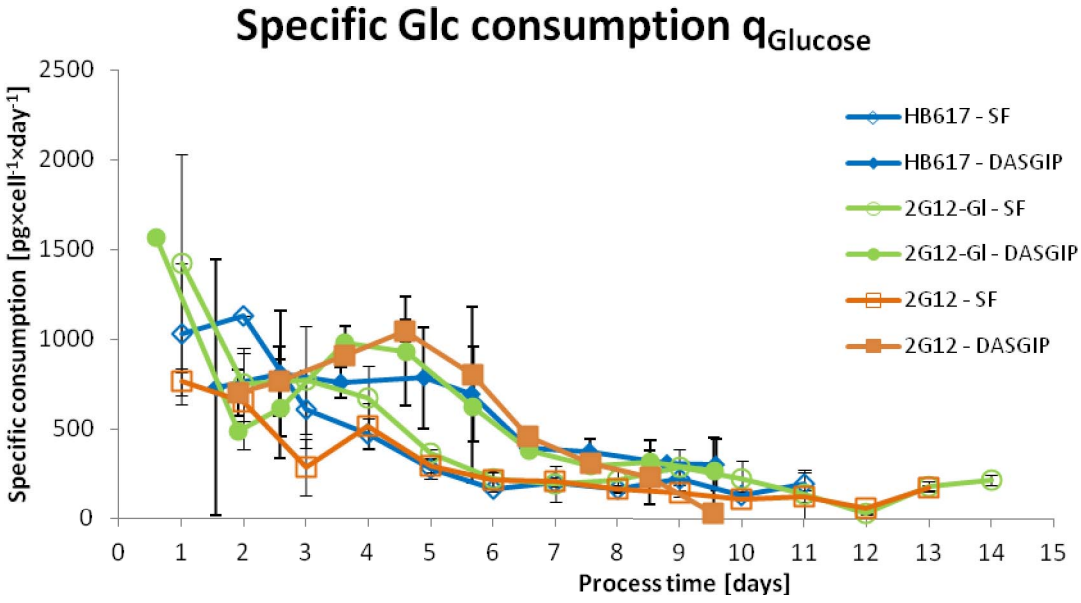

Fig. 7: Specific glucose consumption rates of the respective cell line during fermentation in shaking flasks (empty boxes) and in the DASGIP bioreactor (filled boxes).

As glucose is considered one of the two main nutrients, its specific rate of consumption was also monitored throughout the course of each fermentation. Fig. 7 shows, that cultivation in the DASGIP led to a higher specific consumption of Glc in between days 5 – 8. The high standard deviations during the first days of fermentation render a meaningful interpretation difficult and might be due to very small differences in the total concentration of Glc in the medium and the low initial cell concentrations.

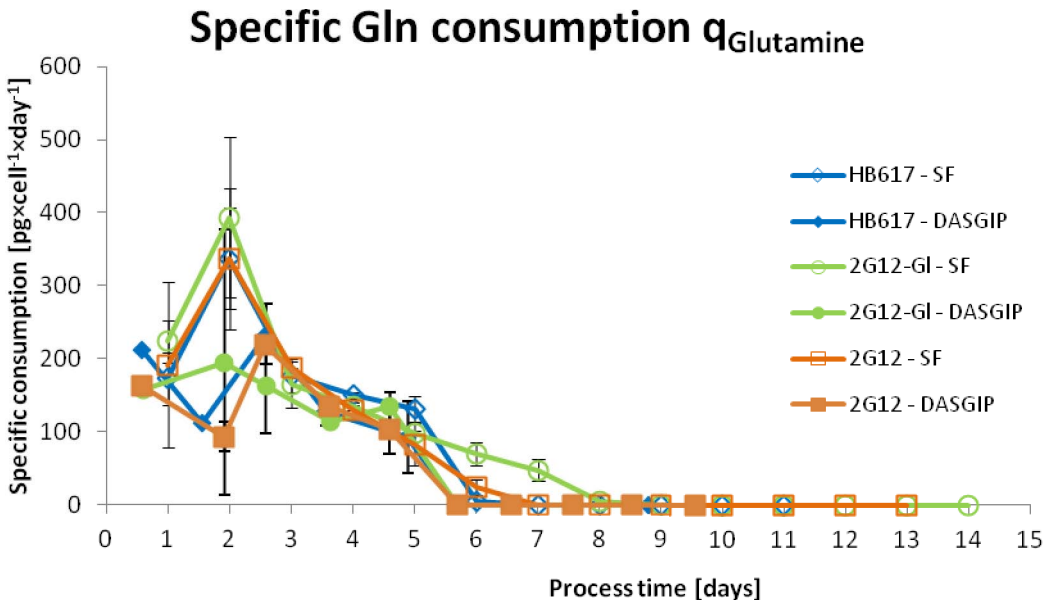

Fig. 8: Specific glutamine consumption rates of the respective cell during fermentation in shaking flasks (empty boxes) and in the DASGIP bioreactor (filled boxes).

Similar to glucose, glutamine is considered to be the second main nutrient. Unlike q_{Glc}, however, Fig. 8 shows no differences of q_{Gln} between shaking flask and DASGIP cultivation except for single days. Depletion of Gln occurs between day 6 and 8 and remained below a detectable level afterwards.

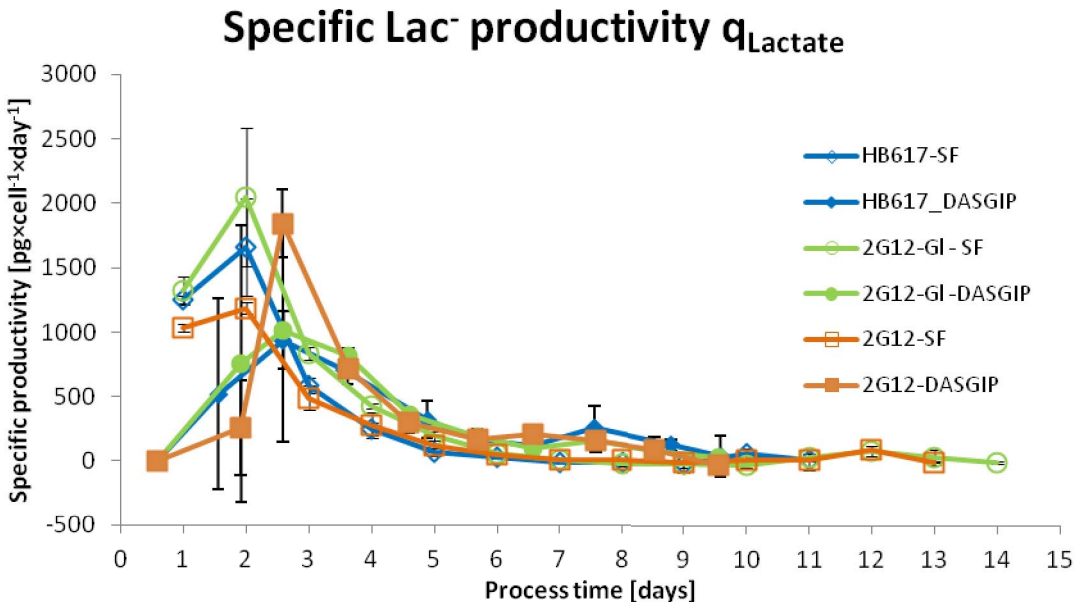

Fig. 9: Specific production rate of lactate of the respective cell line during fermentation in shaking flasks (empty boxes) and in the DASGIP bioreactor (filled boxes).

Another metabolite, lactate, is considered a critical by-product of mammalian cell fermentation. Its production is depicted in Fig. 9. Starting with day 4, the specific lactate production had been higher in the DASGIP than in the shaking flask.

Tab. 11: Short overview over all critical parameters of the investigated cell lines comparing the shaking flasks to the DASGIP bioreactor. Every fermentation was performed in ProCHO5. The growth rate was calculated as averages over all values obtained from the exponential growth phase of the culture (days 1 to 7). The q_P was calculated by dividing the end titre through the accumulated viable cell counts to that time. The process duration is given as the number of days until viability dropped below 80 %.

Cell line	Parameter	DASGIP bioreactor	Shaking flasks	Difference in shaking flask
HB617	Peak cell concentration [cells×mL^{-1}]	$4.10\times10^6 \pm 0.04\times10^6$	$4.94\times10^6 \pm 0.11\times10^6$	+ 20 %
	µ [day^{-1}]	0.39 ± 0.27	0.47 ± 0.17	+ 20 %
	Process duration [days]	7	8	+ 14 %
	End titre [µg×mL^{-1}]	365 ± 43	502 ± 21	+ 38 %
	q_P [pcd]	20.46 ± 1.19	19.79 ± 0.61	- 3 %
2G12-Gl	Peak cell concentration [cells×mL^{-1}]	$4.60\times10^6 \pm 0.64\times10^6$	$2.70\times10^6 \pm 0.43\times10^6$	- 41 %
	µ [day^{-1}]	0.41 ± 0.20	0.40 ± 0.16	- 1 %
	Process duration [days]	9	11	+ 22 %
	End titre [µg×mL^{-1}]	46 ± 9	96 ± 2	+ 110 %
	q_P [pcd]	2.28 ± 0.19	4.13 ± 0.82	+ 81 %
2G12	Peak cell concentration [cells×mL^{-1}]	$4.70\times10^6 \pm 0.01\times10^6$	$5.71\times10^6 \pm 0.28\times10^6$	+ 21 %
	µ [day^{-1}]	0.47 ± 0.16	0.49 ± 0.17	+ 5 %
	Process duration [days]	9	10	+ 11 %
	End titre [µg×mL^{-1}]	32 ± 5	41 ± 3	+ 30 %
	q_P [pcd]	1.56 ± 0.28	1.05 ± 0.06	- 33 %

Tab. 12: Short overview over all metabolic parameters of the investigated cell lines comparing the cultivation vessel, shaking flasks or the DASGIP bioreactor. Every fermentation was performed in ProCHO5. All parameters were calculated as averages over all values obtained from the exponential growth phase of the culture (days 1 to 7).

Cell line	Parameter	DASGIP bioreactor	Shaking flasks	Difference in shaking flask
HB617	$q_{Glucose}$ [pcd]	694 ± 154	555 ± 392	- 20 %
	$q_{Glutamine}$ [pcd]	111 ± 92	139 ± 115	+ 25 %
	$q_{Lactate}$ [pcd]	462 ± 321	550 ± 662	+ 19 %
2G12-Gl	$q_{Glucose}$ [pcd]	797 ± 405	628 ± 426	- 20 %
	$q_{Glutamine}$ [pcd]	153 ± 31	161 ± 118	+ 6 %
	$q_{Lactate}$ [pcd]	540 ± 375	700 ± 758	+ 30 %
2G12	$q_{Glucose}$ [pcd]	780 ± 199	419 ± 225	- 46 %
	$q_{Glutamine}$ [pcd]	143 ± 51	136 ± 115	- 5 %
	$q_{Lactate}$ [pcd]	581 ± 648	452 ± 480	- 22 %

3.1.1.1 Gene copy number

Fig. 10 shows the relative gene copy numbers (GCN) for the HC, LC and the JC genes for the three IgM clones (HB617, 2G12-Gl and 2G12) relative to the housekeeping gene β-actin. It can be inferred from the chart that the LC, HC and JC genes were similarly abundant in each cell line. A higher GCN was determined in HB617 than in 2G12-Gl than in 2G12. Further, the relative GCN correlates roughly with the specific productivities of each cell line (see Fig. 11), with q_P values being HB617 > 2G12-Gl > 2G12.

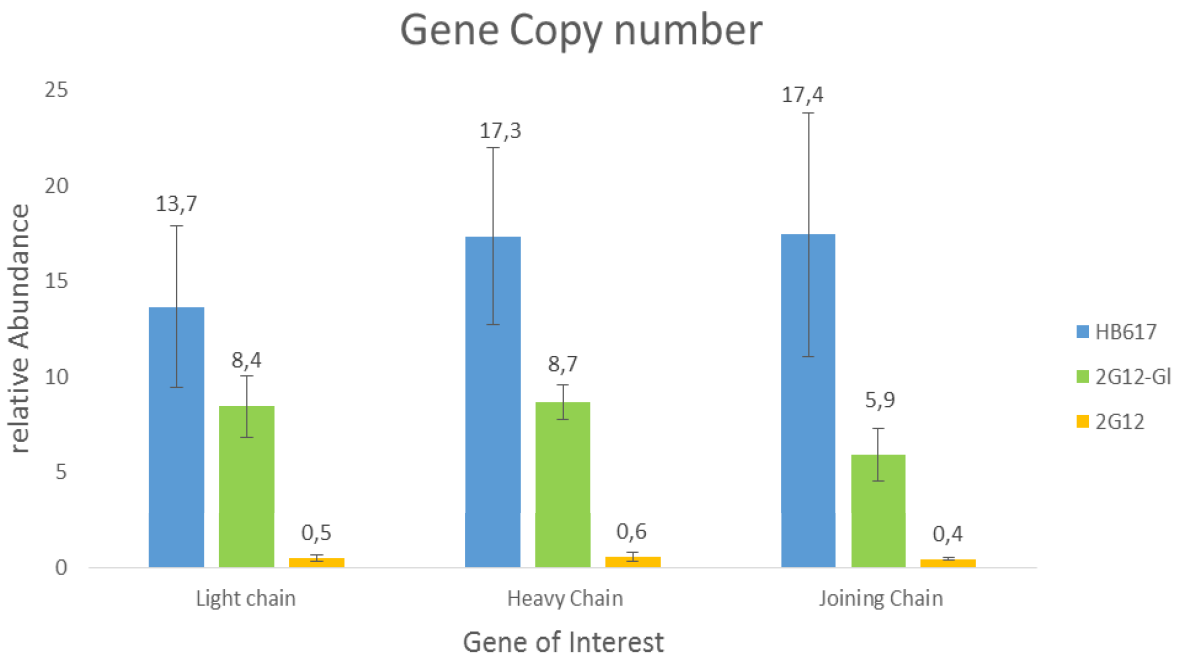

Fig. 10: This bar chart shows the relative GCN for each gene and each cell line investigated. For all genes, the β-actin gene was used as a reference. Each colour represents a different cell line.

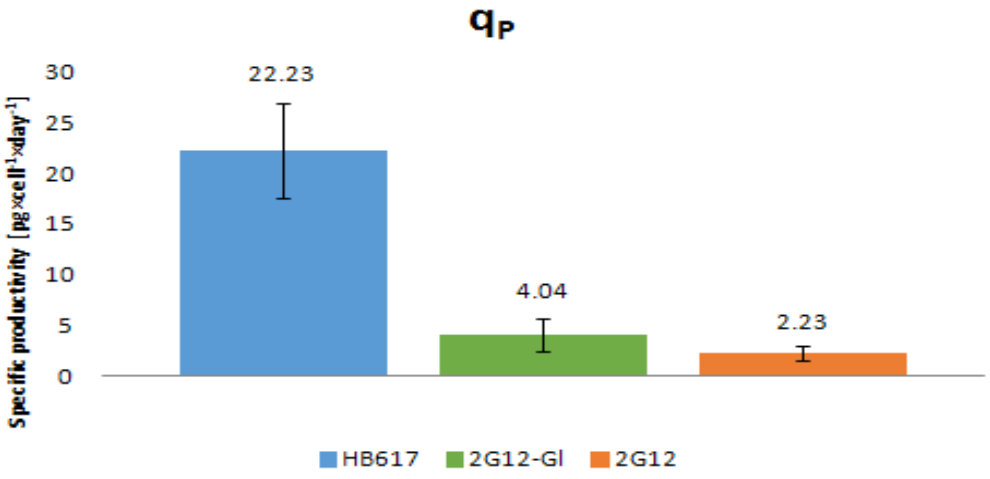

Fig. 11: Averaged q_P values obtained from the DASGIP trial performed in ProCHO5. The values were averaged over the exponential phase of growth in the DASGIP bioreactor (Day 1 to 7).

3.1.2 Influence of fermentation medium (in shaking flasks)

In this section, the two media ProCHO5 and DMEM/Ham's F12 are compared with respect to important fermentation parameters. ProCHO5 is a commercially available medium specifically designed for the cultivation of CHO cells. It is, however, unknown in its chemical composition and rather expensive, while D/H medium would be a cheaper alternative. The composition of D/H medium and its supplements are stated in Tab. 2 and Tab. 3. All trials were done in triplicates and performed in shaking flask at identical conditions.

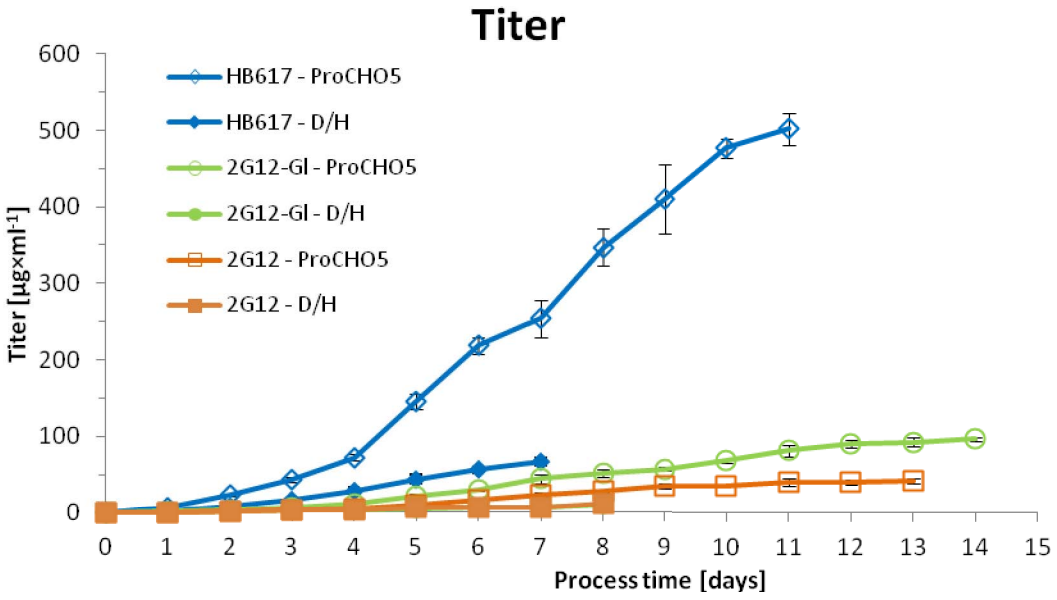

Fig. 12: Product titre of the respective cell lines during fermentation in ProCHO5 (empty boxes) or DMEM-Ham's F12 (filled boxes) medium.

As shown in Fig. 12, final titres of 502 µg×mL^{-1} (HB617), 96 µg×mL^{-1} (2G12-Gl) and 42 µg×mL^{-1} (2G12) were reached when the cells were cultured in ProCHO5 medium. Batch cultures in D/H medium resulted in 87 % (HB617), 94 % (2G12-Gl) and 74% (2G12) lower IgM concentrations.

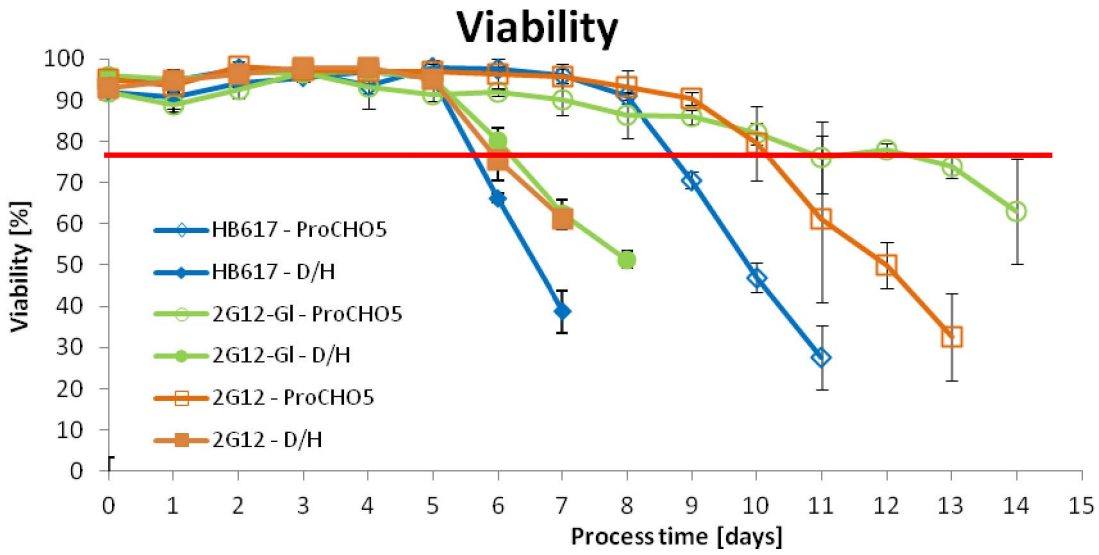

Fig. 13: Viability of the respective cell line during fermentation in ProCHO5 (empty boxes) or DMEM-Ham's F12 (filled boxes) medium. Fermentation was considered finished after viability dropped below an arbitrary threshold (<80%) which is indicated by the horizontal red line.

Fig. 13 clearly shows that when cultivated in D/H medium, the cell culture lost viability three to five days earlier compared to ProCHO5. Furthermore, all cell lines started to drop below 80% viability at the very same time (day 6) when cultivated in D/H medium, unlike in ProCHO5 medium.

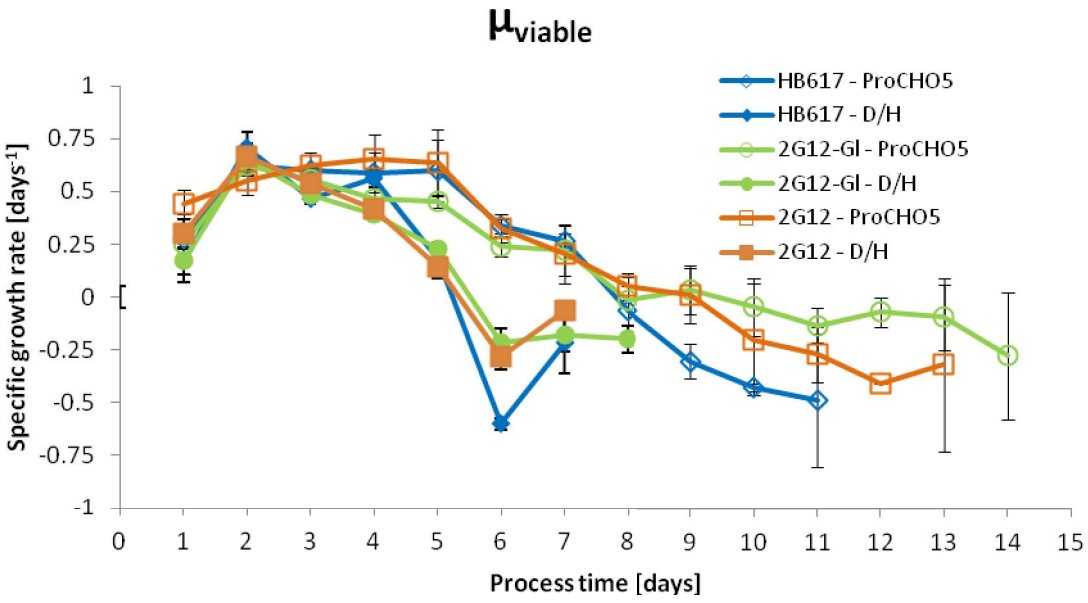

Fig. 14: Growth rate of the respective cell line during fermentation in ProCHO5 (empty boxes) or DMEM-Ham's (filled boxes) medium.

Fig. 14 shows the specific growth rates of all cell lines. On average it was around 0.4 to 0.5 day^{-1} for every cell line independent of the medium used. However, due to the longer culture duration in ProCHO5 also more cells accumulated throughout the batch process. This figure also mirrors the premature loss of viability of the cells in D/H medium compared to ProCHO5 medium.

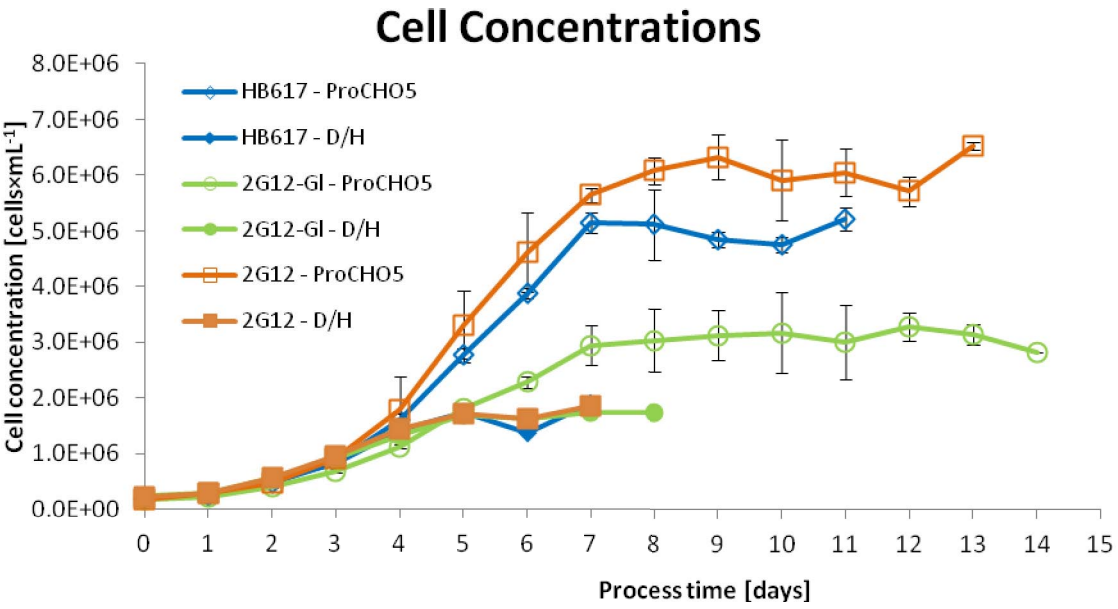

Fig. 15: Cell counts of the respective cell line during fermentation in ProCHO5 (empty boxes) or DMEM-Ham's (filled boxes) medium.

Maximum cell counts in D/H medium were much lower compared to those in ProCHO5 medium (Fig.13). In ProCHO5 peak cell concentrations of 4.94×10^{6} $c\times mL^{-1}$ (HB617), 2.70×10^{6} $c\times mL^{-1}$ (2G12-Gl) and 5.71×10^{6} $c\times mL^{-1}$ (2G12) were reached. Batch cultures in D/H medium resulted in 66 % (HB617), 40 % (2G12-Gl) and 72 % (2G12) lower peak cell concentrations.

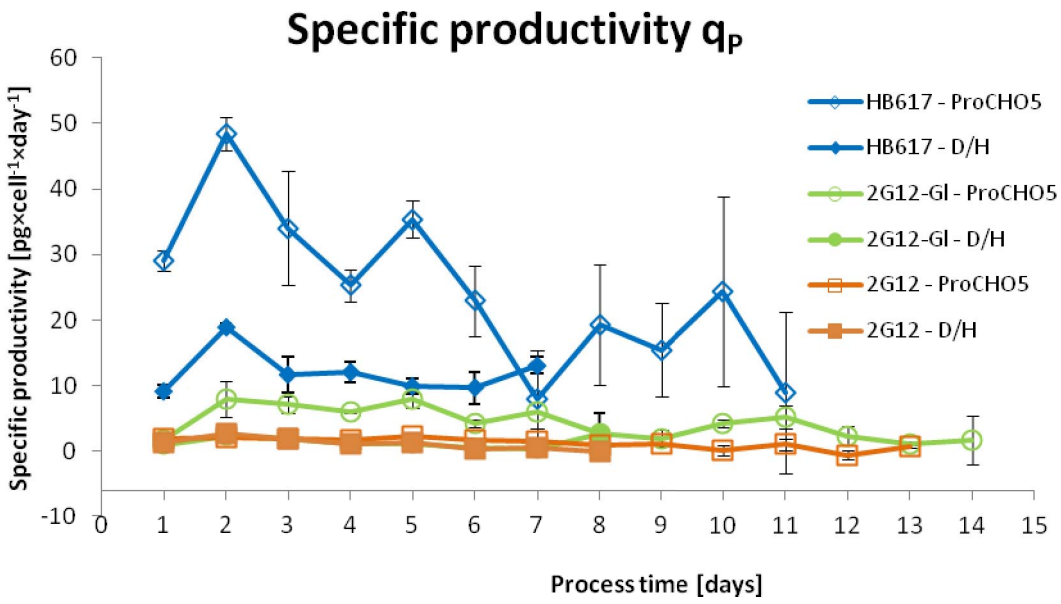

Fig. 16: Specific productivity of the respective cell line during fermentation in ProCHO5 (empty boxes) or DMEM-Ham's (filled boxes) medium.

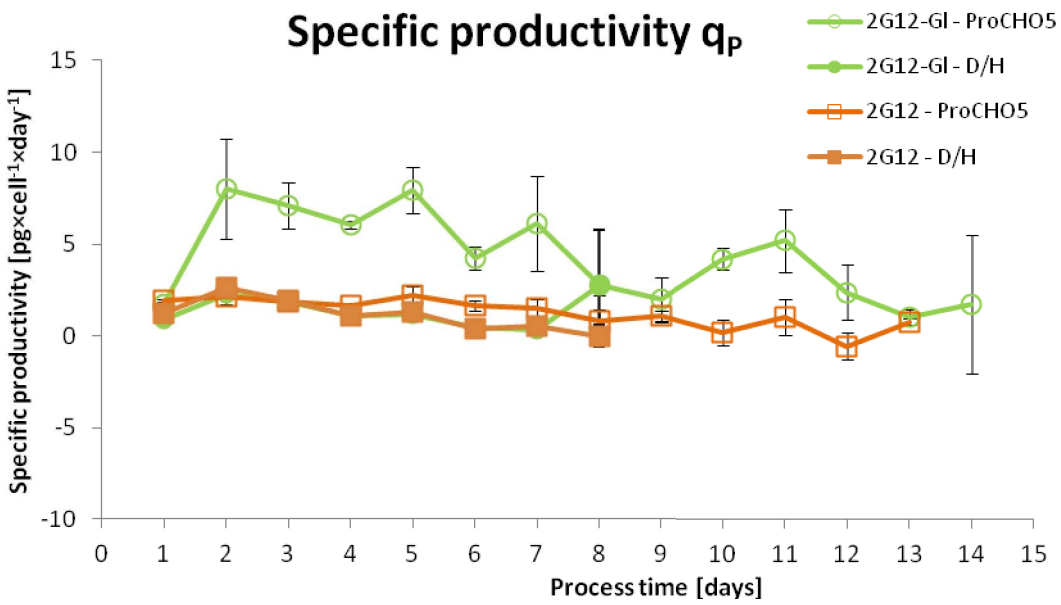

Fig. 17: Close-up of the specific productivity of the respective cell line during fermentation in ProCHO5 (empty boxes) or DMEM-Ham's (filled boxes) medium.

The specific productivity of each cell line is shown in Fig. 16 and Fig. 17. Over the whole course of the fermentation (for D/H and ProCHO5, respectively) q_P-values of 11.43 or 19.79 pcd for HB617, 1.13 and 4.13 pcd for 2G12-Gl and lastly 1.32 and 1.05 pcd for 2G12 were obtained. For direct comparison, see Tab. **13**.

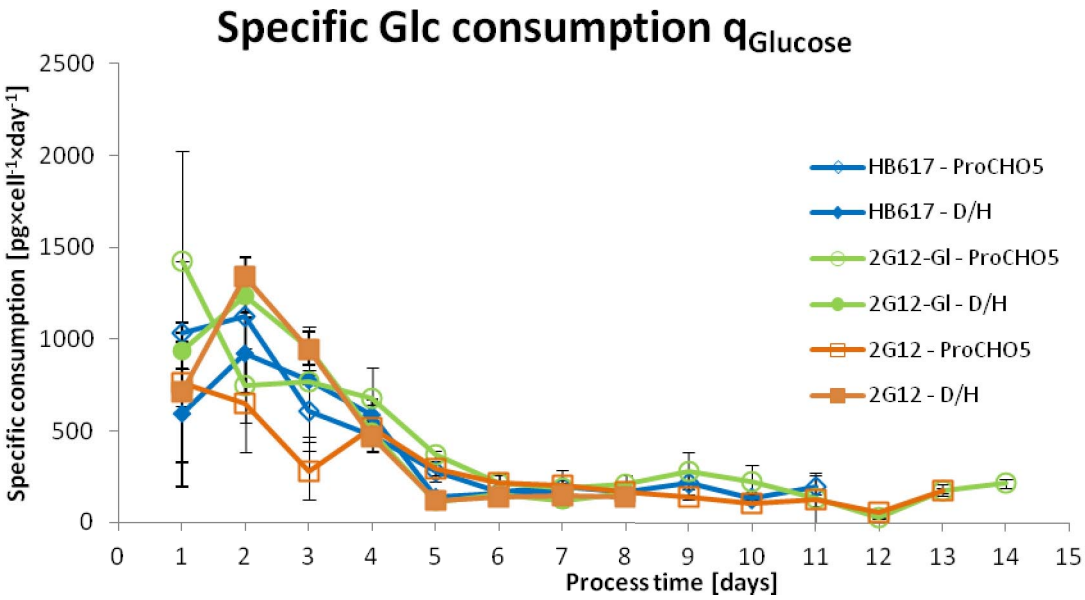

Fig. 18: Specific glucose consumption rate of the respective cell line during fermentation in ProCHO5 (empty boxes) or DMEM-Ham's (filled boxes) medium.

From Fig. 18 it can be inferred that, except for 2G12, which consumed more Glc when cultured in D/H medium, all cell lines showed similar glucose consumption rates independent of the medium.

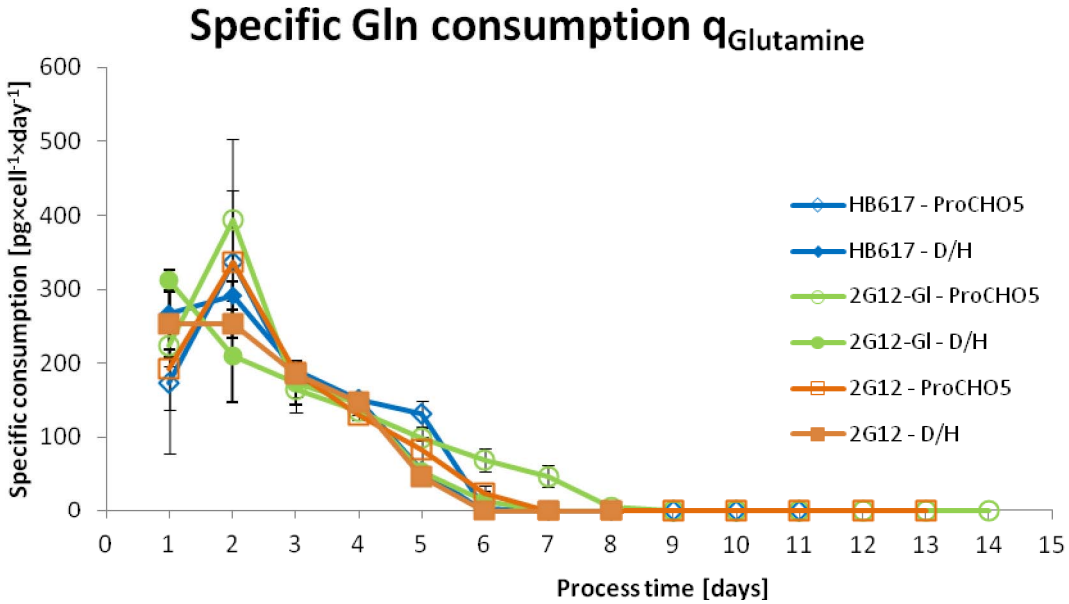

Fig. 19: Specific glutamine consumption rate of the respective cell line during fermentation in ProCHO5 (empty boxes) or DMEM-Ham's (filled boxes) medium.

The above figure shows the q_{Gln}, which was identical for all cell lines except for 2G12-Gl, which consumed glutamine over a longer period of time when cultured in ProCHO5 medium.

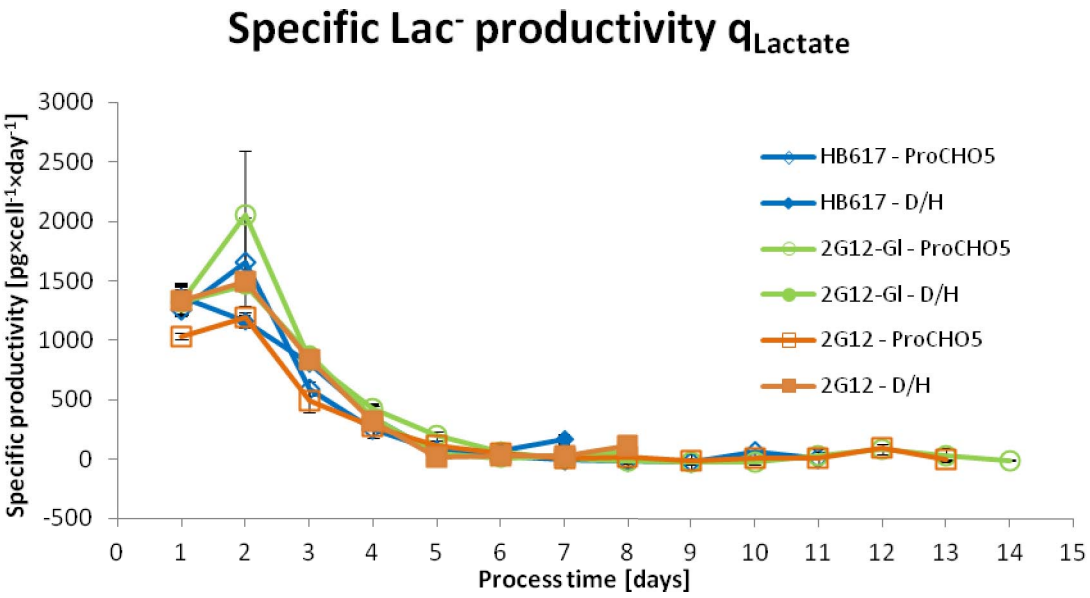

Fig. 20: Specific lactate formation rates of the respective cell line during fermentation in ProCHO5 (empty boxes) or DMEM-Ham's (filled boxes) medium.

Fig. 20 shows that, except for 2G12, which produced more lactate during the first three days of fermentation if cultivated in D/H medium, all cell lines investigated showed the same formation rate of lactate irrespective of the used medium.

Tab. 13: Short overview over all critical parameters of the investigated cell lines comparing two different media, ProCHO5 and D/H. Every fermentation was performed in shaking flasks. The growth rate was calculated as averages over all values obtained from the exponential growth phase of the culture (days 1 to 7 for ProCHO5 and days 1 to 5 for D/H). The q_P was calculated by dividing the end titre through the accumulated viable cell counts to that time.

Cell line	Parameter	D/H	ProCHO5	Deviation in D/H
HB617	Peak cell concentration [cells×mL^{-1}]	1.66×10^6 ± 0.54×10^6	4.94×10^6 ± 0.11×10^6	- 66 %
	µ [day^{-1}]	0.42 ± 0.24	0.47 ± 0.17	- 11 %
	Process duration [days]	5	8	- 38 %
	End titre [µg×mL^{-1}]	67 ± 5	502 ± 21	- 87 %
	q_P [pcd]	11.43 ± 0.76	19.79 ± 0.61	- 42 %
2G12-Gl	Peak cell concentration [cells×mL^{-1}]	1.62×10^6 ± 0.04×10^6	2.70×10^6 ± 0.43×10^6	- 40 %
	µ [day^{-1}]	0.39 ± 0.19	0.40 ± 0.16	- 4 %
	Process duration [days]	6	11	- 45 %
	End titre [µg×mL^{-1}]	6 ± 1	96 ± 2	- 94 %
	q_P [pcd]	1.13 ± 0.36	4.13 ± 0.82	- 73 %
2G12	Peak cell concentration [cells×mL^{-1}]	1.62×10^6 ± 0.04×10^6	5.71×10^6 ± 0.28×10^6	- 72 %
	µ [day^{-1}]	0.41 ± 0.20	0.49 ± 0.17	- 16 %
	Process duration [days]	6	10	- 40 %
	End titre [µg×mL^{-1}]	11 ± 1	41 ± 3	- 74 %
	q_P [pcd]	1.32 ± 0.17	1.05 ± 0.06	+ 25 %

Tab. 14: Short overview over all metabolic parameters of the investigated cell lines comparing two different medium, ProCHO5 and D/H. Every fermentation was performed in shaking flasks. All parameters were calculated as averages over all values obtained from the exponential growth phase of the culture (days 1 to 7 for ProCHO5 and days 1 to 5 for D/H).

Cell line	Parameter	D/H	ProCHO5	Deviation in D/H
HB617	$q_{Glucose}$ [pcd]	604 ± 295	555 ± 392	+ 9 %
	$q_{Glutamine}$ [pcd]	191 ± 95	139 ± 115	+ 38 %
	$q_{Lactate}$ [pcd]	743 ± 547	550 ± 662	+ 35 %
2G12-Gl	$q_{Glucose}$ [pcd]	747 ± 441	628 ± 426	+ 19 %
	$q_{Glutamine}$ [pcd]	179 ± 94	161 ± 118	+ 11 %
	$q_{Lactate}$ [pcd]	810 ± 608	700 ± 758	+ 16 %
2G12	$q_{Glucose}$ [pcd]	717 ± 464	419 ± 225	+ 71 %
	$q_{Glutamine}$ [pcd]	177 ± 86	136 ± 115	+ 30 %
	$q_{Lactate}$ [pcd]	802 ± 636	452 ± 480	+ 77 %

3.1.3 Other culture conditions

Tab. 15: Short overview over all critical parameters of those batch trials performed with different culture conditions. Different alterations of culture conditions were investigated to analyze the influence on crucial culture parameters. n describes the number of repeats of the respective batch, <u>not</u> the days taken into account for the calculation of the respective mean. μ was calculated as averages over all values obtained from the exponential growth phase of the culture (days 1 to 7). The q_P was calculated by dividing the end titre through the accumulated viable cell counts to that time.

Cell line	Parameter	Standard (n=3)	Double inoculum (n=2)	Gln-Feeding (n=2)	Pre-incubation (n=1)
HB617	μ [day^{-1}]	0.47 ± 0.17	0.43 ± 0.21	0.47 ± 0.16	0.46 ± 0.18
	q_P [pcd]	19.79 ± 0.61	18.58 ± 1.04	19.57 ± 2.14	16.63
	End titre [μg×mL^{-1}]	502 ± 21	445 ± 5	431 ± 54	463
	Peak cell concentration [cells×mL^{-1}]	$4.94\times10^6 \pm 0.11\times10^6$	$5.66\times10^6 \pm 0.38\times10^6$	$4.84\times10^6 \pm 0.27\times10^6$	5.15×10^6
2G12-Gl	μ [day^{-1}]	0.40 ± 0.16	0.31 ± 0.21	0.39 ± 0.17	0.35 ± 0.21
	q_P [pcd]	4.13 ± 0.82	3.69 ± 1.14	3.15 ± 0.17	3.96
	End titre [μg×mL^{-1}]	96 ± 2	91 ± 14	86 ± 3	76
	Peak cell concentration [cells×mL^{-1}]	$2.70\times10^6 \pm 0.43\times10^6$	$4.44\times10^6 \pm 0.29\times10^6$	$3.73\times10^6 \pm 0.01\times10^6$	2.54×10^6
2G12	μ [day^{-1}]	0.49 ± 0.17	0.42 ± 0.22	0.53 ± 0.10	0.47 ± 0.25
	q_P [pcd]	1.05 ± 0.06	1.61 ± 0.72	1.07 ± 0.08	1.00
	End titre [μg×mL^{-1}]	42 ± 3	55 ± 11	39 ± 4	37
	Peak cell concentration [cells×mL^{-1}]	$5.71\times10^6 \pm 0.28\times10^6$	$6.98\times10^6 \pm 0.23\times10^6$	$5.80\times10^6 \pm 0.06\times10^6$	5.81×10^6

According to Tab. 15, the batch-wise glutamine feeding showed no improvement in growth rate, specific productivity or product titre compared to the standard conditions. The added glutamine, however, was readily consumed and specific consumption rates of Gln stayed constant after glutamine feeding (not shown). No further effects of Gln-feeding on any other monitored culture parameters could be observed.

Doubling the inoculum size from 2×10^5 to 4×10^5 cells×mL^{-1} led to a shorter fermentation (1-2 days) and a higher peak cell concentration (about 15-30%) compared to cultivation under standard conditions. However, no improvement in respect to other culture parameters such as end titre, specific productivity or growth rate could be observed.

Pre-incubating the medium yielded no difference in any culture parameter such as viability, growth rate or specific productivity and was thus not further investigated after a single run.

3.2 Medium adaptation

The viability, growth rate and specific productivity were consulted to possibly monitor changes as a result of the medium change from a ProCHO5 to D/H. Red vertical bars shown in the figures below indicate transfer events into different medium or vessel.

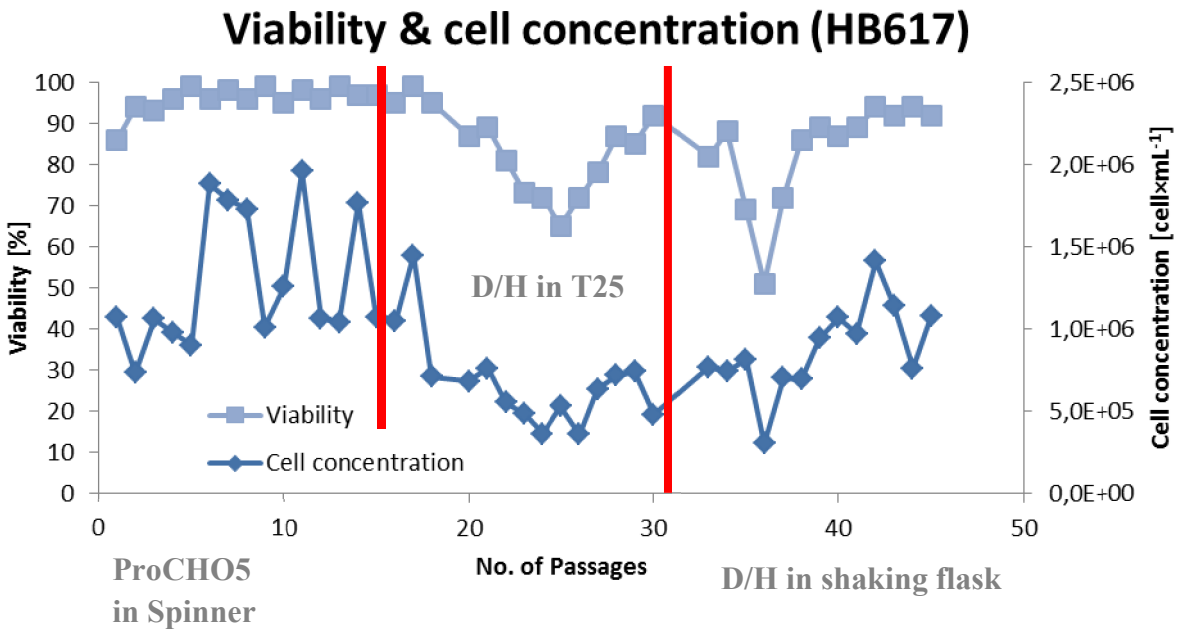

Fig. 21: Course of viability and cell concentration of the cell line HB617 during the adaptation process to D/H medium in shaking flask. Transfers were performed on passage No. 16 and 31.

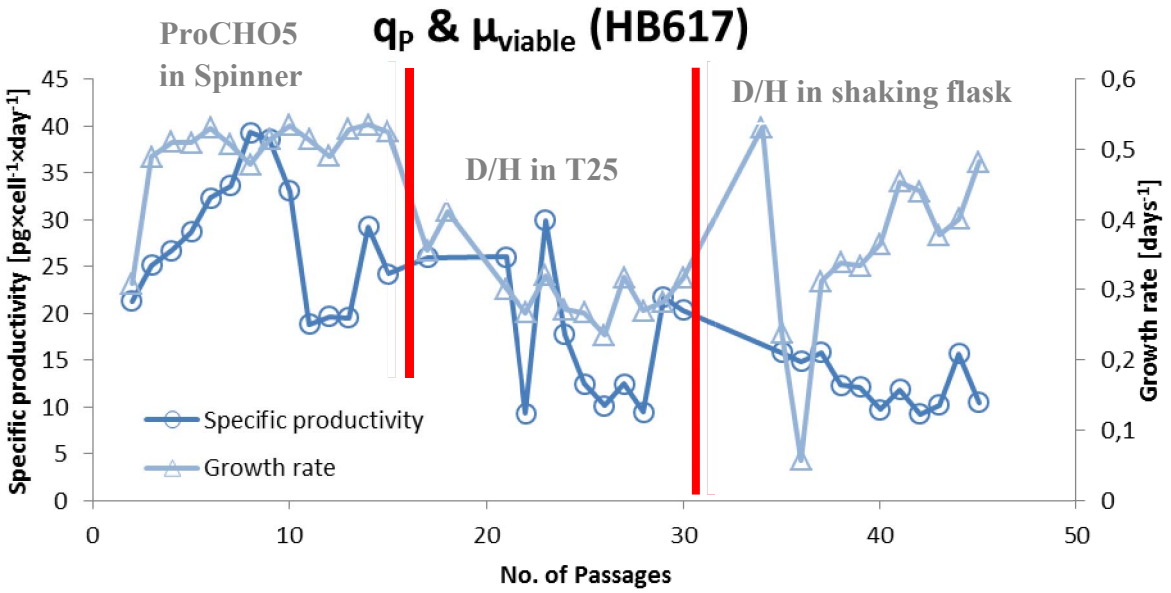

Fig. 22: Course of specific productivity and viable growth rate of the cell line HB617 during the adaptation process to D/H medium in shaking flask. Transfers were performed on passage No. 16 and 31.

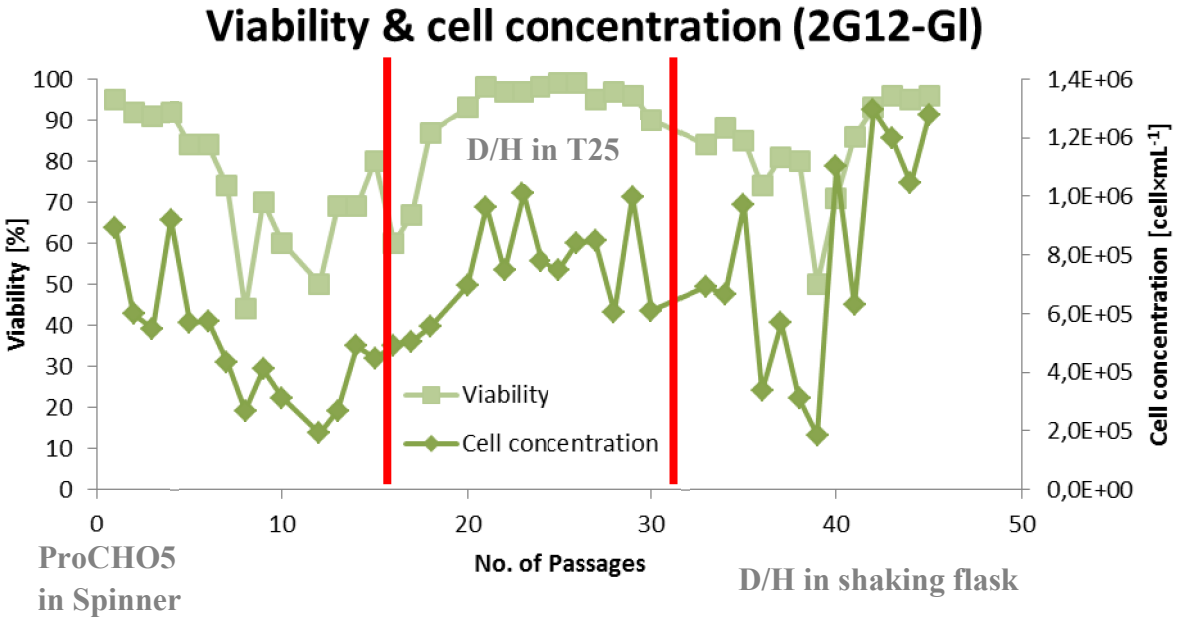

Fig. 23: Course of viability and cell concentration of the cell line 2G12-Gl during the adaptation process to D/H medium in shaking flask. Transfers were performed on passage No. 3 and 31.

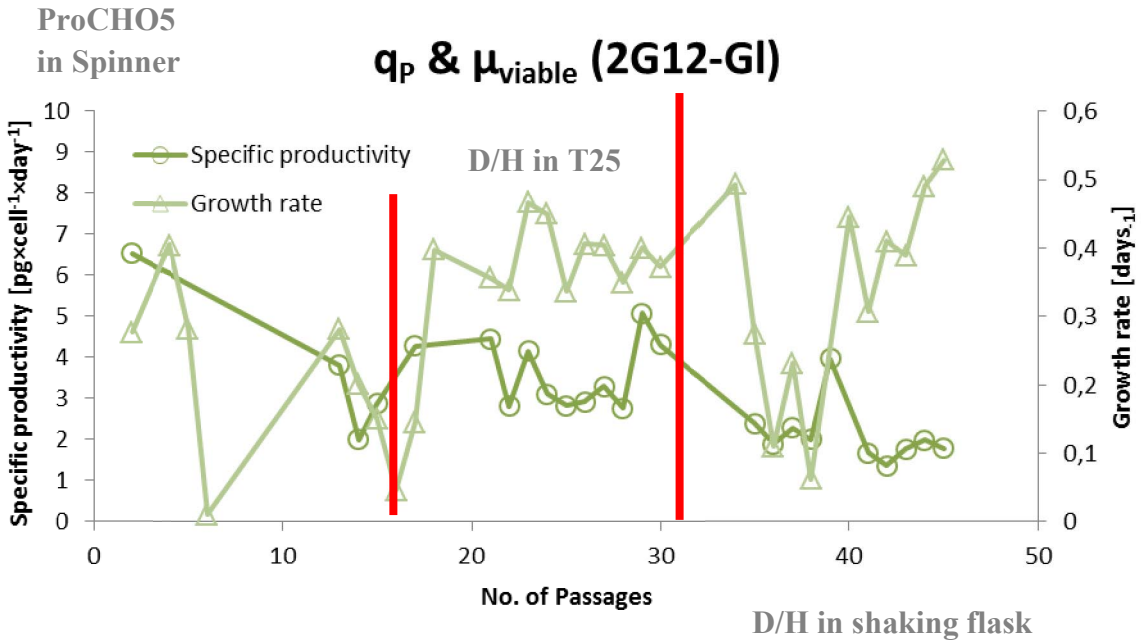

Fig. 24: Course of specific productivity and viable growth rate of the cell line 2G12-Gl during the adaptation process to D/H medium in shaking flask. Transfers were performed on passage No. 3 and 31.

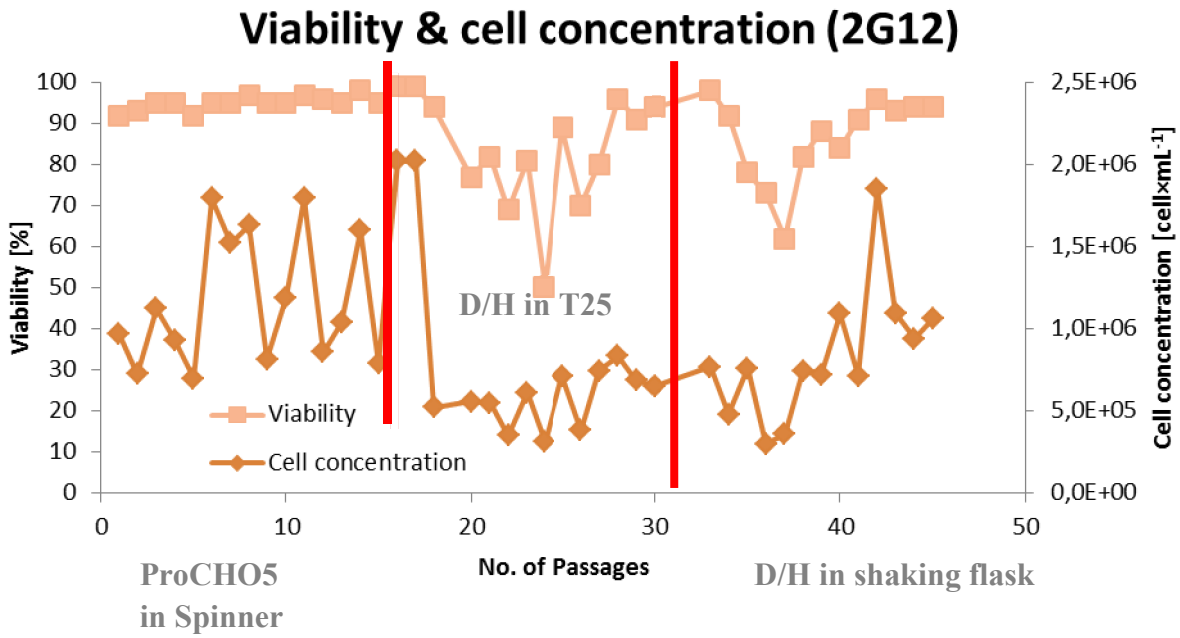

Fig. 25: Course of viability and cell concentration of the cell line 2G12 during the adaptation process to D/H medium in shaking flask. Transfers were performed on passage No. 16 and 31.

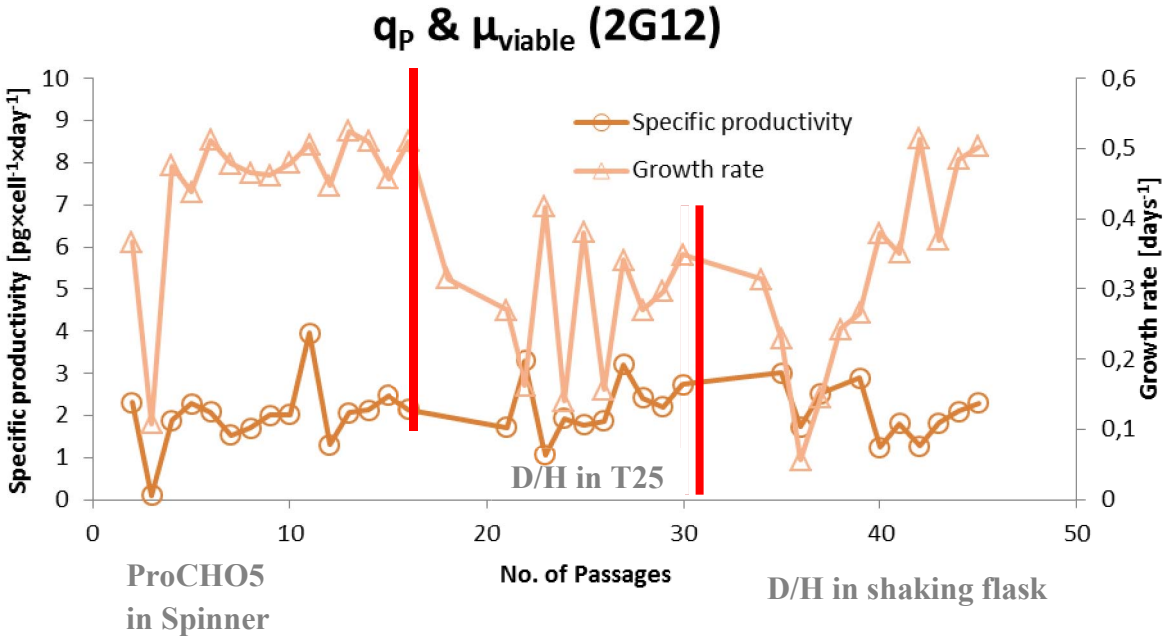

Fig. 26: Course of specific productivity and viable growth rate of the cell line 2G12 during the adaptation process to D/H medium in shaking flask. Transfers were performed on passage No. 16 and 31.

HB617 grew well from the start in the spinner with ProCHO5. Specific productivity rose at first to values of 39 pcd, but then dropped to 19 pcd for unknown reasons from the 8th to the 11th passage. After 15 passages, the full medium change to D/H medium in T25 flasks resulted in a delayed decline in viability (the low point being at 65 %) from passage 20-25 and then recovered to >90 % in the subsequent 5 passages. After this recovery, the culture was transferred into a shaking flask, which led to another delayed decline of viability to 51 %, from which the culture recovered again after 2-3 passages. Over the whole course of adaptation, specific productivities declined steadily from 25.6 pcd (average productivity in ProCHO5 spinner) to 11.5 pcd (average productivity in D/H medium in shaking flask) as can be seen in Tab. 16. The re-occurring loss of viability is contributed to the sudden changes of the medium as well as that of the vessel. In could be shown, however, that the culture was able to recuperate from this change after about 5 passages.

The **2G12-GI** cultures behaved similar to HB617. However, after transferring it from the spinner to the T25 in D/H medium it took this culture much longer (~12 passages) to recover up to a satisfactory viability of > 90 %. Productivity dropped from 7.5 pcd (average

productivity in ProCHO5 spinner) to about 1.7 pcd (average productivity in D/H medium in shaking flask) in the course of adaptation.

The **2G12** culture showed very similar behaviour to HB617 in regard to its viability. In contrast to the other cell lines, however, this cell lines was able to retain its productivity throughout all changes of medium and/or vessel. Its productivity, however, is the lowest of the three cell lines achieving only around 2 pcd. On the other hand, q_P does not seem to deteriorate over the course of adaptation but stays at a constant level.

In conclusion, adapting all three cell lines from the ProCHO5 medium to the D/H medium seemed to have temporary negative effects on the viability, which were compensated after few passages. In regard of q_P, both HB617 and 2G12-Gl lost performance as a result of the adaptation process, while 2G12 retained its (relatively low) specific productivity. Averaged values are stated in Tab. 16.

Tab. 16: Averaged values of q_P and µ for the medium adaptation in continuous cultivation. Values were calculated by averaging all values from a continuous culture after it re-established its initial viability. All adaptation processes started from ProCHO5 in a spinner. The adaptation process progressed from the left to the right column in which the far right column states the relative difference between cultivation in D/H medium in a shaking flask to that in ProCHO5 and in a spinner flask.

Cell line	Parameter	ProCHO5 in spinner flask	D/H in T25	D/H in shaking flask	Difference after adaptation
HB617	q_P [pcd]	25.6 ± 7.4	13.5 ± 4.9	11.5 ± 2.1	- 55 %
	µ [day^{-1}]	0.50 ± 0.05	0.35 ± 0.04	0.40 ± 0.05	- 20 %
2G12-Gl	q_P [pcd]	7.5 ± 2.0	3.0 ± 1.0	1.7 ± 0.23	- 91 %
	µ [day^{-1}]	0.47 ± 0.08	0.38 ± 0.04	0.43 ± 0.08	- 9 %
2G12	q_P [pcd]	2.4 ± 1.0	1.8 ± 1.1	1.9 ± 0.58	- 21 %
	µ [day^{-1}]	0.46 ± 0.09	0.25 ± 0.10	0.41 ± 0.09	- 11 %

3.3 Vessel adaptation

After the successful adaptation of all CHO cell lines to the shaking flask in D/H-medium, we further investigated the suitability of the shaking flask when using ProCHO5 as cultivation medium. The goal was to investigate differences in parameters, such as growth rate, specific productivity and viability between shaking flask and Spinner when attempting to establish a continuous CHO culture in ProCHO5.

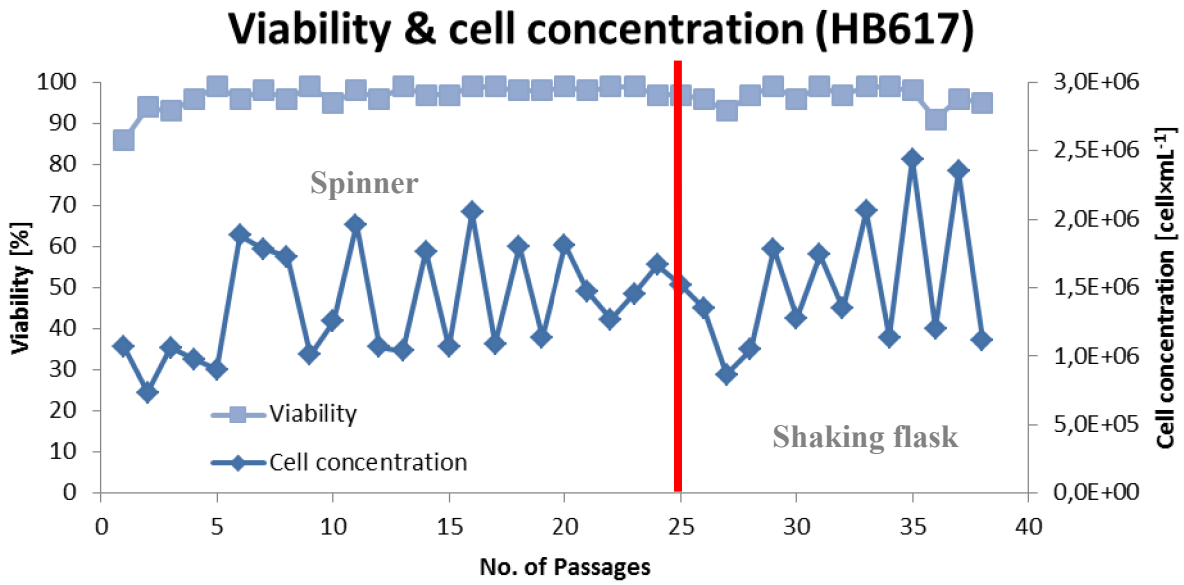

Fig. 27: Course of viability and cell concentration during the adaptation of HB617 from Spinner to shaking flask while cultivated in ProCHO5. Transfer was performed on passage No. 25.

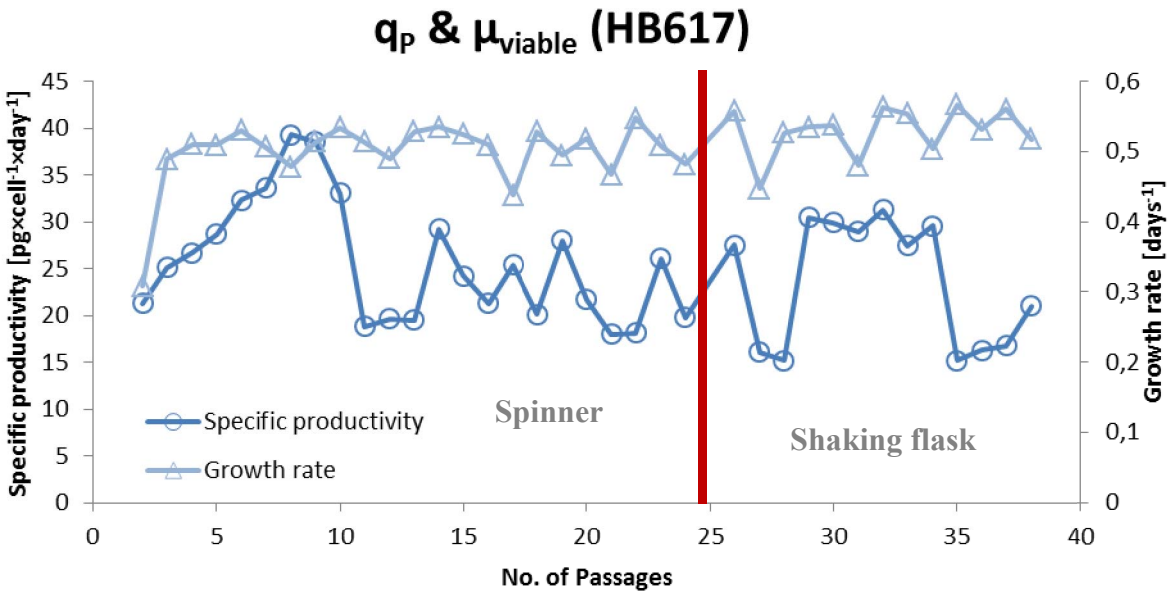

Fig. 28: Course of specific productivity and viable growth rate during the adaptation of HB617 from Spinner to shaking flask while cultivated in ProCHO5. Transfer was performed on passage No. 25.

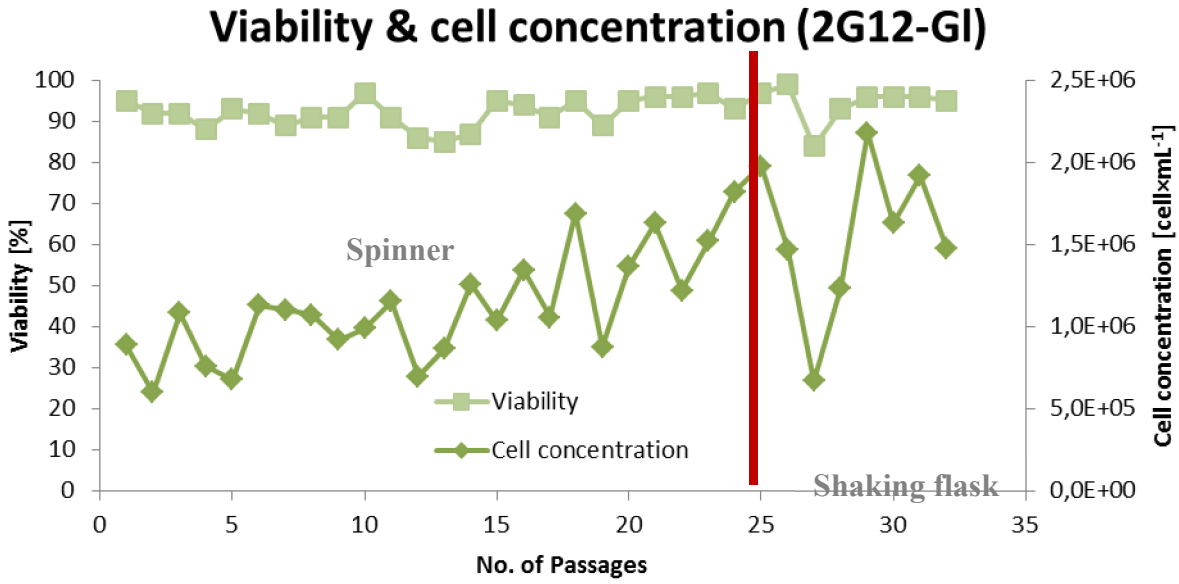

Fig. 29: Course of viability and cell concentration during the adaptation of 2G12-Gl from Spinner to shaking flask while cultivated in ProCHO5. Transfer was performed on passage No. 25.

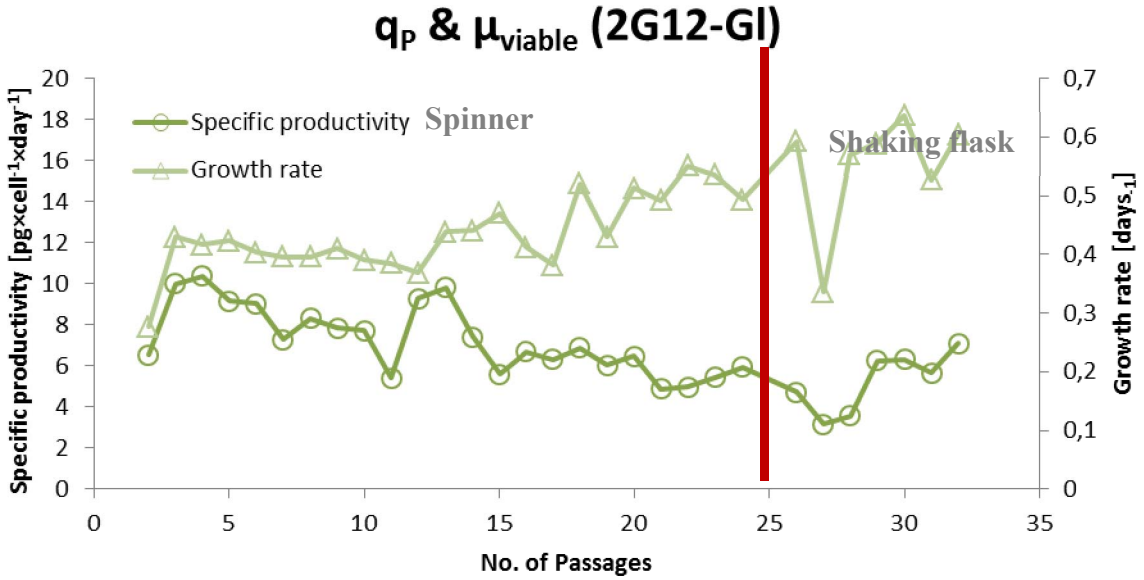

Fig. 30: Course of specific productivity and viable growth rate during the adaptation of 2G12-Gl from Spinner to shaking flask while cultivated in ProCHO5. Transfer was performed on passage No. 25.

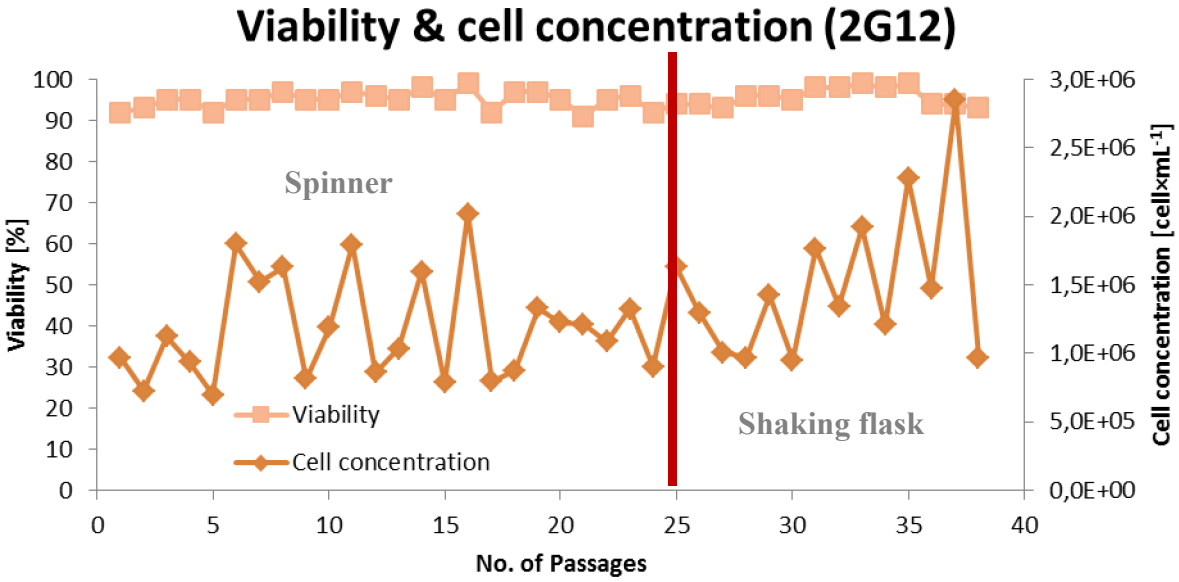

Fig. 31: Course of viability and cell concentration during the adaptation of 2G12 from Spinner to shaking flask while cultivated in ProCHO5. Transfer was performed on passage No. 25.

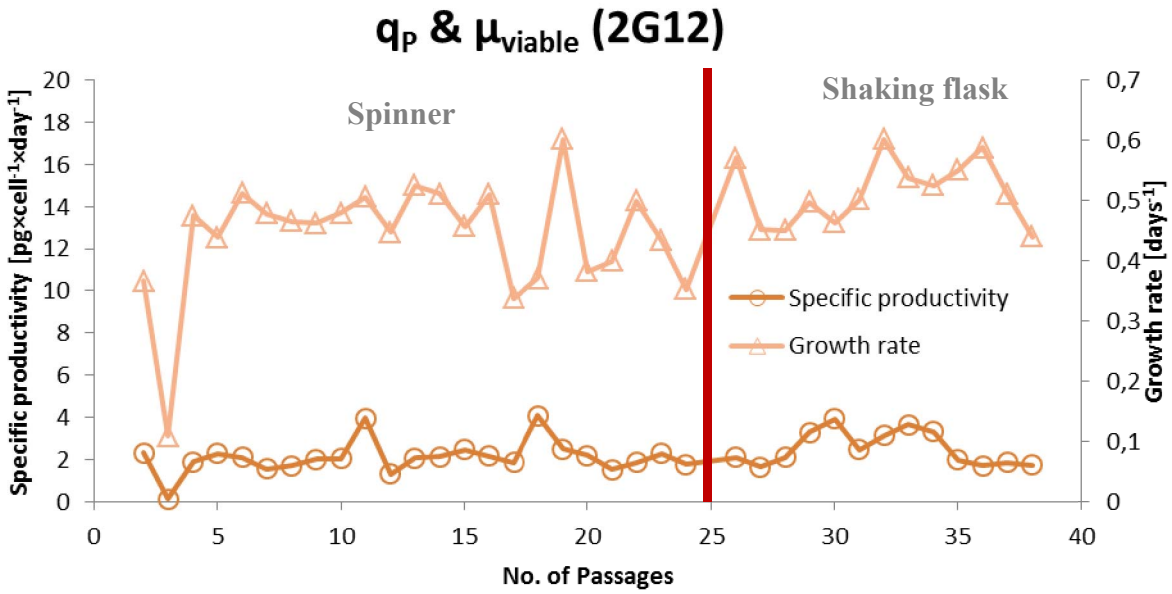

Fig. 32: Course of specific productivity and viable growth rate during the adaptation of 2G12 from Spinner to shaking flask while cultivated in ProCHO5. Transfer was performed on passage No. 25.

The influence of the vessel adaptation on the viability of each cell line can be observed in Fig. 27, Fig. 29 and Fig. 31. It clearly shows that adapting the cell line to a different vessel did not influence the viability, except for 2G12-Gl, which experienced a slight drop of viability, which was, however, quickly overcome after two passages.

Fig. 28, Fig. 30 and Fig. 32 show the growth rate and specific productivity of the cell lines over the course of the adaptation. The q_P seemed mostly unaffected by the adaptation process and stayed constant except for 2G12-Gl, whose q_P started to deteriorate already before the adaptation. In regard of the growth rate, it also stayed constant except for 2G12-Gl. Again, this trend establish itself well before the actual adaptation process happened and thus seems to had occurred independently.

In conclusion, adapting all three cell lines from the Spinner to the shaking flask seemed to have no effect on the μ or q_P when cultivated in ProCHO5 medium. Averaged values are stated in Tab. 17.

Tab. 17: Averaged values of q_P and μ for the vessel adaptation for continuous cultivation. Values were calculated by averaging all values from a continuous culture after it re-established its initial viability. All adaptation processes started from ProCHO5 in a spinner. The adaptation process progressed from the left to the right column.

Cell line	Parameter	ProCHO5 in spinner	ProCHO5 in shaking flask	Difference
HB617	q_P [pcd]	25.6 ± 7.4	23.5 ± 6.7	- 8 %
	μ [$days^{-1}$]	0.50 ± 0.05	0.53 ± 0.04	+ 6 %
2G12-Gl	q_P [pcd]	7.5 ± 2.0	5.3 ± 1.5	- 30 %
	μ [$days^{-1}$]	0.47 ± 0.08	0.55 ± 0.10	+ 17 %
2G12	q_P [pcd]	2.4 ± 1.0	2.5 ± 0.8	+ 4 %
	μ [$days^{-1}$]	0.46 ± 0.09	0.51 ± 0.05	+ 11 %

3.4 FACS

In Fig. 33 to Fig. 35 the results of the FACS are shown. The graphs depict the population of cells who were tested positively for production of the respective subunit (either light or heavy chain of the IgM) on different days of one DASGIP fermentation in ProCHO5. The data was processed to only depict live cells, the agglomerates and dead cells where removed through data processing. The negative control, a non-producing CHO host cell line (K1), was gated by 1% and used as a reference to distinguish non-producing from producing recombinant cells. Those cells which actually contain the respective polypeptide are shown within the border of a horizontal line (labelled with "+") drawn into the pictures. All cells outside of those borders were considered non-producing cells. The logarithmic x-axis shows the intensity of the signal while the linear y-axis shows the number of counts detected. The higher the intensity signal, the higher is the intracellular concentration of heavy or light chain.

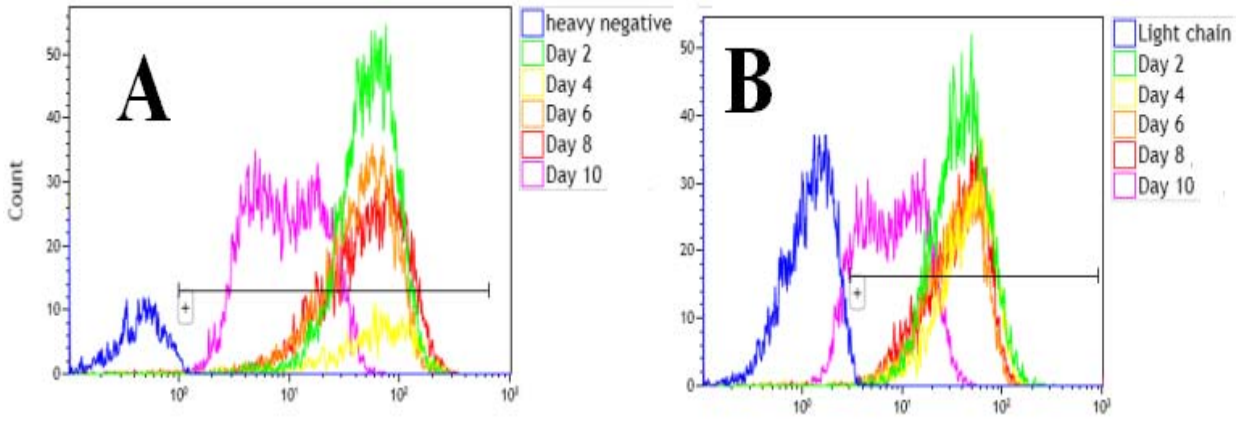

Fig. 33: Intensities obtained from the FACS-chromatogram for the cell line HB617. The blue lines indicate the negative control (non-secreting CHO K1 cell line). A: Heavy chain. B: Light chain.

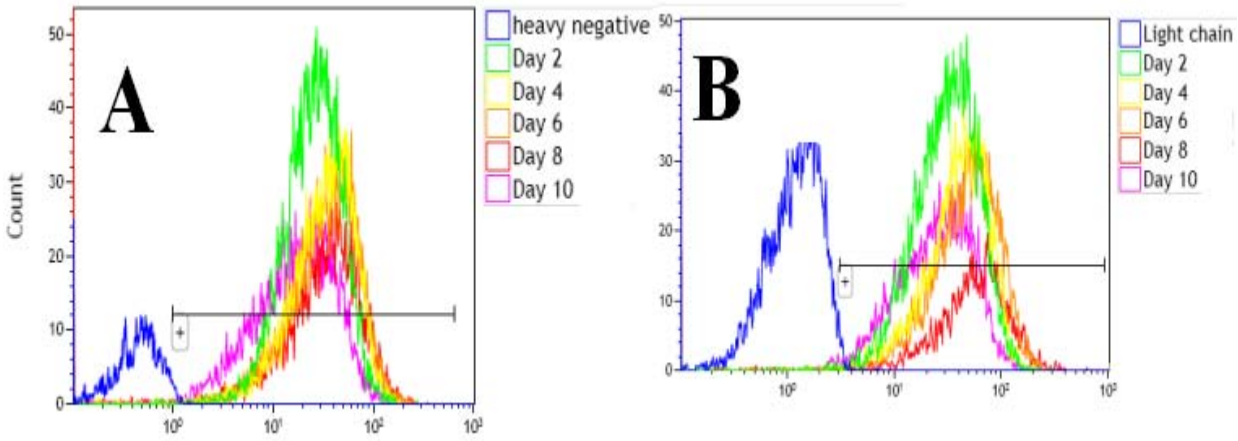

Fig. 34: Intensities obtained from the FACS-chromatogram for the cell line 2G12-Gl. The blue lines indicate the negative control (non-secreting CHO K1 cell line). A: Heavy chain. B: Light chain.

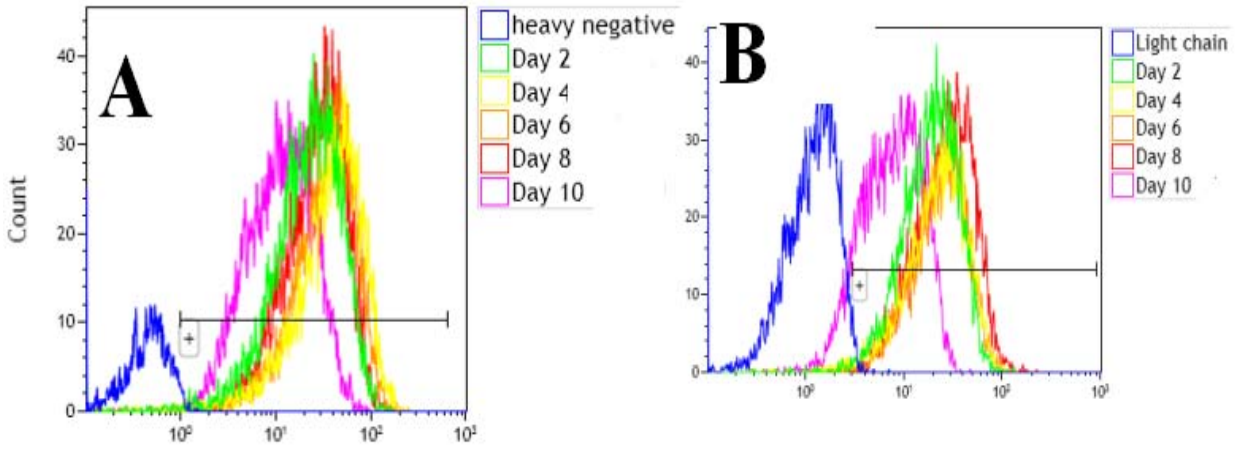

Fig. 35: Intensities obtained from the FACS-chromatogram for the cell line 2G12. The blue lines indicate the negative control (non-secreting CHO K1 cell line). A: Heavy chain. B: Light chain.

As shown in the figures above, almost no non-producers were found with this method. One can observe that the signal distributions of all cell lines and both subunits (LC, HC) are following a normal distribution, though tailing occurred on some occasion for HB617 and 2G12, where the normal distribution seemed to shift towards lower signal intensities on Day 10. On all other days the cell populations showed a homogeneous distribution.

3.5 Agarose gel electrophoresis

3.5.1 Genomic DNA

On Fig. 36, the genomic DNA was applied on a 0.8% agarose gel. In all samples, fragments of a size >10,000 bp were observed.

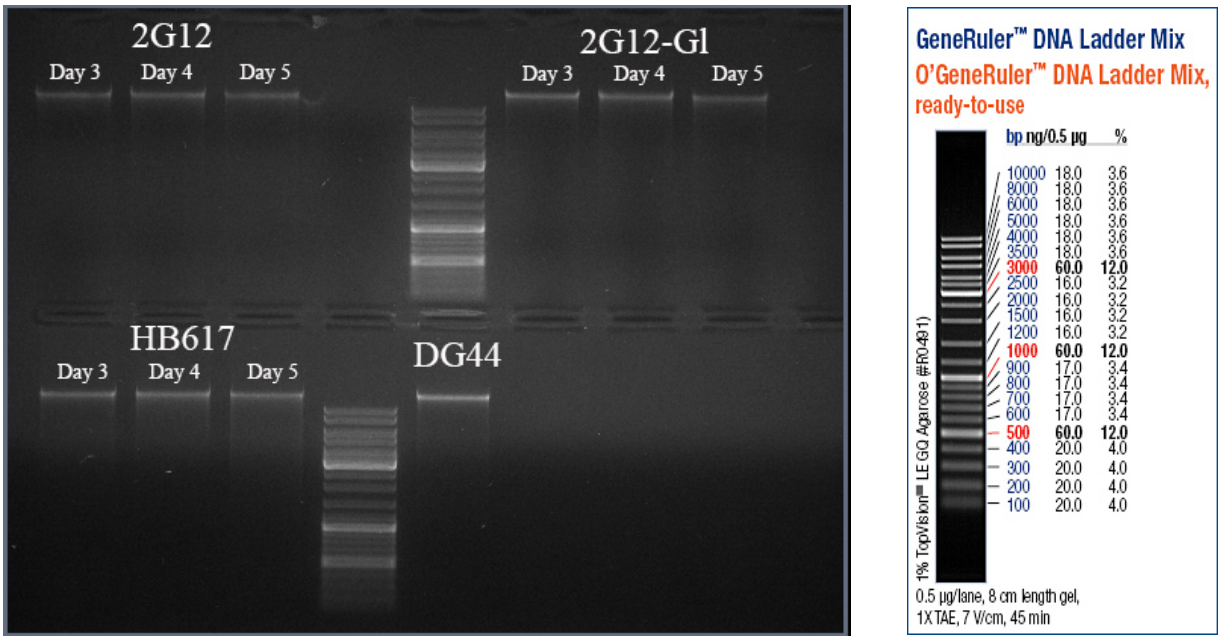

Fig. 36: Picture of the 0.8% agarose gel. Samples applied originate from purified genomic DNA of the respective cell line. The DG44 was the host cell lines from which all investigated cell lines came from originally and was assumed to have a very similar size. Thus, DG44 was used as a positive control.

3.5.2 DNA Amplicons

In Fig. 37, the 2% agarose gel, which was loaded with the qPCR amplicons, is displayed. DG44 was used as a positive control for the β-actin gene. The detected bands for β-actin, HC, LC and JC had sizes of 97 bp, 118 bp, 183 bp and 194 bp respectively. For β-actin, a band below 100 bp occured for each cell line and almost no smear appears on the gel. For HC, all bands lay closely beyond 100 bp and showed some visible smear on each lane. Further, the

intensity of the band for 2G12 seemed to be somewhat lower compared to the other cell lines. For LC, all bands lay closely below 200 bp and show little smear on all lanes. Again, the lane for 2G12 showed a somewhat lower intensity compared to the other cell lines. For JC, all bands showed the same intensity, a visible smear and a band at 200 bp.

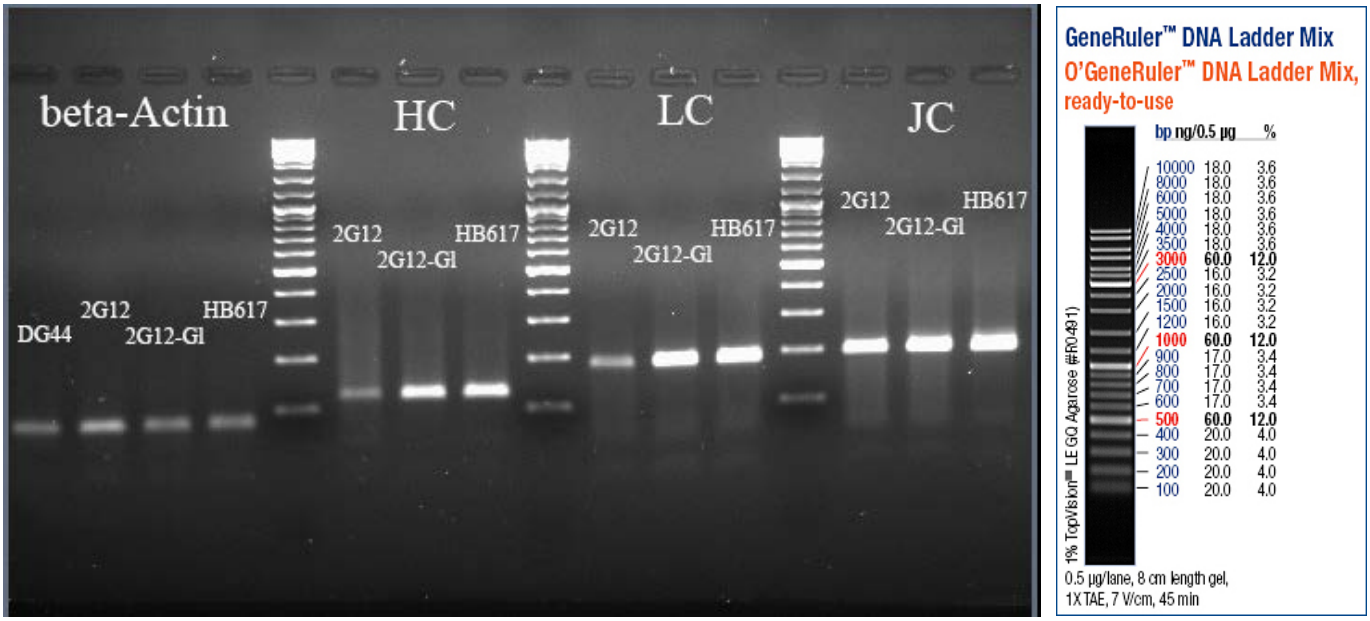

Fig. 37: Picture of the 2% agarose gel with the amplicons of each cell line and each target gene applied. β-actin was used as the "housekeeping gene" to which the relative gene copy number (stated above) refers to.

3.6 Subcloning in D/H medium

Subcloning yielded different results for the different cell lines. While HB617 showed a high variation of IgM-expression between the different subclones, the differences among the single clones for 2G12-Gl was less pronounced and barely mentionable for 2G12 on the first screening. During this first IgM screening in a 96 well plate, the relative standard deviations were high for HB617 (± 65 %) and lower for 2G12-Gl (± 29 %) and 2G12 (± 15 %). Finally, three clones of each cell lines which showed the highest q_P value were selected for further cultivation. However, from these selected clones, only a few kept their viability in the subsequent cultivations and were stored frozen in the cell bank (see Tab. 18).

Tab. 18: Specific productivities and titres of the subclones selected for further cultivation compared to their parental cell line. Both values are mean values obtained from routine cultivations in T25 flasks in D/H-medium. Note that the averages after subcloning consist only of two measured values. The values before subcloning refer to values obtained from the continuous cultivation of the respective cell line (also see Tab. 16).

Cell line	Subclone	Parameter	before Subcloning	after Subcloning	Improvement
HB617	A10	q_P [pcd]	**13.5 ± 4.9**	10.7 ± 2.0	- 21 %
		End titre [µg×mL^{-1}]	**60.3**	27.5	- 54 %
	C7	q_P [pcd]		16.7 ± 1.1	+ 24 %
		End titre [µg×mL^{-1}]		29.4	- 51 %
2G12-Gl	A5	q_P [pcd]	**3.0 ± 1.0**	13.1 ± 1.3	+ 336 %
		End titre [µg×mL^{-1}]	**12.0**	32.7	+ 173 %
	H3	q_P [pcd]		4.9 ± 0.7	+ 63 %
		End titre [µg×mL^{-1}]		11.8	- 2 %
2G12	D6	q_P [pcd]	**1.8 ± 1.1**	8.3 ± 1.4	+ 361 %
		End titre [µg×mL^{-1}]	**7.5**	16.7	+123 %

4 Discussion

4.1 Fermentations

4.1.1 Influence of Fermentation Vessel (in ProCHO5)

In Fig. 4 it can clearly be observed that all investigated cell lines behave totally different in respect to their **peak cell concentrations (pcc)**. In the DASGIP bioreactor, all three cell lines grow to the same cell density of about $4.5x10^6$ cells×mL^{-1}. In the shaking flask, HB617 ($4.9x10^6$ cells×mL^{-1}) and 2G12 reached 20 % higher peak cell concentrations ($5.7x10^6$ cells×mL^{-1}), whereas 2G12-Gl ($2.7x10^6$ cells×mL^{-1}) grew to 40% lower pcc, respectively. It seems that some cell lines are more robust and therefore can cope better with the different fermentation environment than others. Since the DASGIP system homogenizes the bioreactor's content by impeller mixing, it might be that the tested cell lines were not robust enough and could cope better with the low-shear movement in the shake flasks. It should also be considered that the scale-up to a higher volume as well as the control of pH and DO might have influenced the process performance. This difference in the maximum cell counts might also be due to a prolonged phase of high viability in the shaking flask compared to the DASGIP. Fig. 2 depicts the viability during the course of fermentation and shows that the viability drops 1 to 2 days earlier in the DASGIP for 2G12 and HB617 (see also Tab. 11), which represents a drop in longevity for up to 13%. For those two cell lines, it can be inferred from the data that on the very day the viability starts to differ between DASGIP and shaking flask, the same is true for the cell count. Until that day, however, cell counts as well as viability are the same in both vessels, indicating that the maximal cell count is governed by the longevity, which is shorter in the DASGIP. For 2G12-Gl, however, this is not true, since cell counts already start to differ between shaking flask and DASGIP in the early exponential phase of growth on Day 3.

In summary, the different cell counts might be a result of the different longevities in the two compared vessels. In the shaking flask, cell lines HB617 and 2G12-Gl are able to maintain a higher viability than in the DASGIP, while the viability is the same in both vessels for 2G12. It seems that 2G12 can cope better with the DASGIP environment than HB617 and 2G12-Gl. This lower viability of HB617 and 2G12-Gl might be due to enhanced stress exerted on the

cells in the DASGIP or could be due to a not sufficiently long adaptation phase to the dynamic cultivation, as neither the cells for the DASGIP trials nor those for the shaking flask trials were adapted to the shaking flask or the DASGIP environment, respectively. As for the medium the cell cultures for the shaking flask trials were adapted 6 or 15 passages in ProCHO5 medium before being used for the batch experiments, whereas the DASGIP trials were performed later on and thus had already 17 or 22 passages to adapt to the ProCHO5 medium. These different adaption times to the medium might have had influences on the experimental outcome. On the other hand, all cell lines were thawed from samples already adapted to ProCHO5 medium in the spinner flask, so that it could be assumed that these different adaptation times had no influence on the behaviour of the different cell lines.

Apart from the explanation stated above, the poor performance of the DASGIP cultures could be explained by the culture parameters applied. Parameters like pH, stirrer speed or the volumetric rate of gassing were not specifically optimized beforehand. The parameters mentioned were just adjusted to an empirically sound value of pH 7 and 80 rpm, for example. These conditions, however, might not have been optimal for the specific cell lines used and thus might have led to a worse performance in comparison to the shaking flask trials.

The **end titre** [$\mu g \times mL^{-1}$] achieved was considerable higher when using shaking flasks compared to the DASGIP bioreactor. HB617 produced 38 % higher titres, 2G12-Gl improved by 110 % and 2G12 by 30 %. It seems that fermentation in the shaking flasks leads to higher titres than in the bioreactor. As no relevant dilution took place during the trials in the DASGIP bioreactor (no feeding of medium) dilution effects cannot explain these lower titres. It has to be noted, however, that the fermentation in the shaking flask took up to four days longer in the shaking flask, so the higher end titre can partially be attributed to the longer fermentation time.

The **viable specific growth rate** [day^{-1}] seems to be almost identical for both vessels (besides single days where µSF > µDASGIP), which can be observed in Fig. 3 and Tab. 11. Also, the 20 % improvement of µ for HB617 in the shaking flask is probably not significant due to the high standard deviation if the calculation. However, beginning with day 8, µ seems to drop faster in the DASGIP bioreactor than in the shaking flask and coincides with the decreasing viability that drops earlier in the DASGIP bioreactor.

In respect to the **specific productivity** q_P [pcd] (see Fig. 6 and Tab. 11), results are less consistent throughout the investigated cell lines. From the data it can be inferred that 2G12 has a lower q_P in the shaking flask (resulting in a drop of 33 %), unlike 2G12-Gl, which performs better in the shaking flask from day 4 on, resulting in a 81 % increase in q_P compared to cultivation in the DASGIP bioreactor. For HB617, q_P seems to be equal in both vessels (see Fig. 5), except on individual days, where the shaking flask culture performs better, resulting in a minor overall decrease of merely 3 %. One might infer, however, that the shaking flask culture has better specific productivities during the early stage of fermentation but shows no improvement later on. This, however, has to be investigated more closely in order to produce a robust statement. As stated above, the cell line 2G12, which is the only cell line that performs better in the DASGIP bioreactor, might happen to cope better with the higher shear and osmotic stress of the continuously stirred, gassed and pH-adjusted bioreactor.

In regard to **specific glucose consumption** rate $q_{Glucose}$ [pcd], the fermentation in the DASGIP bioreactor seems to be more demanding compared to the shaking flask, as can be seen in Fig. 7 and Tab. 12. The cultures in shaking flasks consume less glucose especially from day 4 on resulting in an overall decrease in consumption rates by 20 % (HB617 and 2G12-Gl) and 46 % (2G12). During the first days of cultivation, however, no clear statement can be made due to the relatively high standard deviations, although it seems that the reverse is true and that the culture in the shaking flask is more demanding than those in the DSAGIP bioreactor. The low cell counts at the time led only to a small change in the absolute Glc concentrations and thus might have been smaller than the technical standard deviation of the method. The measurement methods applied, as described in 2.4.4, seem to have an insufficient sensitivity to describe the nutrient consumption at this early stage of fermentation. More sensitive methods should be applied in future research. It can, however, be stated that on average the cell culture is more demanding in respect of glucose if they're cultivated in the DASGIP bioreactor compared to shaking flask cultivation. This might again be attributed to the enhanced stress cells suffer in the continuously stirred DASGIP bioreactor. Cells might need more nutrients for compensating the elevated stress levels, which might lead to enhanced production of stress response proteins, such as chaperones. This enhanced production of non-target protein could easily lead to enhanced nutrient uptake without an increase in q_P. Keane et al. (2002) found that increasing the shear stress also lead to an increase in glucose

consumption (along with a decrease in productivity). They also observed, however, a decreased lactate productivity, which could not be observed in our investigations, where specific lactate production remained equal (see next paragraph). Overall, more research is needed to elucidate the connection between nutrient consumption and shear stress.

Concerning the **specific glutamine consumption** $q_{Glutamine}$ [pcd], no consistent trend could be observed in Fig. 8. Once again, standard deviations tend to be very high due to eliminated outliers, especially during the first days of the fermentation. Overall, no difference could have been observed between the two vessels in regard to glutamine consumption.

Specific lactate production $q_{Lactate}$ [pcd] seems to be elevated in the shaking flasks until day 3 as can be inferred from Fig. 9. This trend, however, reverses after day 4, where more lactate is produced in the DASGIP. This trend could be observed in all cell lines and might correlate with the enhanced glucose consumption of the cultures in the DASGIP. Over the whole course of the exponential phase, however, no difference in specific lactate production could be observed.

In conclusion, all cell lines perform either equal or worse in the DASGIP environment compared to the shaking flask, as can be seen in Tab. 11. The culture dies 1 to 2 days earlier, glucose consumption is elevated after day 4 and more lactate is produced by the cells after day 4 if they're cultivated in the DASGIP. This elevated accumulation of a waste product might have adverse effects on the performance of the culture and might be responsible for the lower q_P in the DASGIP bioreactor. However, the literature indicates the opposite that lactate actually might improve cell performance. Lao and Toth (97) for example discovered that a CHO cell line producing a recombinant glycoprotein actually increased their q_P by 10 % when adding 60 mM lactate to the medium. Furthermore, the cells own lactate production was completely halted and glucose and glutamine consumption decreased by 15-20%. These effects could not be contributed to the elevated osmolality since their effects were “removed” by comparing the results with a NaCl culture with the same osmolality. Furthermore, Choi et. al (2007) report enhanced erythropoietin end titres and improved longevity of recombinant CHO cells when adding 40 mM sodium lactate to the medium in order to stabilize the pH and decrease the lactate production of the cell. This decreased lactate production coupled with an increased productivity proposes a shift of the carbon metabolism towards protein expression. Thus, the actual effect of lactate on the stress level experienced by the cell remains unclear.

Unfortunately, the high standard deviation (of up to +/- 100 %) and the high fluctuations of q_{Glc}, q_{Gln} and q_{Lac} hamper the creation of a meaningful statement, as most of the observed differences were within these high standard deviations.

The DASGIP environment seemed to have a negative effect on the performance of the cells, which might have been caused through elevated stress levels. Since it has been previously shown that CHO cells producing a therapeutic protein experience elevated stress levels when cultivated in a bioreactor due to elevated shear forces (Trummer et al., 2008), this might be a plausible explanation for the observed effects. The stress could have been caused by several parameters: In the DASGIP bioreactor we adjusted, for example, the pH by adding NaOH. This was done to stabilize the pH but also led to an increase in osmolality and thus might have been beyond the cells tolerated range. The added amount of NaOH is estimated to go up to 40 mM until the end of the fermentation. Takagi et al (2000) found that CHO cells growing in suspension and expressing recombinant tissue plasminogen activator had elevated q_{Glc}, q_{Lac} and q_P values when grown at an osmolality of up to 450 mOsm×kg^{-1} while their growth rate decreased with increasing osmolality. Above this critical value of 450 mOsm×kg^{-1}, however, all these parameters decreased. Thus, a higher osmolality could improve productivity until a certain threshold is reached. As we did not measure the osmolality in our experiments, we cannot determine whether this critical threshold had been exceeded or not thus a clear statement cannot be made.

Furthermore, we gassed the DASGIP system constantly (with altering ratios of O_2 and CO_2) throughout the fermentation, which most definitely introduced shear forces into the system which, together with those exerted by the constant stirring applied, might have been too high. At last, antifoaming agent was added to the DASGIP, when needed. Although the antifoam used was of non-toxic nature, its presence might have negatively influenced the cells capacity to grow and produce efficiently. All these operations were not performed in the shaking flask, where simply constant shaking was applied and might have led to an increased stress level.

4.1.2 Influence of fermentation medium (in shaking flasks)

Cultivation in D/H medium resulted in a reduction of longevity, **peak cell concentration (pcc)** and specific productivity compared to cultivation in ProCHO5 medium. As seen in Fig. 13, the viability of all investigated cell lines drops three to five days earlier in D/H medium

compared to in ProCHO5 medium, which equals a decrease in process time of 38 % (HB617) to 45 % (2G12-Gl). This also led to a reduction in growth and a therefore much lower maximum cell density of $<2\times10\times^{6}$ cells$\times$mL^{-1}, which led to a decrease in pcc of 66 % for HB617, 40 % for 2G12-Gl and 72 % for 2G12. Interestingly, all three cell lines behave very similar in D/H in respect of viability and cell counts, in contrast to the very different behaviour in ProCHO5, which can be observed in Fig. 13 and Fig. 15. These findings indicate that the nutrient poor D/H media cannot support the high cell counts obtained from the ProCHO5 cultivation, which was to be expected as the lower nutrient content limited the cell growth.

The **end titres** [$\mu g\times mL^{-1}$] deteriorated for all cell lines when D/H medium used. HB617 suffers a decline by 87 %, 2G12-Gl loses 94 % and 2G12 loses 74 % of their end titres when comparing them to the ProCHO5 batch trials. It can be inferred from these numbers that using D/H medium for severely reduces the obtained end titres. Again, these effects are attributed to the lower nutrient content of the D/H medium.

The **viable growth rate µ** [days^{-1}], depicted in Fig. 14, seems to be identical in both media until day 4, when it drops rapidly in D/H along with a sudden loss of viability, which becomes apparent on day 5. Comparing the µ during the exponential phase of growth showed no difference between the two different medium.

The **specific productivity** q_P [pcd] is outlined in Fig. 16 and Fig. 17 and shows clearly, that HB617 and 2G12-Gl perform much worse in D/H than in ProCHO5 medium. 2G12, however, seems to behave differently from the other cell lines. HB617 experiences a 42 % drop in q_P and 2G12-Gl performs even 73 % worse. 2G12 on the other hand gains 25 % of q_P when cultivated in D/H medium. The lower end titres of 2G12 despite its higher q_P can only be partially attributed to the shorter process duration but is also governed by the cell counts, which were much lower in the D/H trials. All values are also listed in Tab. 13. These findings indicate that the protein expression per cell is downregulated in HB617 and 2G12-Gl, as the nutrient poor D/H medium apparently does not provide enough energy to perform the energy-intensive expression of a heterologous protein. Furthermore, 2G12 seems to cope better with the altered nutrient supply.

Specific glucose consumption $q_{Glucose}$ [pcd], however, seems to be rather identical in both media, except for 2G12, which seems to consume 71 % more glucose when grown on D/H medium which can be observed in Fig. 18 and Tab. 14.

In regard of the **specific glutamine consumption** $q_{Glutamine}$ [pcd] (see Fig. 19) values are also identical except for 2G12-Gl which seems to consume less Gln in D/H medium after Day 4 (along with a drop in viability, though). It is striking that the consumption rates for Gln reach zero on Day 6 due to depletion of Gln in the medium (data not shown). Unlike in ProCHO5, the cells cultivated in D/H medium simultaneously lose their viability at the same day as Gln gets depleted, while the ProCHO5 cultures seem unaffected by this event. This correlation between Gln depletion and loss-of-viability indicates that the D/H cultures seem to handle the Gln depletion worse than the ProCHO5 cultures. Presumably, the cultures in ProCHO5 can substitute Gln for some other nutrient.

Specific lactate production $q_{Lactate}$ [pcd] again seems rather identical (see Fig. 20), except for 2G12 which seems to produce more lactate during the first three days of fermentation, which is consistent with the increased glucose demands of this culture in this timeframe. Overall, the $q_{Lactate}$ increases for 2G12 by 77 % but stays unaltered for HB617 and 2G12-Gl when cultivated in D/H medium. It can be assumed that the increased lactate production is due to the higher glucose consumption observed in this cell line.

In conclusion, all cell lines, except 2G12, perform much worse in D/H medium compared to ProCHO5 medium, which was to be expected, since ProCHO5 is considered a much richer medium, however, unknown in its composition. The actual drop in performance can be observed in Tab. 13. From the figures, one could infer that a nutrient seems to become limited after day 5 of the fermentation, which then led to a steep decline of viability. However, Glc can be ruled out to be that nutrient since it is still sufficiently available on Day 5 (and still gets consumed afterwards). Gln could be the limiting substrate since its depletion on Day 5 coincides with the sudden drop on viability. Further research on this specific aspect has to be performed to confirm that notion. Taking a look at the specific productivity qP, values are improved in ProCHO5 medium for HB617 and 2G12-Gl but not for 2G12, which actually performs worse. In conclusion, D/H medium is inferior to ProCHO5 medium considering end titre (up to - 94 %), peak cell concentration (up to - 72 %) and the viability (up to 45 % shorter fermentation) of the cultures. On the other hand, this trend is not so clear for qP, as

2G12 is the only cell line which actually increases its specific productivity in D/H medium. This improved qP might be connected to the elevated qGlc and qLac. Unlike the other cell lines, 2G12 consumes much more glucose (+ 71 %) and produces much more lactate (+ 77 %) per cell when cultivated in D/H medium. This elevated nutrient consumption and waste production seems to match these findings and indicate that 2G12 is more versatile than the other cell lines in regard to coping with poor nutrient medium. Lastly, an economic analysis should be made for any large-scale fermentation to evaluate whether this improvement in productivity justifies the higher price of the ProCHO5 medium.

4.1.3 Other culture conditions

Since the investigated culture conditions (double inoculum, pre-incubation of medium, glutamine-feeding) didn't yield any improvement in $q_{P,}$ growth rate or end titre, no further trials were conducted and it was concluded that these culture conditions were not suitable to improve the performance of an IgM-producing CHO cell line.

4.2 Medium adaptation

In regard to adapting the cell lines from ProCHO5 to D/H medium, the following conclusion can be drawn (also seen in Fig. 21 to Fig. 26): First, the establishment of a continuous culture in D/H medium in a spinner flask was not possible as HB617 and 2G12 died after few passages when cultivated under these conditions (data not shown). Secondly, each change in cultivation technique led to temporary drops in viability, which recovered after a few passages of cultivation. This effect has been observed in all investigated cell lines. The specific productivity, however, did not always recover and declined steadily for the cell lines HB617 and 2G12-Gl, yielding 55 % (HB617) and 91 % (2G12-Gl) lower specific productivities when cultured in D/H-medium in a shaking flask compared to the initial cultivation in ProCHO5 in the spinner flask. The cell line 2G12, however, seems to be unaffected in its specific productivity when adapted to D/H medium and retains its low q_P of ~2 pcd over the whole course of the adaptation. Tab. 16 summarizes the results of the adaptation process by comparing the averaged values of all relevant culture parameters.

It thus can be concluded that, though being successful, media adaptation had led to a loss of performance for the cell lines HB617 and 2G12-Gl. 2G12 seems to be mostly unaffected by the adaptation and seems to be the most versatile cell line. These findings match with those obtained in the batch fermentations. All cell lines, however, need some time to adapt to the new conditions, in which they lose much of their viability.

4.3 Vessel adaptation

Adapting the three CHO cell lines from the spinner to the shaking flask in order to establish a continuous culture seemed to have no effect on vital parameters such as viability, μ or q_P, when cultivated in ProCHO5 medium as can be observed in Tab. 16. Except for 2G12-Gl, whose viability shortly declined to 84 %, not even a drop in viability occurred after transfer to the new vessel resulting in constant viabilities beyond 90 %.

In regard to their specific productivities, values also stayed almost constant over the course of the adaptation, resulting in 23.5 pcd (- 8 %) for HB617, 5.3 pcd (- 30 %)for 2G12-Gl and 2.5 pcd (+ 4 %) for 2G12. Due to the high standard deviation of all q_P values, no difference could be stated between the q_P before and after adaptation.

For 2G12-Gl, however, two trends could have been observed referring to Fig. 30. One is that the growth rate seemed to slightly increase while on the same time q_P decreased over the course of the adaptation trial. Both trends, however, were established well before the actual adaptation has been started and are thus believed to have happened independent from the adaptation. It might have been occurred that a certain sub-population (with higher μ but lower q_P) of CHO cells was about to establish itself among the culture. On the other hand, these changes in μ and q_P are small and might have been not significant due to the high standard deviation of the values obtained, as stated in Tab. 17. Thus, no clear statement can be made whether this observation is true or not.

Despite small deviations in μ and q_P for 2G12-Gl, their overall changes were considered to be minor for all investigated cell lines. Presumably, the rich ProCHO5 medium could have had a beneficial effect on the cells, aiding them in adapting to the new cultivation environment. The nutrients in the medium could have been responsible for maintaining a high viability and thus shortened the length of the adaptation process (= the time until the culture is able to maintain a reasonable viability). Another explanation could be that the cells had already been adapted to the shear forces present in the continuously shaken shaking flasks, since they’ve already

been adapted to the Spinner flask, which also exerts some shear forces to the culture. So, unlike the adaptation from the Spinner to a T25, the adaptation from the Spinner to a shaking flask might not lead to a big difference in the stress level exerted on the culture. To prove this statement, however, further investigation on the actual stress level should be done, e.g. via microarray analysis in order to analyse the differential expression of stress-related proteins.

In conclusion, all three cell lines were able to cope much better with the vessel adaptation than with the medium adaptation. No permanent change in the vital cultivation parameters (viability, μ or q_P) had been observed over the course of the adaptation for any of the cell lines. It seems that changing the cultivation vessel had much less impact on the culture than changing the cultivation medium and both vessels under investigation were equally well suited for maintenance cultivation.

4.4 Subcloning

As medium change was presumed to lead to considerable rearrangements within the cell population due to adaptation processes to the different nutrient offer, we assumed that this would lead to the creation of several different sub-populations. Thus, subcloning was performed, which comprised isolation of single cells from an adapted cell population, screening via IgM-analysis, survey of the growth rate and finally expanding the best subclones (= those clones with the highest specific productivities q_P).

After subcloning, we were able to select clones from all three cell lines which showed an increase in specific productivity of merely 24% (HB617) to about 337% (2G12-Gl) or 617% (2G12), compared to before subcloning was performed (see Tab. **18**). This increment was calculated by comparing the averaged q_P values of cultivations in T25 flasks in D/H medium before and after subcloning was performed.

From the results of the initial measurement of IgM titres, the high spread of values for HB617 (± 65 %) indicates the presence of many different subpopulations in the culture. 2G12-Gl and 2G12 experience a much lower spread (± 29 % and ± 15 %, respectively), which implies that the culture is much more homogenous (or that the subclones present do not differ much in their end titres produced). In any case, these findings imply that the subcloning procedure

performed was definitely necessary to (re-)establish a homogeneous culture, especially in the case of HB617.

It has to be noted that the clones selected for subcloning (8 out of initially 384 for each cell line) might not have been representative as the initial screening in 384 well plate only selected those clones who reproduced the best (high μ) and did not consider the titres or the specific productivities, since no IgM measurement was feasible to that time.

4.5 FACS

Fig. 33 to Fig. 35 show counts detected via FACS after removing invalid counts so that all counts shown refer to a cell showing fluorescence either due to conjugation with an anti-HC or an anti-LC-antibody (coupled with a fluorophore). FACS was conducted using labelled antibodies specifically designed to bind to the HC and LC of the IgM's under investigation. Results should show the intracellular concentration of IgM subunits and whether they were different between the cell lines or whether their concentration changed in the course of the fermentation in the DASGIP bioreactor with ProCHO5 (+ supplements).

Fig. 33 shows the results for the **HB617** cells. After 10 days (shown in pink) the peak experienced a distortion to the left, which indicated that the majority of cells showed a weaker signal compared to the days before. This has been observed both for the heavy as well as for the light chain. This indicated that the intracellular production of IgM subunits started to deteriorate at Day 10 in the DASGIP. Data clearly shows that viability dropped below 20% and growth rate was almost stalled on that Day. The specific productivity of the viable cells, however, seemed to have actually increased (see Fig. 5). Data from the FACS, however, indicated that the intracellular concentration of IgM subunits is lower than in previous days. One could assume that the intracellular production of IgM deteriorated in the viable cells of the dying culture, while the assembly of the subunits and the secretion of the finished product seemed (yet) unaffected, which would have faked an increasing productivity.

For **2G12-Gl** this trend could not be observed (see Fig. 34). The signal obtained from the FACS remained constant over the whole course of the fermentation, which indicated a more stable expression of IgM subunits compared to HB617. According to ELISA-analysis, however, specific productivity did decrease over the course of fermentation and almost came

to a halt at Day 10 (see Fig. 6), which would have been expected, as viability of the culture was below 80 % to that time. It could be inferred from the data that the intracellular concentration of subunits remained constant, whereas the extracellular concentration of assembled IgM decreased. We hypothesize, that this phenomenon could have been attributed to degradation processes taking place in the extracellular space (detected by the ELISA measurement), while the intracellular IgM production remained unimpaired (indicated by the FACS analysis).

It can be inferred from Fig. 35 that **2G12** seemed to behave somewhat in between HB617 and 2G12-Gl. While the specific productivity decreased slightly towards the stationary phase (see Fig. 6), intracellular HC- and LC-concentrations also seem to decrease at Day 10, as indicated by the FACS analysis. One could infer that the FACS data thus mirrored the actual extracellular levels of IgM.

Despite the fact that the signals obtained in the FACS decreased towards the end of the DASGIP fermentation, no non-producers were identified on any day in any cell line. This indicates that the cell lines used showed a continuous expression of heterologous protein over the whole course of the fermentation.

4.6 Gene copy number

As shown in Fig. 10 the gene copy number was calculated relative to the housekeeping gene β-actin for each cell line and each target gene. Gene copy number analysis was conducted from a single ProCHO5 fermentation in the DASGIP bioreactor during the exponential phase of growth (Day 3 to 5), because sufficient material had to be harvested for the qPCR, which could not have been provided by the 70 mL shaking flask trials.

Data indicates that HB617 did have the highest copy number of all three relevant genes inserted in its genome of all three investigated cell lines. This is true except for LC, where no significant difference between HB617 and 2G12-GL could have been established. In any case, 2G12 had significantly less gene copy numbers of all three genes compared to the other two cell lines.

Overall we observed that the gene copy numbers followed the pattern HB617>2G12-Gl>2G12 which correlated with the specific productivities of the respective cell lines, which

is shown in Fig. 11. q_P values of the very same DASGIP fermentation can be observed in Tab. 11, where HB617 performed better than 2G12-Gl which again performed better than 2G12. This pattern is representative for about every fermentation trial conducted in the course of this thesis.

The GCN alone, however, seemed not to be fully responsible for the specific productivity since the ratios of gene copy numbers between the cell lines were not identical to those of the q_P values. HB617, for example, did not have a q_p value twice as high as for 2G12-Gl, although the GCN would indicate this. The same is true for every other cell line. This leads to the assumption that the overall productivity must be additionally governed by something other than the mere gene copy number. We suggest that parameters like the number of RNA-transcripts, the availability of secretion vesicles, the assembly rate of the multiprotein complex and the folding speed might have additional influence on the overall production rate of the protein. In fact, research by O'callahan et al. (2008) indicates that the rate of protein folding becomes the limiting step in high-producers. Their research on GS-NS0 cell lines indicates that chaperone expression is increased in cell lines with high q_P, which could not be attributed to merely an unfolded protein response. In conclusion, GCN mirrors the q_P of the respective cell line but not in a quantitative manner.

4.7 Gel electrophoresis

Our analysis of the **genomic DNA** isolation with a 0.8% agarose gel (see Fig. 36) revealed that the isolated DNA was > 10,000 bp which was to be expected. Furthermore, almost no smear was visible on the gel, which indicated a good DNA quality due to the lack of DNA-degradation products. The band intensity observed, however, was different from our expectation. Compared to the intensity of the 3000 bp band of the ladder, which contained 120 ng of DNA, the sample DNA, that contained 200 ng of genomic DNA, emitted much less signal. This could be due to loss of DNA sample during the manipulation steps.

Fig. 37 shows the result for the 2% agarose gel onto which the amplicon DNA from the qPCR were applied. It can be stated that the sizes for the amplicons of the target genes β-actin, HC, LC and JC were within the expected range (see

Tab. 9). However, a noticeable smear appeared on the gel, which indicates degradation products probably caused by the long storage of the samples (~ 2 months at 4°C). In the figure one can also observe different band intensities of the different samples, especially in the case of 2G12, which seems to show lower band intensities for HC and LC. This difference, however, was ignored since the sample for the electrophoresis was extracted from a 48-well-plate, which was subject to a qPCR. Due to the nature of this procedure, the final number of amplifications can be quite random for each sample.

The conclusion drawn from Fig. 37 is that each cell line contained all necessary genes of the correct size. Also, the absence of primer dimers was confirmed, as can be seen in the same figure.

5 Conclusion

In our experiments, we observed that, in our scenario, cultivation in the DASGIP is not superior to cultivation in the shaking flask. This is a rather unexpected result, as the enhanced means of control and monitoring should have yielded improved cultivation parameters, such as μ and q_P. We believe, however, that this bad performance in the DASGIP should not be attributed to the bioreactor itself, but to the enhanced stress level the cells might experience at these conditions. An adaption to the bioreactor might have alleviated the adverse effect on the cells. However, our proposal of stress-induction via the bioreactor should be confirmed with e.g. an expression analysis using microarray technology. If our hypothesis proves to be true, engineering approaches could be applied via inserting genes into the CHO cells, which confer stress-resistance. Butler et al (2012), for example, report that overexpressing genes belonging to the Bcl-2 family increase the longevity of the culture as well as its robustness against changes in pH, osmotic pressure and the presence of lactate and ammonia in the medium. This approach might give rise to more versatile and robust cell lines able to grow in high-stress environments such as the DASGIP bioreactor.

Recently, Butler et al (2012) have reported CHO high producers, which secrete recombinant glycoproteins in magnitudes of up to 50 pcd with titres of up to 5 $g \times L^{-1}$ in a fed batch system. The q_P and end titres achieved in this thesis (20 pcd an 0.5 $g \times L^{-1}$) are much below these values but one has to take into account that the cell lines investigated in this thesis were, firstly grown in a batch mode without feeding strategies (which certainly would have

improved productivities and titres) and were secondly secreting IgM's, which are among the more complex complex proteins used in mammalian cell culture due to their large size (ca. 900 kDa) and complex glycosylation pattern. More comparable values are reported by Tchoudakova et al. (2009) who state that the highest productivities for a recombinant CHO cell line secreting IgM is 30 pcd. They further described an IgM secreting PER.C6 cell line that showed a q_P of greater than 20 pcd when grown in a seven day batch process. They further argue that the glycosylation patterns of PER.C6 are identical to that of the human species, while CHO cell lines do have minor deviations from human glycosylation patterns. These safety considerations should be always taken into account when producing therapeutic recombinant proteins. Butler et al (2012) further report that HEK293 cell lines produce up to 20 pcd of a recombinant protein while HeLa cell lines are reported to produce low titres (10 $ng \times mL^{-1}$) and rat hybridoma cell lines producing titres up to 300 $mg \times L^{-1}$ in batch cultivation (Tchoudakova et al.; 2009). In this thesis, a maximum q_P of 19.79 ± 0.61 pcd and end titres of 0.5 $g \times L^{-1}$ were achieved when cultivating HB617 in a batch process using shaking flasks and ProCHO5 as a medium. Thus, this cell line is able to compete with other IgM secreting cell lines though not being the best producer currently available. As stated at the beginning of this thesis, the safety consideration regarding the use of CHO cells can be considered minor, although in some cases, the use of PER.C6 cells might be more appropriate.

Another finding of this thesis is, that the cell line 2G12, though having the worst q_P of all three cell lines, seem to be the most versatile one, as it was able to cope better with the high stress conditions in the DASGIP bioreactor and the poor nutrient offer of the D/H medium. Thus, this cell line has proven its use despite its low productivity.

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