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Biphasic cultivation strategy to avoid Epo-Fc aggregation and optimize protein expression

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ABSTRACT

In biphasic cultivations, the culture conditions are initially kept at an optimum for rapid cell growth and biomass accumulation. In the second phase, the culture is shifted to conditions ensuring maximum specific protein production and the protein quality required. The influence of specific culture parameters is cell line dependent and their impact on product quality needs to be investigated. In this study, a biphasic cultivation strategy for a Chinese hamster ovary (CHO) cell line expressing an erythropoietin fusion protein (Epo-Fc) was developed. Cultures were run in batch mode and after an initial growth phase, cultivation temperature and pH were shifted. Applying a DoE (Design of Experiments) approach, a fractional factorial design was used to systematically evaluate the influence of cultivation temperature and pH as well as their synergistic effect on cell growth as well as on recombinant protein production and aggregation. All three responses were influenced by the cultivation temperature. Additionally, an interaction between pH and temperature was found to be related to protein aggregation. Compared with the initial standard conditions of 37 °C and pH 7.05, a parameter shift to low temperature and acidic pH resulted in a decrease in the aggregate fraction from 75% to less than 1%. Furthermore, the synergistic effect of temperature and pH substantially lowered the cell-specific rates of glucose and glutamine consumption as well as lactate and ammonium production. The optimized culture conditions also led to an increase of the cell-specific rates of recombinant Epo-Fc production, thus resulting in a more economic bioprocess.

1. Introduction

Product aggregation is a commonly observed issue during recombinant protein manufacturing. Aggregated proteins are highly undesirable as they can compromise a drug's quality, safety and efficacy (Vázquez-Rey and Lang, 2011). In extreme cases, such as the aggregation of the anemia drug erythropoietin (EPO), product aggregation was one of the factors implicated in EPO-derived immunogenicity that caused pure red cell aplasia and subsequently fatalities in patients (Schellekens and Jiskoot, 2006; Buchanan et al., 2013). As a consequence, aggregates have to be separated from the final product. Aggregate removal can be achieved during protein purification, however, due to similar characteristics of monomer and aggregates the separation is often challenging and typically reduces the final yields. Therefore, maintaining cells at conditions that minimize the formation of protein aggregates is desirable. A number of cell culture parameters have been investigated for their effect on expression level and quality of the recombinant protein. A particular focus has been the cultivation temperature.

Although reducing the temperature below the standard value of 37 °C decreases the specific growth rate, other positive effects have been reported e.g. extended culture longevity, reduced nutrient consumption, improved viability and decreased protease activity (Woo et al., 2008; Kou et al., 2011). Additionally, a lower cultivation temperature may affect the cell cycle and can lead to an increase in

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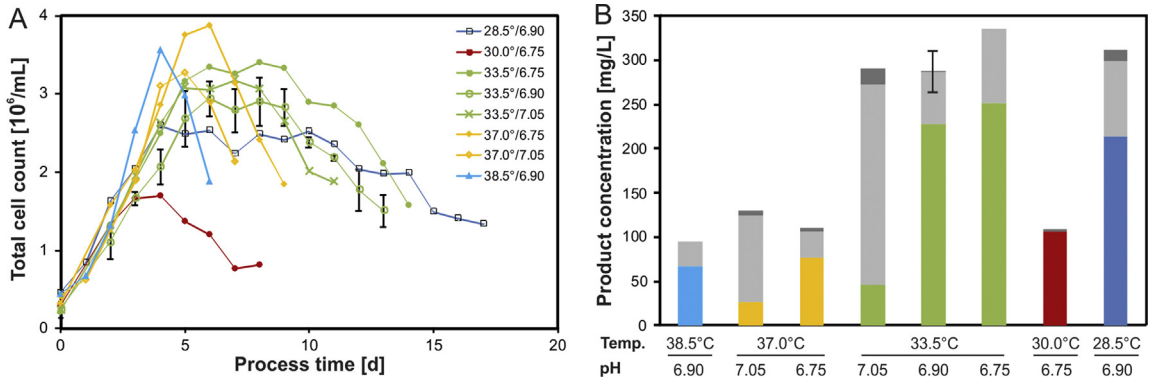


Fig. 1. (A) Total cell concentrations and (B) peak poduct concentrations under different cultivation conditions (parameter shift after 48 h). The graphs show 9 parameter settings. The error bars show the standard deviation for the triplicate center point. (B) The bars represent the total Epo-Fc concentration; monomeric product of experiments conducted at the same temperature are represented in the same color. Aggregated product is shown in light greyscale bars and fragmented product is depicted in dark greyscale bars.

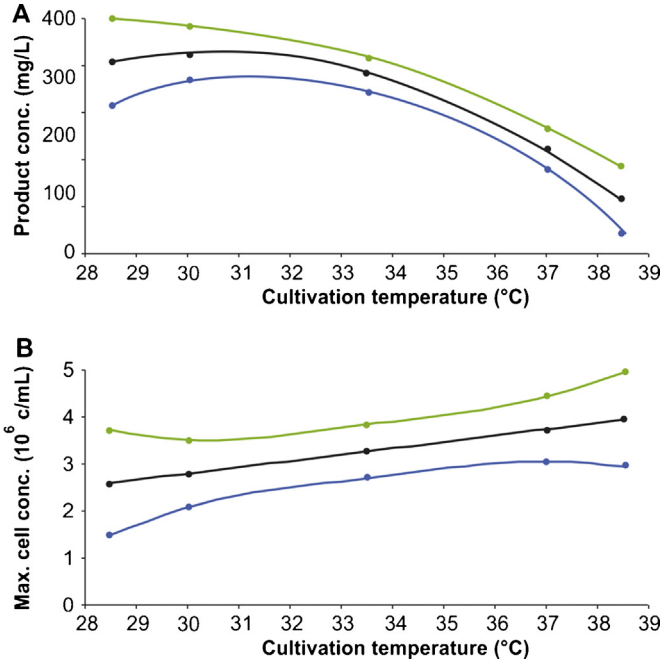


Fig. 2. Prediction plots for (A) predicted product titer versus cultivation temperature, (B) predicted peak cell concentration versus cultivation temperature. The respective confidence intervals are 95%. Colored lines indicate the calculated average (black), upper confidence interval (green) and lower confidence interval (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the specific productivity, although this effect is cell line dependent. A temperature range of about 25–39 °C has been investigated in the literature but there is a clear focus on 30–37 °C (Yoon et al., 2005; Trummer et al., 2006a).

To a lesser extent cultivation pH has also been used as a parameter to influence cell growth and productivity in mammalian cell culture. Typical pH set points for CHO cells are neutral to slightly alkaline (7.0–7.2). It has been shown that the cultivation pH has an influence on growth rate and nutrient consumption. In general, cells tend to grow more slowly at a slightly acidic pH (6.8–7.0) and exhibit a reduced cell metabolism (Yoon et al., 2005; Trummer et al., 2006a). At a slightly alkaline pH (7.2 and higher) the metabolism of CHO cells tends to be more active. The influence of pH on recombinant protein production is cell line specific. Some reports claim higher specific productivities at alkaline pH values of 7.6–7.8 (Borys et al., 1993). Other groups report fairly constant levels over a broad pH range of 6.8–7.3 (Trummer et al., 2006a) or 6.85–7.8 (Yoon et al., 2005).

Another important criterion for the economic feasibility of a production process is the required cultivation time. Low cultivation temperatures and an acidic pH can offer a positive effect on cell specific protein production but can also decrease the cellular growth rate and consequently reduce volumetric productivity. To find a compromise between process duration and optimal environmental conditions for recombinant protein production, the concept of biphasic cultivation has been suggested (Yoon et al., 2005; Trummer et al., 2006a). In this mode a process is run in two distinct phases. Initially biomass is generated at 37 °C and the pH optimum for growth of the respective cell line; the culture is then shifted to temperature and pH conditions ensuring the maximum specific protein production and the protein quality required. Thereby, a high protein output and acceptable process duration can be achieved. The focus of most studies on biphasic cultivation was the increase of volumetric productivity and minimal formation of catabolites (Butler, 2005; Trummer et al., 2006a) but far less attention has

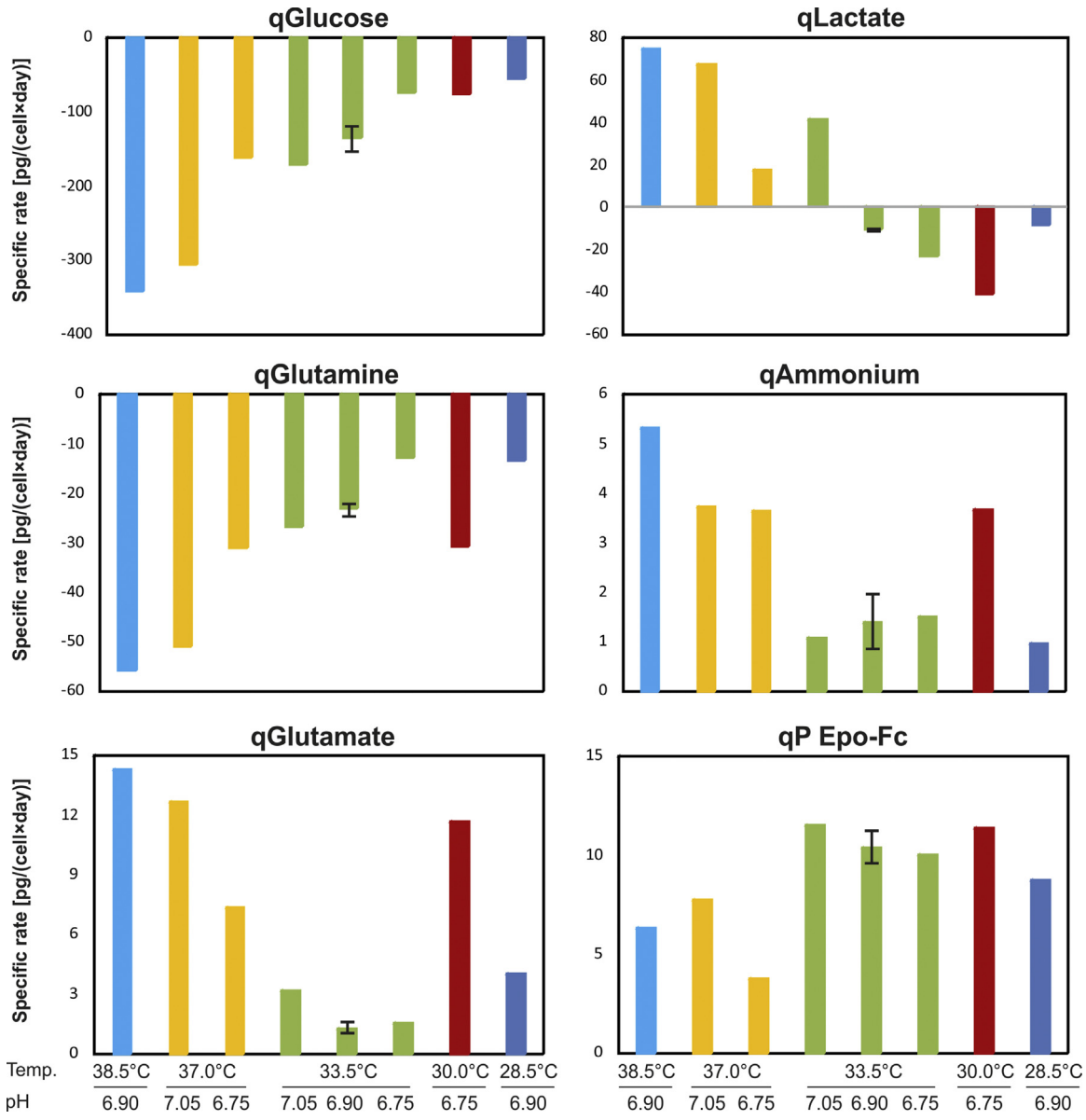


Fig. 3. Cell-specific rates of glucose, lactate, glutamine, ammonium, glutamate and Epo-Fc production or consumption. All cell-specific metabolic rates were calculated starting from the day of the parameter shift until culture termination. The specific metabolic rates were designated negative for consumption and positive for production. Experiments conducted at the same temperature are represented in the same color. The error bar shows one standard deviation of triplicate center point experiments.

been paid to the impact of parameter shift on the quality of the recombinant protein.

In this study, we have employed a DoE (Design of Experiments) approach to systematically investigate simple process parameters such as temperature and pH individually as well as their synergistic effect on expression level and aggregation of a recombinantly produced homodimeric Fc-fusion protein in CHO cells during biphasic batch cultivation in bioreactors.

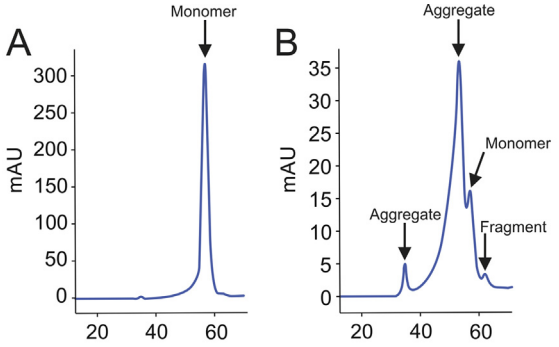
2. Materials and methods

2.1. Cell line and medium

A recombinant CHO DUKX-B11 (ATCC CRL-9096) cell line expressing a homodimeric fusion protein Epo-Fc (clone C14F2) was originally established by Lattenmayer et al. (2007). Cells were thawed from a research cell bank and propagated in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 (Biochrom) supplemented with 0.6 g/L L-glutamine, 10 g/L protein-free additive (Polymun Scientific), 2.5 g/L soy peptone (HyPep^{TM} 1510, Sheffield Bioscience), 1 g/L Pluronic F-68 (Sigma Aldrich) and 192 nM methotrexate (Sigma Aldrich). The cell line was maintained in a 125 mL spinner flask (Techne) operated at a stirring speed of 50 rpm. The routine culture was passaged twice per week and seeded at working volume of 50 mL and a starting cell concentration of 3×10^5 c/mL.

2.2. Bioreactor and biphasic cultivation parameters

Inoculum for the batch experiments was prepared in a Cellbag 10 L (GE Healthcare, General Electric Company) using DMEM/Ham's medium supplemented to a final concentration of 5 g/L D-glucose, 0.9 g/L L-glutamine, 10 g/L protein-free additive, 2.5 g/L soy peptone, 1 g/L Pluronic F-68 and 192 nM methotrexate. All experiments were conducted with media and additives derived from the same batches to guarantee consistency. The culture was run at standard



Experiment [#]	Cultivation parameters	Monomers [%]	Aggregates [%]	Fagments [%]
9	pH 6.90, 28.5°C	68.5	27.4	4.1
3	pH 6.75, 30.0°C	96.5	0.7	2.8
7	pH 6.75, 33.5°C	74.8	25.2	0.0
1, 5, 10*	pH 6.90, 33.5°C	78.9 ± 0.6	20.5 ± 1.5	0.6 ± 0.8
6	pH 7.05, 33.5°C	16.0	77.8	6.2
8	pH 6.75, 37.0°C	69.4	27.3	3.3
2	pH 7.05, 37.0°C	20.3	75.7	4.0
4	pH 6.90, 38.5°C	70.4	29.6	0.0

Fig. 4. Size exclusion chromatograms after protein A capture showing different Epo-Fc conformations depending on cultivation conditions. The chromatograms depict product samples from cultivation at (A) 30.0 °C and pH 6.75 or (B) 37.0 °C and pH 7.05. The percentages of monomers, aggregates and fragments are shown below for all investigated culture conditions. All experiments were randomized as indicated by the respective experimental numbers. *Center point experiments performed in triplicates.

Table 1
Parameter settings and experimental results of Epo-Fc titers, peak cell concentrations, viable cumulative cell days (VCCD), specific Epo-Fc productivities (qP) and volumetric productivities (space time yield; STY) for all investigated culture conditions. All experiments were randomized as indicated by the respective experimental numbers.

Experiment [#]	Cultivation parameters	Titer [mg/L]	Peak cell conc. [10^6 c/mL]	VCCD [10^6 c/mL]	qP [pg/c/d]	STY [mg/L/d]
9	pH 6.90, 28.5 °C	312.0	2.60	33.49	8.7	18.5
3	pH 6.75, 30.0 °C	109.6	1.70	8.84	11.4	14.5
7	pH 6.75, 33.5 °C	336.2	3.40	34.24	10.0	28.3
1, 5, 10[a]	pH 6.90, 33.5 °C	288.1 ± 23.0	2.94 ± 0.57	26.82 ± 3.96	10.3 ± 0.8	21.9 ± 1.6
6	pH 7.05, 33.5 °C	291.0	3.18	24.72	11.5	26.3
8	pH 6.75, 37.0 °C	109.7	3.87	21.47	3.8	10.9
2	pH 7.05, 37.0 °C	129.7	3.27	14.53	7.7	16.8
4	pH 6.90, 38.5 °C	94.5	3.56	12.27	6.3	17.0

[a] Center point experiments performed in triplicates.

cultivation conditions of pH 7.05 and 37 °C. Biphasic batch experiments were conducted in Minifors (Infors) stirred-tank bioreactors. The bioreactors were inoculated with exponentially growing cells at concentrations of 3–4 × 10^5 c/mL in a final working volume of 2 L. The process parameters were initially set to standard conditions of 37 °C, 30% DO, pH 7.05 and a stirring speed of 35 rpm. At cell concentrations of 1.2–1.5 × 10^6 c/mL the cultures were shifted to their respective pH and temperature set points according to Table 1. The investigated temperature set points were 28.5 °C, 30.0 °C, 33.5 °C, 37.0 °C and 38.5 °C. The examined pH set points were 6.75, 6.90 and 7.05. The batch cultivations were terminated once the viability dropped below 70%.

2.3. Sample analysis

During the bioreactor culture a 10 mL sample was withdrawn daily to determine cell concentration, viability, antibody titer and metabolite concentrations of glucose, glutamine, glutamate as well as lactate and ammonium. Total cell concentration was measured by nuclei count after cell lysis using a Coulter counter (Multisizer™ II, Beckman Coulter). Cell viability was determined using the trypan blue exclusion method and cell count in a haemocytometer (C-Chip PS, NanoEnTek Inc.). Metabolite concentrations were analyzed in a BioProfile® 100 Plus (Nova Biomedical). Recombinant protein concentration was quantified by ELISA. Briefly, polyclonal goat anti-human ɣ-chain antiserum (Sigma; I-7883) was used for coating and product capture. Detection was performed with a horseradish peroxidase-conjugated goat anti-human ɣ-chain antiserum (Zymed; 62-8420). The concentration of bound detection antibody was visualized with o-phenylenediamine (Merck). Plates were measured with an Infinite® M1000 microplate reader (Tecan) at a wavelength of 492 nm and a reference wavelength of 620 nm.

Additionally, protein quality was determined from a 20 mL cell culture suspension at the time of harvest (criterion was a viability of <70%). The sample was centrifuged and the cell-free supernatant was filtered through a 0.2 μm filter (Millipore) and subsequently purified by protein A chromatography (MabSelect SuRe™, General Electric Company) on an ÄKTA Purifier (General Electric Company). The conformation of Epo-Fc was then analyzed by size exclusion chromatography (SEC) on a Superose 6 resin (General Electric Company).

2.4. Design of experiment (DoE)

A central composite design at three temperature and pH levels (Table 1) was used to evaluate the impact of these parameters on cell growth, recombinant protein production and aggregation of Epo-Fc. As a nonlinear response to temperature was expected, the design was extended by two additional cultures at 28.5 °C and 38.5 °C to investigate curvature in the response to temperature. A fractional factorial design with “Resolution V” was used. This design allows estimation of the main effects as well as two factor

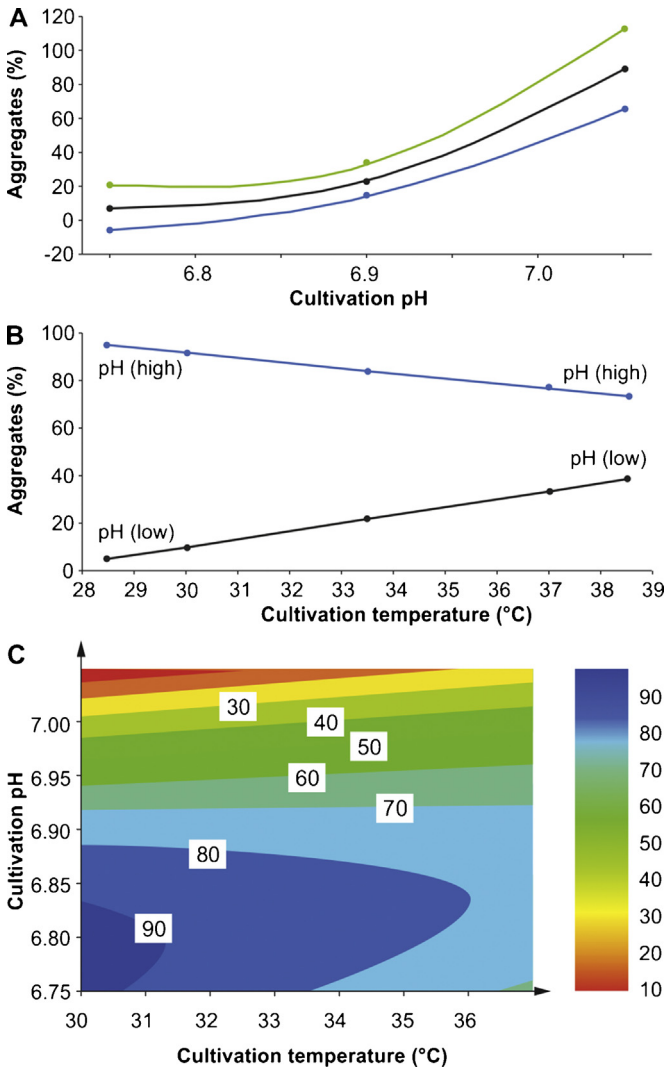


Fig. 5. Prediction plots for (A) predicted percentage of aggregates versus pH at a cultivation temperature of 30.0 °C, and (B) predicted percentage of aggregates versus pH and temperature. The respective confidence intervals are 95%. (C) Monomer concentration in percent versus pH and temperature. Colored lines indicate the calculated average (black), upper confidence interval (green) and lower confidence interval (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

interactions. The experimental variability was assessed by three independent center point experiments.

3. Results

The cultivation temperature had a pronounced effect on cell growth. While no significant increase in the growth rate was observed at 38.5 °C, temperatures lower than 37 °C resulted in slower cell growth and a substantially lower peak cell concentration (Fig. 1A; Table 1). However, cultivation at lower temperature extended the process time by up to 8 days resulting in a substantially increased viable cell integral. At lower temperatures the final product concentration usually increased compared with the initial standard conditions (37 °C, pH 7.05), as shown in Fig. 1B and Table 1. Upon the parameter shift, the cell-specific recombinant protein production was constant at about 11 ± 1 picogram Epo-Fc per cell and day (pcd) in the temperature intervals of 33.5 °C and 30 °C (Table 1). Outside this temperature range the mean specific productivities were 20% lower at 28.5 °C, and 30–60% reduced at the commonly applied standard cultivation temperature of 37 °C or higher. The prediction plot for the cultivation temperature shows a clear curvature and the maximum product concentration was predicted for the low end of the investigated temperature range (Fig. 2A). In contrast, the highest peak cell concentrations are projected at the higher cultivation temperatures as shown in the prediction plot in Fig. 2B. Because of the extended plateau phase, the volumetric productivity (space-time yield; STY) of the cultures was higher at temperatures lower than the standard of 37 °C. As shown in Table 1, the mean volumetric productivity at 33.5 °C was 22–28 mg/(L × d) but 15–60% lower or 11–19 mg/(L × d) when the bioreactor was operated at higher or lower temperatures. The cultivation pH did not have a significant influence on the titer.

Metabolite concentrations of glucose, lactate, glutamine, glutamate and ammonium were followed throughout the biphasic batch process after parameter switch. The cell-specific metabolic rates after parameter shift are compared in Fig. 3. Culture temperature and pH affected glucose metabolism in a synergistic manner. Glucose and lactate metabolism steadily increased with increasing temperature and pH. More specifically the cellular glucose consumption rates (qGlc) were lowest at 28.5 °C (pH 6.90) and increased by 30% when the temperature was elevated to 30 °C (pH 6.75). Glucose consumption was similar at 30 °C and 33.5 °C, however, in this case, higher pH values resulted in an up to 2.3-fold rise of the specific glucose consumption rates. Compared to the minimal observed rate at 28.5 °C, a standard cultivation temperature of 37 °C resulted in a twofold increase of the cell specific glucose consumption at pH 6.75 and cultures grown at near neutral pH of 7.05 also showed a further increase of the consumption rate. In accordance with this trend the highest cell-specific rates of glucose consumption were found when the bioprocess was operated at 38.5 °C and pH 6.90. It should be noted that only in two cultures did the glucose concentrations drop below 0.5 g/L, however, this coincided with the day of termination (data not shown). Overall, the synergistic effect of temperature and pH resulted in an up to 6.3-fold increase in the cell-specific glucose consumption rates.

During the initial phase of the culture, lactate accumulated in all experiments. However, following the parameter shift, cells consumed the built-up lactate if grown at temperatures up to 33.5 °C, while they continued to produce lactate at higher temperatures (Fig. 3). Again, a synergistic effect of pH was observed with the highest lactate consumption at an acidic pH. Increasing the pH from 6.75 to 6.90 reduced the specific lactate consumption rate by about 50%. Further increasing the pH to 7.05 resulted in lactate production.

Glutamine consumption and corresponding ammonium production increased at elevated temperature and pH values (Fig. 3). Maintaining the pH at 7.05 but increasing the temperature from 33.5 °C to 37 °C doubled the specific glutamine consumption rates (qGln) from 27 pcd and 51 pcd. At the same time cell-specific ammonium production rates (qNH_4^+) increased from 1.1 pcd (33.5 °C) to 3.7 (37 °C). A similar effect was observed when increasing the pH while growing cell cultures at a temperature of 33.5 °C. Cell-specific glutamine consumption rates rose from 13 pcd (pH 6.75) to 23 pcd (pH 6.90) and 27 pcd (pH 7.05). Likewise, at 37 °C, a pH increase from 6.75 to 7.05 elevated glutaminolysis by 40%. The highest glutamine consumption and corresponding ammonium production were observed at 38.5 °C and pH 6.90. In summary, a synergistic effect of temperature and pH was observed and increased temperature combined with a slightly alkaline pH resulted in 4-fold and 6-fold increase of glutamine consumption and ammonium production, respectively.

The production of glutamate was inversely related to glutamine consumption as shown in Fig. 3. As a consequence, increased glutaminolysis rates elevated glutamate production. In general, the lowest cell-specific rates of glutamate production were observed at 33.5 °C. Elevating or decreasing the temperature resulted in higher production rates.

Aggregation of Epo-Fc was strongly dependent on the cultivation pH and varied between 78% and 0.7% in the experiments. All peaks eluting earlier than Epo-Fc homodimers (i.e. monomers) in the SEC chromatograms were summarized as aggregates. The experiments indicated that an acidic pH resulted in low aggregate concentrations (Fig. 4). A shift to low cultivation temperatures further reduced Epo-Fc aggregation, demonstrating a synergistic interaction between the two process parameters. The influence of the culture conditions on product quality is also illustrated in the SEC chromatograms in Fig. 4, comparing cultivation at 30 °C and pH 6.75 with the initial standard conditions of 37 °C and pH 7.05. Under low temperature and low pH conditions, about 3% of the recombinant protein was detected as fragments. The monomer peak contained more than 96% of the recombinant protein and less than 1% was detected in aggregates. At higher temperature and a neutral pH, a similar fragment fraction of about 4% was observed. However, only about 20% of the total recombinant protein was detected as monomer, while more than 75% was found in aggregates. The prediction plot shown in Fig. 5A depicts a clear curvature with the lowest percentage of aggregates at an acidic pH. The beneficial effect of a low pH to reduce product aggregation was most pronounced in combination with a low cultivation temperature (Fig. 5B). The synergistic effect of pH and temperature is represented in the contour plot in Fig. 5C in which the maximum monomer concentration was predicted for a parameter shift to a cultivation temperature below 31 °C and a pH below 6.85.

One experiment was removed from the model predicting monomer concentration after outlier identification by residual analysis using a normal probability plot. A statistically valid model was obtained after excluding this experiment. No definite root cause for the outlier was established, however, the 10 remaining experiments were sufficient to support the reported model.

4. Discussion

Cultivation at hypothermic conditions is frequently performed to maximize product concentration. This is usually a result of extended culture longevity. Additionally, an increase in cell-specific recombinant protein production has been observed, however, this response was shown to be cell line-specific (Becerra et al., 2012). Regarding Epo-Fc sialylation, Trummer et al. (2006a) have previously reported that cultivation pH (investigated range: 6.8–7.3) did not affect the sialic acid content when using the same cell line as in this project. Product sialylation did not change between cultivation temperatures of 39–35 °C, but decreased by 20% at 33 °C and 40% at 30 °C. However, Trummer et al. (2006b) later showed that reduced sialylation at cell culture temperature of 33 °C and 30 °C was prevented when used in combination with slightly acidic pH levels (pH set point 6.90). Therefore, no decrease in Epo-Fc sialylation is anticipated at low cultivation temperature (30 °C) and acidic pH (6.75), which resulted in the lowest levels of product aggregation (0.7%). The impact of low cultivation temperatures on higher specific productivities but reduced sialylation could be compensated by lowering the pH set point. Since most cell lines grow faster at slightly alkaline pH values, a pH change during the production phase highlights another advantage of biphasic cultivation processes.

An overall reduction of cell metabolism at low temperature settings is probably the main reason behind the maintained cell viability and the extended process time – in our case an increase from 6 to 17 days. The cell metabolism was significantly affected by the applied culture temperature and pH value (Fig. 3). The increase of both process parameters resulted in elevated cell-specific rates for glucose and glutamine consumption as well as lactate and ammonium production. These observations are in agreement with previously published work (Fogolín et al., 2004; Yoon et al., 2005; Trummer et al., 2006a). Compared to a standard cultivation process at 37 °C and pH 7.05, a sole temperature reduction to 33.5 °C (pH 7.05) decreased the cell-specific glucose and glutamine consumption roughly by half and the ammonium production to more than a third of its original value. The lactate production was 40% lower than at the standard conditions. A sole pH reduction from 7.05 to 6.75 (37 °C) decreased the qGln by 40%, qGlc by 50% and qLac by 70%. A pH shift from 7.05 to 6.90 and 6.75 at a constant temperature of 33.5 °C decreased the metabolic rates in a similar way. Altogether, parameter shifting was demonstrated to enable bioprocess optimization to reduce the cell-specific rates of nutrient consumption rates as well as by-product formation.

In our study, a temperature reduction from 37 °C to 33.5 °C or lower increased the final Epo-Fc concentration about 2.5-fold (Fig. 1B). This was caused by an extended process time (6 to 17 days) as well as higher specific productivities. A similar temperature-dependent response of Epo production has also been shown by Yoon at al. (2005). However, protein aggregation was not investigated during these studies despite alternative disulphide bond formation being a major issue in Epo product quality.

Protein aggregation is a commonly observed issue during recombinant protein manufacturing. In general, protein aggregates may compromise a drug's quality, safety and efficacy (Vázquez-Rey and Lang, 2011) and therefore need to be separated from the final product. This can be challenging and typically reduces the final yields. Therefore, maintaining cells at conditions that minimize aggregate formation during the bioproduction process is beneficial. Although several reports investigate cell culture conditions and additives to achieve this (Jing et al., 2012), the process should be as simple as possible and not result in additional purification requirements to remove additives. The impact of cultivation temperature on protein aggregation has been addressed in several studies but led to contrary outcomes. On the one hand, mild hypothermia reduced the fraction of high molecular weight species (Rodriguez et al., 2005; Sunley et al., 2008) and supports the theory of the beneficial effect of a longer residence time of the synthesized protein in folding pathways in a more stable environment (Cromwell et al., 2006). On the other hand, hypothermic cultivation has been shown to increase protein aggregation (Rodriguez et al., 2010; Jing et al., 2012). Jing and colleagues (2012) expressed an IgG fusion protein in CHO cells. A temperature reduction from 37 °C to 34 °C and 32 °C significantly decreased protein productivity and increased product aggregation by up to 25%. As a reason for this the authors suggested altered intracellular mechanisms for aggregation or extracellular product oxidation. In the study of Jing et al. (2012) the pH change had no significant influence on aggregation, but this was probably due to the narrow pH range of 7.0-7.1 which was investigated. Rodriguez et al. (2010) produced recombinant human interferon-beta in a biphasic batch process using CHO cells and accumulated up to 80% aggregates. In this case, the authors argued that the higher level of product aggregation was due to a higher concentration of product and an extended residence time. Unlike their study, we observed a synergistic effect of cultivation temperature and pH value resulting in drastically reduced product aggregation. In general, we found lower amounts of aggregates at lower temperatures (Figs. 1 B and 4). Furthermore, aggregates were substantially reduced at a lower cultivation pH. For the process investigated, the pH value had a substantial influence on product aggregation, which was dependent on the applied cultivation temperature. The influence was less pronounced at higher temperatures, however, a combination of low cultivation temperature and more acidic pH resulted in the lowest concentration of protein aggregates (<1%) compared to more than 75% during standard conditions at 37 °C and physiological pH. Proteins that do not have their native configuration or are aggregated are usually retained during

the secretion process (Ellgaard and Helenius, 2003; Reinhart et al., 2014). Chaperones specifically interact with exposed hydrophobic regions of incompletely folded proteins and thereby assist in folding, stabilization and prevent aggregation. If proper protein folding is inhibited, aggregated protein species cannot proceed further in the secretion process and accumulate in the ER. As a consequence the cell activates a signal transduction network (i.e. unfolded protein response; UPR) for protein degradation. Therefore, aggregation most probably occurred after secretion of Epo-Fc and thus was unrelated to the protein-folding process itself. The Epo-Fc expressing cell line herein used is known to produce non-disulphide-linked aggregates under certain conditions (Schriebl et al., 2006). Additionally, Epo contains four cysteines giving rise to two disulphide bonds (Taschwer et al., 2012). It is prone to form aggregates due to reduction and subsequent random re-oxidation of inter-molecular thiol groups (Way et al., 2005; Wang et al., 2013). The sulfhydryl groups in the Fc domain are less involved in aggregation due to their solvent inaccessibility (Brych et al., 2010; Wang et al., 2013). It seems likely that the conditions in the culture supernatant affected the recombinant protein and caused aggregation which was also shown previously (Wang et al., 2013). Investigating a pH range of 7.0–7.7, it was found that more alkaline conditions facilitate the oxidation of free thiols and consequently increase protein aggregation. Therefore, it seems likely that cell cultivation at a lower pH can further reduce the aggregation of Epo-Fc.

Way et al. (2005) reported an alternative way to reduce Epo-Fc aggregation by rearranging the protein's disulphide bonding pattern to enhance the structural stability. This improved Epo-Fc expression as non-aggregated homodimer from 65% to 90%. However, care needs to be taken when introducing changes to the amino acid sequence to maintain the protein's activity and prevent the introduction of additional T_H cell epitopes that could increase the immunogenicity of the therapeutic molecule (Buchanan et al., 2013).

5. Conclusion

We developed a biphasic process for the production of an aggregation-prone Epo-Fc fusion protein in a CHO cell batch culture. Using a DoE approach, a synergistic effect of cultivation temperature and pH was found and parameters were established to reduce protein aggregation to levels below 1%. Additionally, it was shown that under optimized conditions the cell metabolism was reduced, leading to lower nutrient consumption and by-product accumulation. The optimized culture conditions also led to an increase of the cell-specific rates of recombinant Epo-Fc production, thus resulting in a more economic bioprocess. Furthermore, we demonstrated that the optimization of cultivation temperature and pH in a biphasic process can provide an easy and cost-efficient strategy to increase product yields and significantly reduce protein aggregation without the need of protein engineering.

Disclosure statement

The authors have declared no conflict of interest.

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References

Becerra, S., Berrios, J., Osses, N., Altamirano, C., 2012. Exploring the effect of mild hypothermia on CHO cell productivity. Biochem. Eng. J. 60, 1–8.

Borys, M.C., Linzer, D.I., Papoutsakis, E.T., 1993. Culture pH affects expression rates and glycosylation of recombinant mouse placental lactogen proteins by Chinese hamster ovary (CHO) cells. Biotechnology (N.Y.) 11, 720–724.

Brych, S.R., Gokarn, Y.R., Hultgen, H., Stevenson, R.J., Rajan, R., Matsumura, M., 2010. Characterization of antibody aggregation: role of buried, unpaired cysteines in particle formation. J. Pharm. Sci. 99, 764–781.

Buchanan, A., Clementel, V., Woods, R., Harn, N., Bowen, M.A., Mo, W., Popovic, B., Bishop, S.M., Dall'Acqua, W., Minter, R., Jermutus, L., Bedian, V., 2013. Engineering a therapeutic IgG molecule to address cysteinylation, aggregation and enhance thermal stability and expression. MAbs 5, 255–262.

Butler, M., 2005. Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. Appl. Microbiol. Biotechnol. 68, 283–291.

Cromwell, M.E., Hilario, E., Jacobson, F., 2006. Protein aggregation and bioprocessing. Am. Assoc. Pharm. Sci. J. 8, E572–9.

Ellgaard, L., Helenius, A., 2003. Quality control in the endoplasmic reticulum. Nat. Rev. Mol. Cell Biol. 4, 181–191.

Fogolín, M.B., Wagner, R., Etcheverrigaray, M., Kratje, R., 2004. Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells. J. Biotechnol. 109, 179–191.

Jing, Y., Borys, M., Nayak, S., Egan, S., Qian, Y., Pan, S.-H., Li, Z.J., 2012. Identification of cell culture conditions to control protein aggregation of IgG fusion proteins expressed in Chinese hamster ovary cells. Process Biochem. 47, 69–75.

Kou, T., Fan, L., Zhou, Y., Ye, Z., Zhao, L., Tan, W., 2011. Increasing the productivity of TNFR-Fc in GS-CHO cells at reduced culture temperatures. Biotechnol. Bioproc. Eng. 16, 136–143.

Lattenmayer, C., Loeschel, M., Schriebl, K., Steinfellner, W., Sterovsky, T., Trummer, E., Vorauer-Uhl, K., Müller, D., Katinger, H., Kunert, R., 2007. Protein-free transfection of CHO host cells with an IgG-fusion protein: selection and characterization of stable high producers and comparison to conventionally transfected clones. Biotechnol. Bioeng. 96, 1118–1126.

Reinhart, D., Sommeregger, W., Debreczeny, M., Gludovacz, E., Kunert, R., 2014. In search of expression bottlenecks in recombinant CHO cell lines-a case study. Appl. Microbiol. Biotechnol. 98, 5959–5965.

Rodriguez, J., Spearman, M., Huzel, N., Butler, M., 2005. Enhanced production of monomeric interferon-beta by CHO cells through the control of culture conditions. Biotechnol. Prog. 21, 22–30.

Rodriguez, J., Spearman, M., Tharmalingam, T., Sunley, K., Lodewyks, C., Huzel, N., Butler, M., 2010. High productivity of human recombinant beta-interferon from a low-temperature perfusion culture. J. Biotechnol. 150, 509–518.

Schellekens, H., Jiskoot, W., 2006. Erythropoietin-associated PRCA: still an unsolved mystery. J. Immunotoxicol. 3, 123–130.

Schriebl, K., Trummer, E., Lattenmayer, C., Weik, R., Kunert, R., Müller, D., Katinger, H., Vorauer-Uhl, K., 2006. Biochemical characterization of rhEpo-Fc fusion protein expressed in CHO cells. Protein Expr. Purif. 49, 265–275.

Sunley, K., Tharmalingam, T., Butler, M., 2008. CHO cells adapted to hypothermic growth produce high yields of recombinant beta-interferon. Biotechnol. Prog. 24, 898–906.

Taschwer, M., Hackl, M., Hernández Bort, J.A., Leitner, C., Kumar, N., Puc, U., Grass, J., Papst, M., Kunert, R., Altmann, F., Borth, N., 2012. Growth, productivity and protein glycosylation in a CHO EpoFc producer cell line adapted to glutamine-free growth. J. Biotechnol. 157, 295–303.

Trummer, E., Fauland, K., Seidinger, S., Schriebl, K., Lattenmayer, C., Kunert, R., Vorauer-Uhl, K., Weik, R., Borth, N., Katinger, H., Müller, D., 2006a. Process parameter shifting: part I. effect of DOT pH, and temperature on the performance of Epo-Fc expressing CHO cells cultivated in controlled batch bioreactors. Biotechnol. Bioeng. 94, 1033–1044.

Trummer, E., Fauland, K., Seidinger, S., Schriebl, K., Lattenmayer, C., Kunert, R., Vorauer-Uhl, K., Weik, R., Borth, N., Katinger, H., Müller, D., 2006b. Process parameter shifting: part II biphasic cultivation-a tool for enhancing the volumetric productivity of batch processes using Epo-Fc expressing CHO cells. Biotechnol. Bioeng. 94, 1045–1052.

Vázquez-Rey, M., Lang, D.A., 2011. Aggregates in monoclonal antibody manufacturing processes. Biotechnol. Bioeng. 108, 1494–1508.

Wang, T., Fodor, S., Hapuarachchi, S., Jiang, X.G., Chen, K., Apostol, I., Huang, G., 2013. Analysis and characterization of aggregation of a therapeutic Fc-fusion protein. J. Pharm. Biomed. Anal. 72, 59–64.

Way, J.C., Lauder, S., Brunkhorst, B., Kong, S.M., Qi, A., Webster, G., Campbell, I., McKenzie, S., Lan, Y., Marelli, B., Nguyen, L.A., Degon, S., Lo, K.M., Gillies, S.D., 2005. Improvement of Fc-erythropoietin structure and pharmacokinetics by modification at a disulfide bond. Protein Eng. Des. Sel. 18, 111–118.

Woo, S., Jeon, J., Jeong, Y., Seung, J., Sung, K., 2008. Effect of culture temperature on erythropoietin production and glycosylation in a perfusion culture of recombinant CHO cells. Biotechnol. Bioeng. 101, 1234–1244.

Yoon, S.K., Choi, S.L., Song, J.Y., Lee, G.M., 2005. Effect of culture pH on erythropoietin production by Chinese hamster ovary cells grown in suspension at 32.5 and 37.0Â°C. Biotechnol. Bioeng. 89, 345–356.