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

Evelyn Trummer,[1,3] Katharina Fauland,[1,3] Silke Seidinger,[1,3] Kornelia Schriebl,[1,3] Christine Lattenmayer,[1,3] Renate Kunert,[1,3] Karola Vorauer-Uhl,[1,3] Robert Weik,[2] Nicole Borth,[1] Hermann Katinger,[1,2] Dethardt Müller[1,2,3]

[1]Department of Biotechnology, Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences Vienna; Muthgasse 18, 1190 Vienna, Austria; telephone: +43 1 36006 6230; fax: + 43 1 3697615; e-mail: dethardt.mueller@boku.ac.at
[2]Polymun Scientific Immunbiologische Forschung GmbH, Nussdorfer Laende, Vienna, Austria
[3]Austrian Center of Biopharmaceutical Technology, Muthgasse, Vienna, Austria

Received 21 November 2005; accepted 18 April 2006

Published online 30 May 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.21013

Abstract: The impact of process environment changes on process performance is one of the most crucial process safety issues when cultivating mammalian cells in a bioreactor. In contrast, directed shifting of process parameters can also be used as an optimization tool providing higher cell and product yields. Compared to other strategies that also aim on the regulation of cell growth and protein expression process parameter shifts can be easily performed without reagent addition or even genetic modification of the host cell line. However, a successful application of changing process conditions implies a profound understanding of the provoked physiological changes within the cells. In a systematic approach we varied the dissolved oxygen tension (DOT), pH, and temperature of CHO cultures in controlled bioreactors and investigated the impact on growth, productivity, metabolism, product quality and cell cycle distribution using a recombinant CHO cell line expressing the highly glycosylated fusion protein Epo-Fc. We found the reduction of cultivation temperature and the reduction of (external) pH to exert the most significant effects on process performance by mainly reducing cell growth and metabolism. With respect to the cell line used we identified a set of parameters capable of affecting cell proliferation in favor of an increased specific productivity and total product yield. The well directed alteration of the process environment has emerged as a tool adequate for further process optimization applying a biphasic cultivation strategy. © 2006 Wiley Periodicals, Inc.

Keywords: recombinant CHO cells; process parameter shift; integral of viable cells; specific productivity; product quality; controlled bioreactor

Correspondence to: D. Müller

Contract grant sponsor: ACBT (Austrian Center of Biopharmaceutical Technology)

INTRODUCTION

Several recent studies show that environmental process control strategies can be used to affect the performance of Chinese hamster ovary (CHO) cells regarding cell growth, specific recombinant protein production, glycosylation, cell metabolism and viability (Butler, 2005). Process parameters such as pH, temperature and dissolved oxygen tension (DOT) have been shown to exert a significant effect on cell line performance. In contrast to reports where improvements in process performance have been achieved by the addition of nutrient supplements (Oh et al., 2005; Sung et al., 2004) or by means of metabolic or genetic engineering (Fussenegger and Betenbaugh, 2002), the improvement of process performance by simple parameter optimization offers a cheap and valuable strategy for process optimization. In this context, the pH is a parameter known to significantly influence cell growth, recombinant protein production, cell metabolism and protein glycosylation (Borys et al., 1993; Kurano et al., 1990; Link et al., 2004; Miller et al., 2000; Yoon et al., 2005; Zanghi et al., 1999b). The dissolved oxygen tension (DOT) in the culture medium has also been shown to affect productivity, cell metabolism and glycosylation (Kunkel et al., 1998, 2000; Kurano et al., 1990; Lin et al., 1993; Link et al., 2004; Ozturk and Palsson, 1991). However, compared to pH and DOT the influence of temperature has been studied even more intensively (Andersen et al., 2000; Bloemkolk et al., 1992; Chuppa et al., 1997; Fogolin et al., 2004; Fox et al., 2004; Furukawa and Ohsuye, 1998; Kaufmann et al., 1999; Moore et al., 1997; Rodriguez et al., 2005; Roessler et al., 1996; Sureshkumar and Mutharasan, 1991; Yoon et al.,

© 2006 Wiley Periodicals, Inc.

WILEY InterScience® DISCOVER SOMETHING GREAT

HALOZYME - EXHIBIT 1021

2003a, 2003b, 2005). Low temperature cultivation has been shown to significantly increase specific recombinant production rates and to arrest growth, while maintaining or improving product quality and reducing cell metabolism. Although the technological relevance of such investigations is evident for process optimization only few systematic studies on the effect of process parameter variation on recombinant CHO cells cultivated under controlled process conditions in bioreactors have been published so far. In addition, many of these studies were performed using cultivation systems like T-flasks or spinner flasks without pH and DOT control, in some studies cultivations were actually dependent on the presence of fetal calf serum (FCS) which is known to increase the tolerance towards physiochemical variations (Maurer, 1992) and protects cells against apoptosis (Zanghi et al., 1999b).

In this work, we systematically investigated the effect of pH, DOT and temperature on a recombinant CHO cell line expressing Epo-Fc, a highly glycosylated fusion protein. Cells were cultivated under controlled process conditions in batch bioreactors, also referred to as controlled (batch) bioreactors, in protein-free suspension.

MATERIALS AND METHODS

Cell Line and Culture Medium

Dihydrofolate reductase-deficient Chinese Hamster Ovary cells (DUKX-B11, ATCC CRL-9096) were co-transfected with genes for dihydrofolate reductase (DHFR) and the homodimeric fusion protein Epo-Fc, which was generated by genetic fusion of the Epo coding region upstream to the human IgG_1-Fc gene. Transfected cells were selected for growth in the presence of 0.096 μM MTX. After revitalization from a research cell bank, cells were cultivated in suspension in Dulbecco's Modified Eagle's Medium DMEM/HAM's F-12 (1:1 mixture), supplemented with 0.58 g/L L-glutamine, an in-house developed protein-free supplement (proprietary formulation), 0.25% soy peptone, 0.1% Pluronic F68 and 0.096 μM MTX. Within 2 weeks the CHO cells were adapted in spinner flasks (Techne, Staffordshire, UK) to an in-house developed production medium (Polymun Scientific, Vienna, Austria) of proprietary formulation.

Cell Propagation and Inoculation of Seed Fermenter

Cells were maintained as suspension cultures in 125 and 500 mL spinner flasks agitated on a magnetic stirring platform (Techne; Integra Biosciences, Chur, Switzerland) at 50 rpm and 37°C. Exponentially growing cells at concentrations in a range between 1.0×10^6 and 1.5×10^6 cells/mL were transferred from the spinner flask into the bioreactor (Chemap AG, Maenndorf, Switzerland) and were diluted to achieve a working volume of 2 L at an initial cell concentration of 2.0×10^5–3.0×10^5 cells/mL. Cells were cultivated under standard conditions (T 37.0°C, pH 7.10, DOT 30%) in repeated batch mode over the course of the experimental program. The dissolved oxygen tension was controlled by sparging pure oxygen, culture pH by addition of 0.5 M NaOH and CO_2, respectively. During repeated batch cultivation, the initial cell concentration was adjusted to an initial value of 2.0×10^5 cells/mL.

Batch Cultivations at Different Cultivation Parameters

Batch cultivations at different pH, DOT and temperature levels were performed in the Sixfors multireactor system (Infors AG, Bottmingen, Switzerland). Exponentially growing cells were taken from the 5 L bioreactor at a cell concentration of 1.0×10^6–1.5×10^6 cells/mL to inoculate each Sixfors bioreactor using a working volume of 400 mL with an initial cell concentration between 2.5×10^5 and 3.0×10^5 cells/mL under standard conditions (T 37.0°C, pH 7.10, DOT 30%). At the end of the first batch (after 3–4 days) cells were split and cultivation parameters were changed according to the experimental setup (Table I). At the end of the second batch cells were split again and a third batch was performed until the viability dropped below the specified stop criterion of 70%. The stop criterion was set based on analyses, which have revealed that the sialylation of Epo-Fc analyzed from cultures with viabilities of less than 80% is notably impaired (data not shown). In each series one process was performed under standard conditions (control). During repeated batch cultivation cell concentrations were adjusted to an initial value of 2.0×10^5 cells/mL.

In-Process Control

Samples of 10 mL each were taken aseptically from the bioreactor with a syringe via bypass sampler (Sartorius BBI Systems, Göttingen, Germany). The reduction of the working volume due to sampling was around 30% at the end of each process, though it has not emerged to be critical for process performance.

A Multisizer™ 3 Coulter Counter® (Beckman Coulter, Fullerton, CA) was used to determine the total cell concentration by counting the cell nuclei after cell lysis.

Table I. Experimental setup; investigated process parameters and corresponding bioreactors.

	Series		
Bioreactor	1 (pH)	2 (DOT)	3 (Temperature, °C)
1	6.80	10%	30.0
2	6.90	30%[a]	33.0
3	7.00	50%	35.0
4	7.10[a]	70%	37.0[a]
5	7.20	90%	38.0
6	7.30	100%	39.0

[a]Control.

DOI 10.1002/bit

10970290, 2006, 6, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bit.21013 by Tracy Fritz , Wiley Online Library on [20/06/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Culture viability was determined by the Trypan blue dye exclusion method. Concentrations of glucose, lactate and ammonium were measured using the multiparameter bioanalytical system YSI 7100 MBS (YSI Life Sciences, Yellow Springs, OH).

Epo-Fc Determination

The secreted Epo-Fc concentration was quantified using an in-house developed enzyme-linked immunosorbent assay (ELISA). For this purpose, 96-well plates were pre-coated with a goat-anti-human-γ-chain antibody (I-7883 Sigma, St. Louis, MO) and bound Epo-Fc was quantified in a 2^8 dilution series with a goat-anti-human-γ-chain antibody horse radish peroxidase (HRP) conjugate (62-8420 Zymed Laboratories, San Francisco, CA). Purified Epo-Fc was used as a standard.

Amino Acid Analysis

Amino acid analyses of the culture supernatant were performed by HPLC (Hewlett Packard, Vienna, Austria) with a reversed phase column (C18; 3 µm; 4×100 mm, Agilent, USA). Amino acid derivatization prior to HPLC analysis was performed using OPA (o-phtalaldehyde)/mercaptoethanol. Detection was carried out at 350 and 450 nm, respectively, with a fluorescent detector (Shimadzu, Duisburg, Germany). Proline and cysteine concentrations could not be determined with this method.

Cell Cycle Analysis

To assess the effect of different process parameters on cell cycle distribution, cells were analyzed by flow cytometry. Cells taken from a bioreactor were centrifuged and approximately 1.0×10^6 cells were fixed with 1 mL of 70% ice-cold ethanol. The fixed cells were stored at $+4^\circ$C for subsequent analysis. For analysis, fixed cells were washed twice with a Tris buffer, pH 7.4 (0.1 M Tris/HCL, 0.1% Triton X-100, 2 mM $MgCl_2$) and once with Tris buffer containing 20% fetal calf serum (FCS). Cells were then resuspended in 200 µL Tris/FCS solution containing anti-human IgG gamma-FITC (F-0132 Sigma), and incubated for 1 h at 37°C. Finally, after adding 1 mL of Tris buffer the suspension was centrifuged (170*g*, 10 min). The cells were then stained with 200 µL DAPI containing Tris buffer (3 mg/mL final concentration). Flow cytometric analysis was carried out using a FACS Vantage (Becton Dickinson, Franklin Lakes, NJ) flow cytometer. FITC was excited with 250 mW from an Argon laser turned to 488 nm and measured using a 530/30BP filter. DAPI was excited with 100 mW multiline UV-light from a second Argon laser and measured using a 424/44 BP filter. The software program Verity ModFit (Verity Software House, Topsham, ME) was used for cell cycle analysis.

Determination of Sialic Acid Content of Purified Epo-Fc

The Epo-Fc secreted from CHO cells was purified by Protein A affinity chromatography. The quantity of sialic acid was measured by an in-house developed enzymatic method (Schriebl et al., 2006). In this three-step method the sialic acid was first cleaved by neuraminidase, was subsequently transformed to pyruvic acid by NANA-aldolase and pyruvic acid was finally reduced to lactic acid by lactate dehydrogenase in the presence of NADH. The decrease in NADH was measured spectrofluorometrically. A repeat determination of the complete procedure was performed for each sample.

Evaluation of Specific Growth Rate, Integral of Viable Cells, Volumetric Productivity and Cell Specific Production and Consumption Rates

Following the biomass balance equation for batch conditions $dx/dt = \mu \cdot x$, the solution for µ is shown in Equation 1, where x_2 and x_1 are cell concentrations determined at the corresponding time points t_2 and t_1.

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \tag{1}$$

Since the accumulation of a secreted product is directly related to the integral of viable cells and not to the (viable) cell concentration, the specific productivity q_P and other specific metabolic rates q_m were determined as a function of the cumulative viable cell days (CD) (Dutton et al., 1998) which represents the integral of viable cells at a constant specific growth rate. The solution for CD within the time interval from t_1 to t_n at distinct time points t_i is shown in Equation 2.

$$\begin{aligned} \mathrm{CD} &= \sum_{i=1}^{n} \left(\frac{x_{i+1} - x_i}{\ln(x_{i+1}) - \ln(x_i)} \times (t_{i+1} - t_i) \right) \\ &= \sum_{i=1}^{n} \frac{x_{i+1} - x_i}{\mu} \end{aligned} \tag{2}$$

The specific productivity (q_P) and other specific metabolic rates (q_m) were calculated as shown in Equation 3, where $c_{m,n}$ and $c_{m,1}$ are the metabolite or product concentrations at the time points t_n and t_1.

$$q_m = \frac{c_{m,n} - c_{m,1}}{\mathrm{CD}} \tag{3}$$

All specific metabolic rates were signed positive for consumption and negative for production, except those for lactate and ammonia, which were already denoted as ''specific production rates'' and therefore signed positive.

The volumetric productivity STY (space-time yield) was calculated as the accumulated product concentration relating to the process time and is shown in Equation 4, where c_p is the product concentration at time point t and c_p^0 is the initial

10970290, 2006, 6, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bit.21013 by Tracy Fritz , Wiley Online Library on [20/06/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

product concentration at the beginning of the batch process (t_0).

$$\mathrm{STY} = \frac{c_P - c_P^0}{t - t_0} \quad (4)$$

RESULTS

Reproducibility of Batch Processes Under Standard Conditions

In order to detect potential variations in process performance throughout the experimental program the initial batch phases of all processes within the three series (n = 18 processes) were performed under standard conditions. Table II represents the results for selected metabolic parameters. The variation of cell line performance was in a range of 7%–11% (standard deviations) related to growth, productivity and metabolic activity, whereas the variation of the means of each series was only about 3% (standard errors). These data indicate that variations in process performance associated with the generation time of the cell line or with the individual bioreactor set-up were low compared to the variations which were subsequently provoked by changing process parameters.

Influence of pH, DOT, and Temperature on Batch Cultivation of CHO Cells

Effects of pH, DOT, and Temperature on Cell Growth

The progression of viable cell concentration and cell viability during batch cultivation (third batch) is presented in Figure 1A and B. In comparison to the control cultures, changes in pH and temperature did significantly affect cell growth and viability, whereas changes in DOT did not. With regard to pH, a maximum viable cell concentration of 2.0×10^6 cells/mL was reached after 5 days of cultivation in the control culture. On day 7 of cultivation, the cell viability dropped below the specified stop criterion of 70%. At reduced pH levels (6.90 and 6.80) the cultivation time could be prolonged to 9 and 12 days, respectively, whereas the increase to pH 7.30 resulted in a reduction of cultivation time by 1 day. The cultivation length could be directly related to the depletion of glucose. At increased pH levels an increase in specific glucose consumption was observed. The specific growth rate of the cells was similar in a range between pH 7.00 and pH 7.30 (0.5/day). The maximum viable cell concentration was therefore only dependent on cultivation time. However, at pH levels below 7.00 the specific growth rate was significantly reduced (0.47/day at pH 6.90, 0.38/day at pH 6.80). The maximum viable cell concentration therefore depended on both cultivation time and specific growth rate. Regarding temperature, growth was suppressed when the temperature was lowered to 33°C and 30°C, whereas both the reduction to 35°C and the increase to 38°C had no effect on growth and viability. At 30°C cell growth was almost completely arrested. At 33°C the specific growth

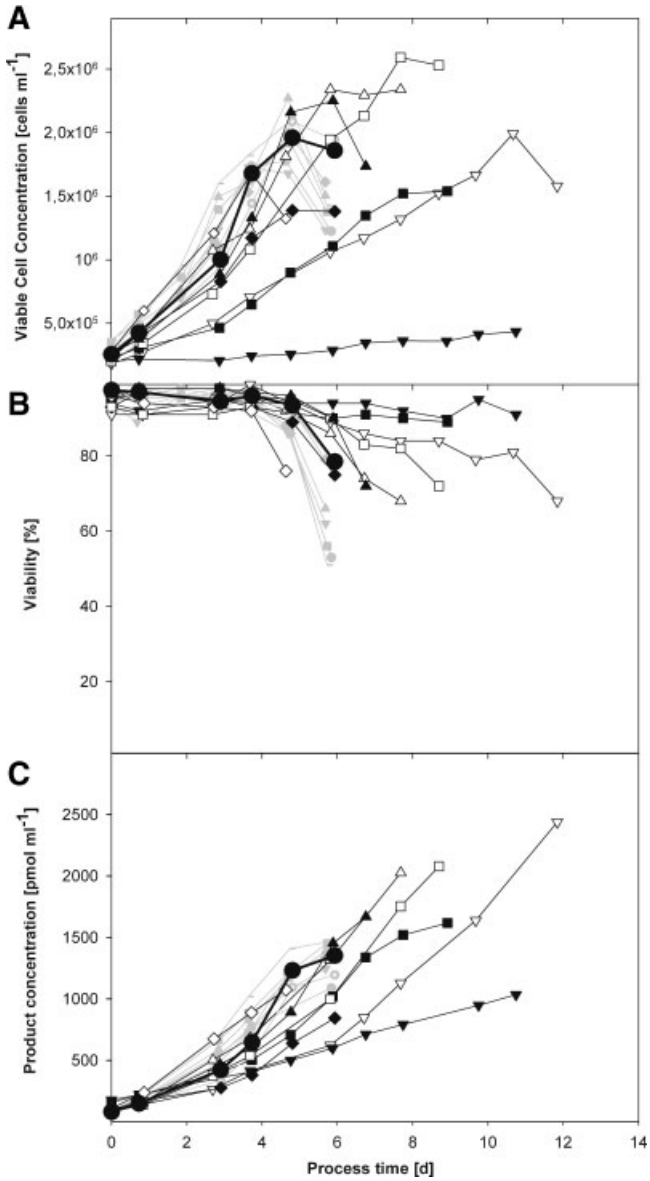


Figure 1. Progression of viable cell concentration, viability and product concentration at tested process parameters (**A**) Viable cell concentration. **B**: Cell viability. **C**: Product concentration. (●, control culture; ▽, pH 6.80; □, pH 6.90; △, pH 7.00; ◇, pH 7.30; ▼, 30°C; ■, 33°C; ▲, 35°C; ◆, 39°C; grey symbols and lines represent unaffected cultures (●, pH 7.20; ▼, DOT 10%; ▲, DOT 50%; ◆, DOT 70%; ■, DOT 90%; —, DOT 100%; ○ 38°C).

Table II. Statistical evaluation of batch processes under standard conditions.

Parameter	n	$\tilde{x}$	s	s_x
μ [/day]	18	0.55	0.04	0.01
q_P [amol/cell/day]	18	197.3	22.3	5.3
q_{Glc} [ng/cell/day]	12[a]	0.95	0.10	0.03
q_{Lac} [ng/cell/day]	12[a]	0.84	0.10	0.03

n, number of processes analyzed; $\tilde{x}$, arithmetic mean; s, standard deviation; s_x, standard error.
[a]Series 3 not analyzed.

DOI 10.1002/bit

10970290, 2006, 6, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bit.21013 by Tracy Fritz , Wiley Online Library on [20/06/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

rate was reduced to 0.28/day which was similar to cultivating cells at pH 6.80. However, at 33°C cultivation time was only extended to 9 days resulting in a lower final cell concentration of 1.5×10^6 cells/mL. The increase in temperature to 39°C led to a delayed decrease in the specific growth rate after 4 days, whereas viability and cultivation time were not affected compared to the control. Growth and viability were mostly unchanged at different DOT levels. The reduced maximum viable cell concentration (1.5×10^6 cells/mL) found at 10% air saturation was probably due to the incipient limitation in oxygen availability. The integral of viable cells was also reduced as a result of the shortened cultivation time which was due to the premature depletion of glucose at a higher specific glucose consumption rate.

Effects of pH, DOT, and Temperature on Product Accumulation and Specific Productivity

Figure 1C shows the corresponding Epo-Fc concentration profiles during the batch cultivation shown in Figure 1A. The maximum achievable Epo-Fc concentration at standard conditions was around 1,400 pmol/mL. At pH 6.80 the maximum Epo-Fc concentration could be increased by a factor of 1.7, mainly due to a prolonged cultivation time. A 1.5-fold increase was also found at pH 6.90 and pH 7.00, whereas the increase in pH to values higher than 7.10 led to a reduction by 20%. Regarding temperature, cells cultivated at 30°C and 33°C continued to produce Epo-Fc at reduced growth rates. Although cells at 30°C did not grow, they accumulated 1,035 pmol/mL Epo-Fc after 11 days of batch cultivation. The maximum Epo-Fc concentration of around 1,600 pmol/mL was achieved at 33°C and 35°C after 9 days and 7 days, respectively. No significant difference in production kinetics could be observed at 38°C (1,200 pmol/mL), whereas cultures subjected to 39°C showed a reduction by 37% (845 pmol/mL). The specific productivity at different DOT levels remained mainly unaffected. The reduction in final Epo-Fc concentration at a DOT of 10% was clearly associated with the reduced integral of viable cells. With regards to specific productivity, the plot of the Epo-Fc concentration as function of the integral of viable cells is presented in Figure 2. At all tested pH and DOT (not shown) levels cells exhibited the same specific productivity which is indicated by the same slope (q_P). The high maximum Epo-Fc concentration at reduced pH values was directly related to the prolongation of cultivation time and the resulting increase of the integral of viable cells. However, the reduction of temperature to 30°C and 33°C affected both batch cultivation time and specific productivity. Accordingly, more than a 1.7-fold increase in specific productivity was achieved by lowering the culture temperature to 30°C and a 1.4-fold increase was obtained at 33°C. In contrast, the specific production rates at 39°C were reduced by 20%. In addition, the increased specific productivity at reduced temperatures remained constant throughout batch cultivation, whereas at 37°C the specific productivity decreased.

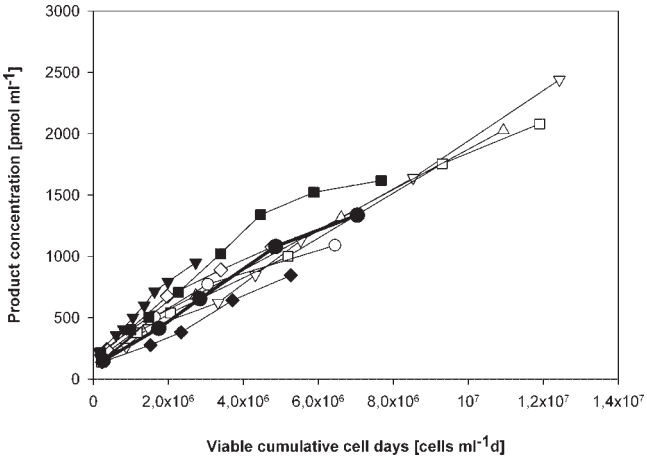


Figure 2. Epo-Fc concentration as a function of the integral of viable cells (●, control culture; ▽, pH 6.80; □, pH 6.90; △, pH 7.00; ○, pH 7.20; ◇, pH 7.30; ▼, 30°C; ■, 33°C; ◆, 39°C).

Effects of pH, DOT, and Temperature on Cell Metabolism

Glucose, lactate, glutamine, and ammonium concentrations were measured during the growth phase and the corresponding metabolic rates were determined. Culture pH and temperature significantly affected glucose metabolism (Fig. 3), whereas different DOT levels did not. Specific rates for glucose consumption and lactate production gradually increased with elevated levels of pH and temperature. This behavior is in accordance to the differences found in cultivation time at different pH levels with glucose as limiting factor. The increase in specific glucose consumption rate was similar for both parameters pH and temperature. The glucose consumption rate increased two-fold when the pH level was increased from 6.80 to 7.20 and 1.7-fold with an increase in temperature from 30°C to 38°C. The increase in specific lactate production was less pronounced at increased temperatures (0.65–0.9 ng/cell/day), but reached higher levels compared to elevated pH levels (0.46–0.78 ng/cell/day). In all cases, the maximum metabolic rate was achieved at 38°C and pH 7.20, respectively. The yield coefficient for lactate from glucose reached its maximum at pH 7.00 and was decreased for both lower and higher pH levels, whereas it was steadily decreasing from 30°C to 39°C.

Even the glutamine metabolism was significantly influenced by pH and temperature (Fig. 4), but followed a completely different trend when compared to the glucose metabolism. Regarding pH, a specific glutamine consumption rate of 91.2 pg/cell/day was observed at standard conditions, resulting in a specific ammonia production rate of 12.8 pg/cell/day. The specific glutamine consumption rates were slightly decreased at low pH values (81.7 pg/cell/day at pH 6.80, 82.9 pg/cell/day at pH 6.90) and displayed a slight increase with elevated pH levels (103.2 pg/cell/day at pH 7.30). Remarkably, higher specific glutamine consumption rates were not associated with correspondingly high specific ammonia production rates (q_{Amm}). In contrast, q_{Amm}

10970290, 2006, 6, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bit.21013 by Tracy Fritz , Wiley Online Library on [20/06/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

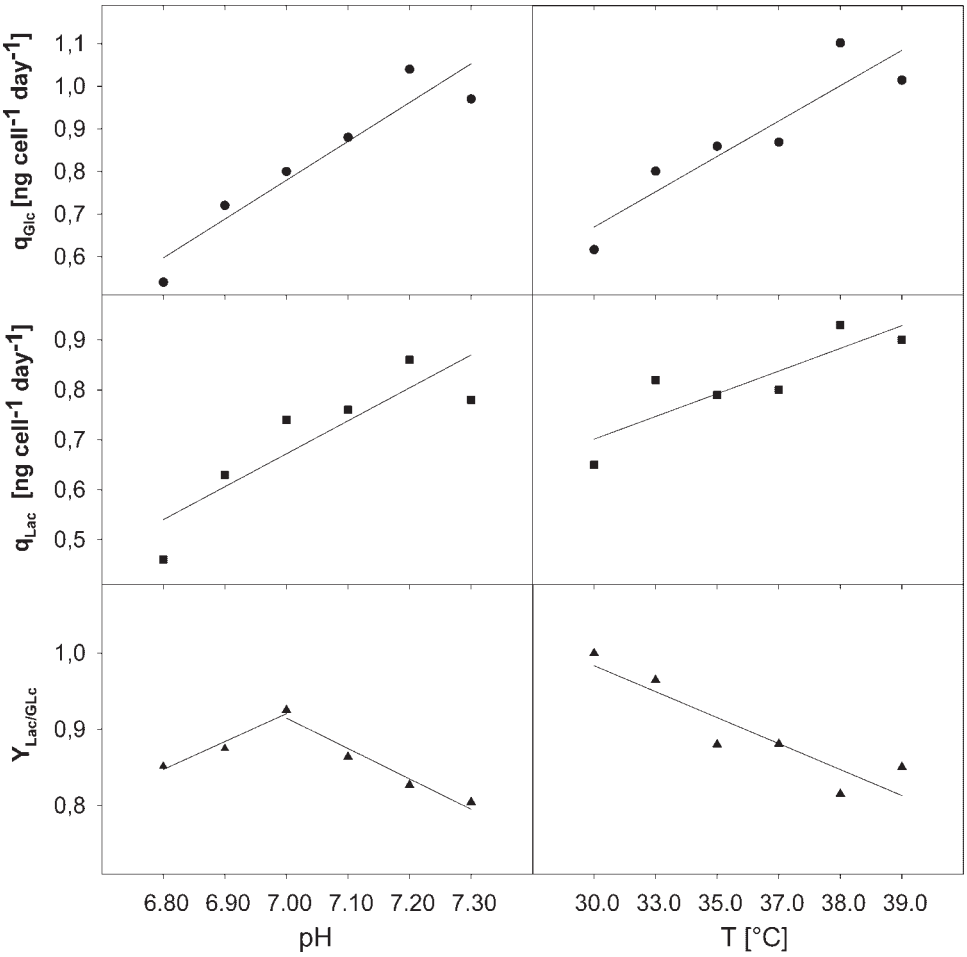


Figure 3. Effect of pH and temperature on specific glucose consumption rate (q_{Glc}), specific lactate production rate (q_{Lac}) and the lactate yield from glucose ($Y_{Lac/Glc}$).

significantly decreased with elevating pH levels from 24.1 ng/cell/day at pH 6.80 to 12.8 ng/cell/day at pH 7.10. The ammonia yield from glutamine exhibited pH dependence at values lower than 7.10. The ammonia yield from glutamine was increased by a factor of 2 at pH 6.80 and 1.8-fold at pH 6.90, respectively.

Temperature exerted a different effect on glutamine metabolism in comparison to pH. In the control culture a specific glutamine consumption rate of 75.4 pg/cell/day and a specific ammonia production rate of 14.5 ng/cell/day were observed. Remarkably, in comparison to cultivation at standard conditions, specific glutamine consumption rates gradually increased for both lower temperatures (85.6 pg/cell/day at 35°C, 93.0 pg/cell/day at 33°C, 110.0 pg/cell/day at 30°C,) and higher temperatures (91.2 pg/cell/day at 38°C, 124.3 pg/cell/day at 39°C). The glutamine consumption at low temperatures was not associated with a corresponding production of ammonia. A 2.2-fold lower ammonia yield from glutamine was observed compared to the control culture. The ammonia yield from glutamine further increased for cultures exposed to higher temperatures (0.23 at 38°C and 0.27 at 39°C). In addition, specific metabolic rates for other amino acids were also determined during the growth phase (Fig. 5A). The data indicate a substantial consumption of glutamine (Gln), asparagine (Asn) and lysine (Lys) at pH 6.80 and pH 6.90 whereby aspartic acid (Asp) was only consumed at pH 6.80. All the other amino acids were formed or remained unaffected. Higher pH levels (pH 7.20 and pH 7.30) did not exert a significant effect on amino acid metabolism compared to the control culture at pH 7.10. Generally, there was a significantly enhanced net utilization of amino acids at pH levels above 6.90. The increase in glutamine consumption coincided with an increase in alanine production. Figure 5B displays the utilization patterns of amino acids at various culture temperatures. The utilization pattern of most amino acids at 30°C and 33°C was very different to cultures exposed to higher temperatures. Remarkably, at 30°C only asparagine and glutamine were consumed, whereas all other amino acids were formed. However, the glutamine consumption rate increased by 46% in comparison to 37°C. The culture at 33°C showed a utilization pattern similar to the culture at 30°C. In addition to asparagine and glutamine, aspartic acid was consumed. Interestingly, the cultivation at 30°C and 33°C led to a significant production of lysine, whereas in all other cultures lysine was consumed. The cultivation at 39 °C also exerted a remarkable effect on the utilization pattern of most amino acids. Glutamine (Gln), asparagine (Asn), glycine

DOI 10.1002/bit

10970290, 2006, 6, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bit.21013 by Tracy Fritz , Wiley Online Library on [20/06/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

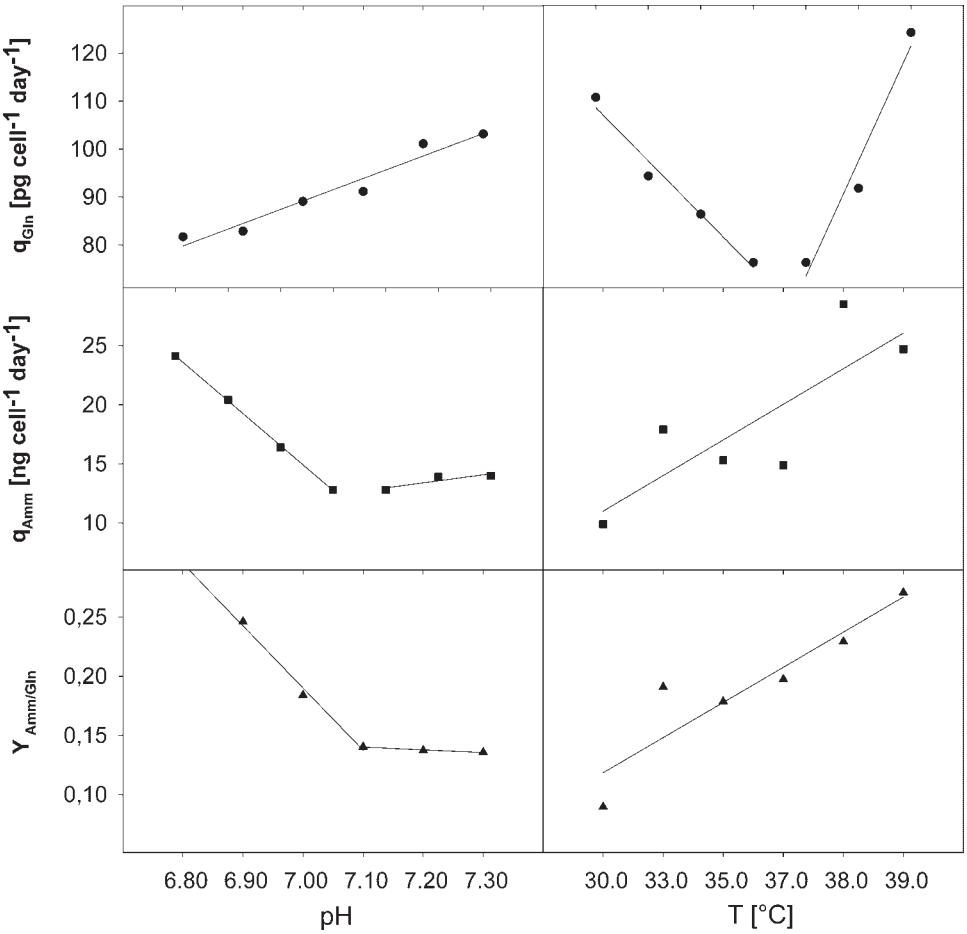


Figure 4. Effect of pH and temperature on specific metabolic rates for glutamine (q_{Gln}) and ammonium (q_{Amm}) and the ammonium yield from glutamine ($Y_{Amm/Gln}$).

(Gly) and lysine (Lys) were consumed, whereas all other amino acids were formed.

Effects of pH, DOT, and Temperature on Epo-Fc Sialylation

As presented in Figure 6, culture pH and most DOT levels tested exerted no significant effect on Epo-Fc sialylation, since 11–14 mol sialic acid/mol Epo-Fc were found under all conditions. This range represents the normal variation found for different samples from cultivations under standard conditions (data not shown). At a DOT of 100% air saturation a loss of 4–6 mol sialic acid/mol Epo-Fc was observed. As indicated, cultivation at low culture temperatures exerted a negative effect on Epo-Fc sialylation. Product sialylation was reduced at 30°C and 33°C by 40% and 20%, respectively. At increased temperatures no significant effect was found.

Effects of pH, DOT, and Temperature on Volumetric Productivity

The data in Figure 7 reveal that the volumetric productivity was clearly decreased at low pH levels and low temperatures. This was mainly due to a comparably low accumulation of viable cells at a prolonged cultivation time. Even if the specific productivities were significantly increased at 30°C and 33°C, the volumetric productivities were decreased by a factor of 3.1 and 1.5, respectively. As a consequence, a volumetric productivity of around 250 pmol/mL/day (control) could not be exceeded at any of the different cultivation conditions tested. Additionally, cultivation at increased temperatures (39°C) and increased pH levels (7.20) led also to a reduction in volumetric productivity by a factor of 1.7 and 1.4, respectively.

Effects of Temperature on Cell Cycle

To further investigate growth suppression at reduced temperatures, we quantified the cell cycle distribution by flow cytometric analysis. Under standard conditions around 45% of the cells were in G_0/G_1 phase (Table III). Cultivation of this cell line at 30°C resulted in a rapid increase of cells in G_0/G_1 phase. Remarkably, only the cultivation at 30°C led to an enhanced cell cycle arrest in G_0/G_1. FACS analysis showed that approximately 60% of cells accumulated in the G_0/G_1 phase of the cell cycle, concomitant with a reduction of S-phase cells by 40% in comparison to cultivation temperatures higher than 30°C. Thus, cultivation of cells at

10970290, 2006, 6, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bit.21013 by Tracy Fritz , Wiley Online Library on [20/06/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

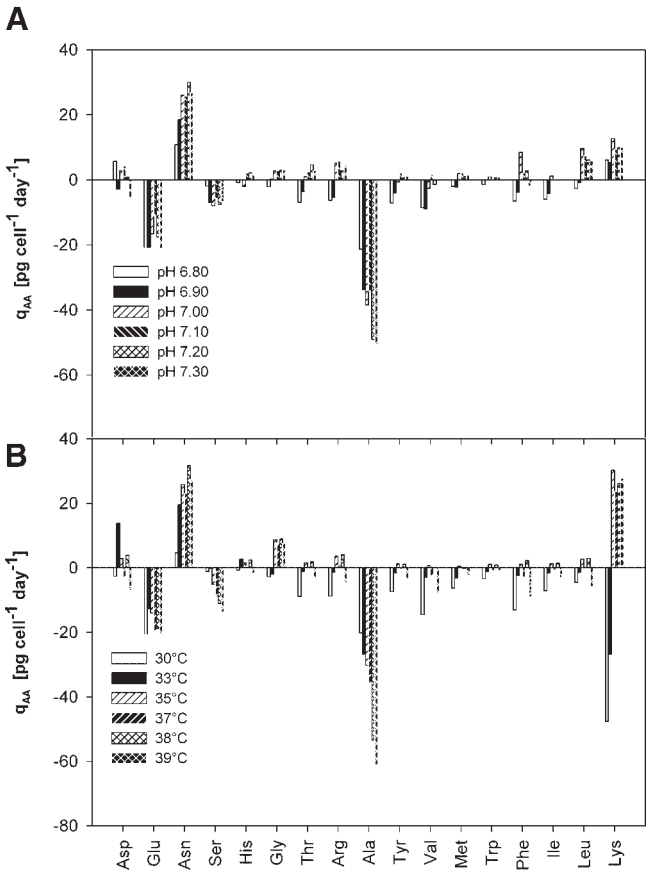


Figure 5. Effect of pH (**A**) and temperature (**B**) on specific metabolic rates for amino acids (q_{AA}) (except for glutamine).

temperatures higher than 30°C had no effect on cell cycle distribution.

DISCUSSION

Effect of pH

Lowering the culture pH led to a significant reduction of the specific growth rate and glucose metabolism, but cell viability remained high for a prolonged cultivation time resulting in a higher integral of viable cells at pH levels lower than 7.10. The highest specific growth rates were observed at pH levels of 7.10 and higher, whereas at pH 7.20 and pH 7.30 the increased specific glucose consumption rate led to a reduced cultivation time due to glucose limitation. The optimal pH for growth of this cell line was at pH 7.10 matching the broad range (pH 6.80–pH 7.60) which was reported for a variety of CHO cell lines (Kurano et al., 1990; Link et al., 2004; Yoon et al., 2005).

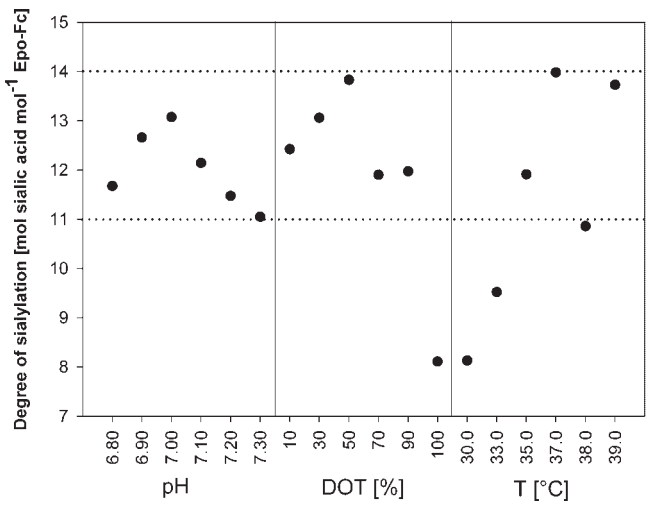


Figure 6. Degree of sialylation in batch cultures at various process parameters; dotted lines indicate normal variation (12.5 ± 1.5).

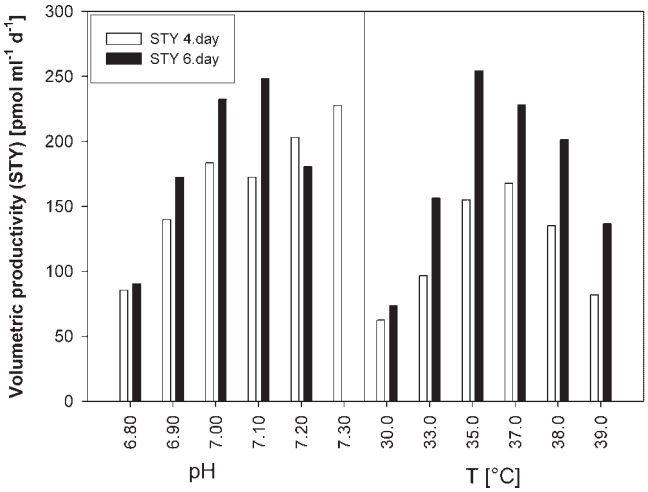


Figure 7. Volumetric productivity in batch cultures at different pH and temperature levels.

The maximum Epo-Fc concentration, which was obtained at pH 6.80, was 1.8-fold higher than at pH 7.10. This increase in product yield at lower pH levels was not due to increased specific production rates, but rather due to an increased integral of viable cells caused by a prolonged cultivation time. In contrast, the cultivation time was clearly reduced at pH 7.20 and 7.30, thus resulting in a 0.8-fold lower product yield. Since the specific production rate remained unchanged at different pH levels, the overall product yield was directly related to the integral of viable cells. This is in accordance with observations made by Yoon et al. (2005), who found no effect of pH on specific productivity of an Epo producing CHO cell line at 37°C in a pH range of 6.85–7.80. Whereas, Borys et al. (1993) reported that the specific productivity may be improved at pH levels higher than 7.60. We therefore deduce that the optimal pH for growth and recombinant protein production differs for different cell lines and has to be determined, respectively.

Table III. Effect of temperature on cell cycle distribution.

Cultivation mode (°C)	G_0/G_1-phase cells (%)	G_2/M- and S-phase cells (%)
30	58.7	41.3
33	47.7	52.3
35	46.9	53.1
37	42.2	57.8
38	44.2	55.8
39	48.0	52.0

DOI 10.1002/bit

10970290, 2006, 6, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bit.21013 by Tracy Fritz , Wiley Online Library on [20/06/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Changes in specific metabolic rates were much more pronounced than for any other parameter. Specific glucose consumption rates and lactate production rates gradually increased with elevated pH levels, reaching a maximum at pH 7.20. The specific glucose consumption and lactate production rates increased nearly 2-fold by elevating the pH value from pH 6.80 to pH 7.20. This increase in glucose and lactate metabolism at higher pH values seems to be a well-known phenomenon (Yoon et al., 2005) also in hybridoma cell lines (Miller et al., 2000; Ozturk and Palsson, 1991). Specific glutamine consumption rates also increased from pH 6.80 to 7.30, but to a lesser extent than specific glucose consumption rates, whereas the specific ammonia production was decreased by elevating pH levels from pH 6.80 to 7.10 and remained constant at pH levels above 7.10. Parts of these results are in contrast to the work of Yoon et al. (2005), who investigated the pH in a range from 6.85 to 7.80 and found no significant influence on glutamine and ammonia metabolism below pH 7.60.

Regarding amino acid metabolism, we found a substantial influence at pH values below 7.00 leading to a net formation of not less than 14 amino acids. This effect was not observed at higher pH levels. Hence, the lower specific consumption of the main carbon sources and of mostly all amino acids as well as the lower specific formation of metabolic by-products offers a strategy for the optimization of processes operated at high cell concentrations.

Providing cell culture conditions that consistently enable maximal product sialylation is one prerequisite for the production of therapeutic glycoproteins. In the tested pH range the degree of sialylation was not affected. Several recent studies, however, showed that a suboptimal pH may alter the glycosylation pattern (Borys et al., 1993; Yoon et al., 2005). We therefore assume that the optimal pH for glycosylation of recombinant proteins is also cell line dependent.

Effect of DOT

Culture performance was not significantly influenced by DOT levels between 30% and 90% air saturation. A DOT of 10% resulted in the lowest viable cell and product concentration. The lower viable cell yield was a result of a reduced cultivation time and the premature entry of cells into death phase. This is in accordance with findings of many other groups (Kurano et al., 1990; Link et al., 2004; Ozturk and Palsson, 1991), where cell growth was depressed at a very low DOT mainly due to oxygen limitation. No effect of the dissolved oxygen tension on specific productivity could be observed likewise. This is different to findings of few reports, in which the specific productivity of recombinant CHO cells was influenced by changing the DOT (Chotigeat et al., 1994; Link et al., 2004).

The metabolism of glucose as well was only influenced at 10% DOT, whereas the metabolism of glutamine and other amino acids was not affected at all (data not shown). Metabolic rates for glucose consumption and lactate production increased by 20% at a DOT level of 10% air saturation. In contrast, Kurano et al. (1990) found both highest specific growth rates and lowest metabolic rates of a CHO cell line between 50%–100% air saturation.

The effect of DOT on the glycosylation of different recombinant proteins has been studied by different groups. In contrast to reports by Chotigeat et al. (1994), who observed a positive correlation between sialyltransferase activity in CHO cells and dissolved oxygen tension, we did not observe any increase in recombinant protein sialylation at elevated DOT levels. Quite contrary to an improvement, we observed a loss in sialylation at a DOT of 100%. Reduced DOT levels showed no effect on Epo-Fc sialylation, which is in accordance to previous studies investigating the effect of hypoxic or anoxic conditions on *N*-glycosylation of recombinant proteins produced by CHO cells (Lin et al., 1993) and BHK cells (Gawlitzek et al., 1995).

Effect of Temperature

Temperature is considered as an important parameter that can be used to improve process performance. In contrast to other environmental factors such as DOT and pH, temperature has been studied intensively (Bloemkolk et al., 1992; Chuppa et al., 1997; Fogolin et al., 2004; Fox et al., 2004; Furukawa and Ohsuye, 1998; Kaufmann et al., 1999; Moore et al., 1997; Rodriguez et al., 2005; Roessler et al., 1996; Sureshkumar and Mutharasan, 1991; Yoon et al., 2003a,b, 2005). In our experiments, process temperature exerted a significant effect on cell growth. Temperature reduction to 33°C led to a remarkable decrease in the specific growth rate, whereas at 30°C growth arrest occurred. The reduction of the specific growth rate at reduced temperatures is a well known phenomenon, since many different investigations with different cell lines confirmed this correlation (Andersen et al., 2000; Bloemkolk et al., 1992; Chuppa et al., 1997; Fogolin et al., 2004; Fox et al., 2004; Furukawa and Ohsuye, 1998; Kaufmann et al., 1999; Moore et al., 1997; Rodriguez et al., 2005; Roessler et al., 1996; Sureshkumar and Mutharasan, 1991; Yoon et al., 2003a,b, 2005). In contrast, the effect of elevated temperatures has been discussed quite inconsistently (Furukawa and Ohsuye, 1998; Jenkins and Hovey, 1993; Sureshkumar and Mutharasan, 1991). In our experiments, the exposure to 39°C led to a significant reduction in viable cell yield due to decreased growth rates along with a reduction of the volumetric productivity. This effect was additionally enhanced by the decrease of the specific productivity. As observed for cell growth, cultivation of cells at 35°C and 38°C had no effect on specific productivity. At lower culture temperatures (30°C, 33°C) we could show that the productivity of this cell line was not associated with growth, since under conditions of remarkably reduced (33°C) or arrested (30°C) growth we found rather enhanced specific productivities which could be maintained even until the end of the batch cultivation, whereas the specific production rate decreased steadily during cultivation at 37°C. In several recent studies the same findings of

10970290, 2006, 6, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bit.21013 by Tracy Fritz , Wiley Online Library on [20/06/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

increasing productivity at low temperatures were made (Ducommun et al., 2002; Fogolin et al., 2004; Fox et al., 2004; Furukawa and Ohsuye, 1999; Kaufmann et al., 1999; Rodriguez et al., 2005; Schatz et al., 2003; Yoon et al., 2003a, 2005). While the delayed viability decrease and the reduction of specific growth at low temperatures appear to be general phenomena, the effect of temperature on specific productivity seems to depend on the cell line or even on the integration site of the recombinant genes (Yoon et al., 2004).

Cell metabolism regarding carbon sources and amino acids was also strongly affected by low cultivation temperature. Previous investigations showed significantly reduced glucose metabolism (Fogolin et al., 2004; Sureshkumar and Mutharasan, 1991) as well as reduced glutamine consumption rates at a cultivation temperature lower than 37°C (Chuppa et al., 1997; Furukawa and Ohsuye, 1998; Weidemann et al., 1994; Yoon et al., 2005). Lowering the culture temperature to 30°C led to a significant decrease in the specific metabolic rates for glucose and lactate. In contrast to many reports, this Epo-Fc expressing cell line showed a significant increase in specific glutamine consumption at reduced temperatures which was accompanied by decreased specific glucose consumption. Glutamine appeared to substitute for glucose at low temperatures. Cultures subjected to temperatures higher than 37°C showed increased specific glucose and glutamine consumption rates, indicating a less efficient metabolism with respect to carbon sources. Furthermore, the utilization pattern of amino acids at 30°C, 33°C and 39°C was altered compared to 37°C indicating that low temperature cultivation and exposure to higher temperatures (39°C) significantly influenced cell metabolism. We found the specific metabolic rates of most amino acids reduced, some were even switched from consumption to formation.

Cultivation at low culture temperatures (30°C and 33°C) resulted in an enhanced specific productivity, but it negatively affected Epo-Fc sialylation which was reduced by 40% and 20% at 30°C and 33°C, respectively. The degradation of glycan structures by glycosidases released from dead cells could be excluded since cell viabilities were maintained above 90%. Furthermore, the accumulation of ammonia, known to increase heterogeneities of Epo glycoforms (Yang and Butler, 2000, 2002), could also be excluded. We preliminary investigated the effect of ammonia and found that terminal product sialylation of Epo-Fc produced in this cell line was not affected at ammonia concentrations up to 9 mM (data not shown), whereas ammonia concentrations in this study did not exceed 6 mM during batch cultivation. To our knowledge, no data have been published so far showing a negative influence of low cultivation temperature on product quality. In contrast, many reports showed an unchanged or even better product quality under these conditions (Bollati-Fogolin et al., 2005; Chuppa et al., 1997; Kaufmann et al., 2001; Yoon et al., 2003b). The data obtained in this work may argue for a correlation between increased specific productivities and reduced levels of sialylation caused by an incomplete glycosyl transfer at higher specific productivities. To date, there has been no unanimous conclusion, whether the expression rate of a recombinant glycoprotein affects the glycoform pattern of the secreted protein in CHO cells. A negative correlation between protein production rate and associated glycosylation of the target protein has also been shown by other authors (Santell et al., 1999; Shelikoff et al., 1994). In contrast, different studies show that protein sialylation was improved at reduced temperatures at even enhanced specific productivities (Kaufmann et al., 2001; Yoon et al., 2003b). Further studies investigated the influence of growth rate on protein sialylation. Mueller et al. (1999) found a correlation between cell proliferation rate and terminal sialylation of glycoproteins, whereas Andersen et al. (2000) revealed a consistent relationship between site occupancy and the proportion of cells in G_0/G_1 phase of the cell cycle. However, these observations are in contrast with findings for our cells, which showed a significantly reduced capability to sialylate Epo-Fc at low temperatures and reduced growth rates.

Cultivation of this cell line at 30°C led to an arrest in the G_0/G_1 phase of the cell cycle. In contrast to other observations (Yoon et al., 2003b), even at 33°C no accumulation of cells in the G_0/G_1 phase was observed. Additionally, quite contrary to our results, limited exposure to 39°C in batch cultures has been reported to arrest temperature sensitive CHO cells in G_1 phase resulting in an up to four-fold increase in specific productivity (Jenkins and Hovey, 1993). The enhancement of specific productivity has often been attributed to a cell cycle arrest in the G_0/G_1 phase. Low temperature-induced growth inhibition was shown to be G_1-phase-specific (Kaufmann et al., 1999; Moore et al., 1997; Yoon et al., 2003a,b). However, the effect of low cultivation temperature on specific recombinant protein productivity varied among different studies (Yoon et al., 2003a). Thus, arresting cells at G_0/G_1 phase of the cell does not consequently lead to enhanced specific productivities. Remarkably, although the specific productivity could be enhanced at 30°C and 33°C, cell growth arrest in the G_0/G_1 phase occurred unexceptionally at 30°C. Additionally, at both temperatures a remarkable decline in the degree of sialylation was observed. In this case, Epo-Fc production and product sialylation could not be correlated in any way to G_0/G_1 arrest or proliferation rate. As already discussed for temperature reduction, these observations may argue for a correlation between high specific production rates and low levels of sialylation.

Taken together, in a systematic approach we primarily varied the dissolved oxygen tension (DOT), pH, and temperature in controlled bioreactors to investigate the impact on growth, productivity, metabolism, product quality, and cell cycle distribution using an Epo-Fc expressing CHO cell line. Low cultivation temperature and low pH conditions exerted the most significant effects on cell line performance by mainly reducing cell growth and metabolism leading to an increased viable cell integral. In addition, cultivating cells at 30°C and 33°C led to an enhancement of specific productivity, whereas the degree of protein sialylation dropped.

DOI 10.1002/bit

10970290, 2006, 6, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bit.21013 by Tracy Fritz , Wiley Online Library on [20/06/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

However, parameters which exerted a positive effect on process performance yielded a reduced volumetric productivity due to an extended cultivation time. The enhancement of the volumetric productivity of a process in turn is one key for successful process development as it is maximizing final product concentrations. An improvement of the volumetric productivity could be achieved using a biphasic cultivation strategy providing an initial phase of rapid growth and a subsequent production phase at reduced growth maintaining productivity. Compared to a simple batch cultivation, this strategy of controlling cell proliferation would improve process performance leading to higher cell and product yields in a relatively short development time. In order to systematically examine the potential of a biphasic cultivation strategy, we will couple the positive effects of temperature (prolonged viability, reduced metabolism, increased specific productivity) and pH reduction (prolonged viability, reduced metabolism, unaffected sialylation) in different cultivation experiments based on biphasic cultivation.

To conclude, this controllable slowdown of cell metabolism at reduced temperatures and decreased pH values represents a valuable tool for optimizing production processes even in fed-batch and perfusion mode.

This research was funded by the ACBT (Austrian Center of Biopharmaceutical Technology), a competence center supported by the Federal Ministry for Economy and Labor, the federal states of Vienna and Tyrol. The authors are grateful to Annalisa Lasagna for the analysis of product concentrations, Katharina Vasicek for amino acid analysis and Friedemann Hesse for reviewing the manuscript.

References

Andersen DC, Bridges T, Gawlitzek M, Hoy C. 2000. Multiple cell culture factors can affect the glycosylation of Asn-184 in CHO-produced tissue-type plasminogen activator. Biotechnol Bioeng 70(1): 25–31.

Bloemkolk JW, Gray MR, Merchant F, Mosmann TR. 1992. Effect of temperature on hybridoma cell cycle and MAb production. Biotechnol Bioeng 40(3):427–431.

Bollati-Fogolin M, Forno G, Nimtz M, Conradt HS, Etcheverrigaray M, Kratje R. 2005. Temperature reduction in cultures of hGM-CSF-expressing CHO cells: Effect on productivity and product quality. Biotechnol Prog 21(1):17–21.

Borys MC, Linzer DI, Papoutsakis ET. 1993. Culture pH affects expression rates and glycosylation of recombinant mouse placental lactogen proteins by Chinese hamster ovary (CHO) cells. Biotechnology (N Y) 11(6):720–724.

Butler M. 2005. Animal cell cultures: Recent achievements and perspectives in the production of biopharmaceuticals. Appl Microbiol Biotechnol 68(3):283–291.

Chotigeat W, Watanapokasin Y, Mahler S, Gray PP. 1994. Role of environmental conditions on the expression levels, glycoform pattern and levels of sialyltransferase for hFSH produced by recombinant CHO cells. Cytotechnology 15(1–3):217–221.

Chuppa S, Tsai Y, Yoon S, Shackleford S, Rozales C, Bhat R, Tsay G, Matanguihan C, Konstantinov K, Naveh D. 1997. Fermenter temperature as a tool for control of high-density perfusion cultures of mammalian cells. Biotechnol Bioeng 55(2):328–338.

Ducommun P, Ruffieux PA, Kadouri A, von Stockar U, Marison IW. 2002. Monitoring of temperature effects on animal cell metabolism in a packed bed process. Biotechnol Bioeng 77(7):838–842.

Dutton RL, Scharer JM, Moo-Young M. 1998. Descriptive parameter evaluation in mammalian cell culture. Cytotechnology 26(2):139–152.

Fogolin MB, 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(1–2):179–191.

Fox SR, Patel UA, Yap MG, Wang DI. 2004. Maximizing interferon-gamma production by Chinese hamster ovary cells through temperature shift optimization: Experimental and modeling. Biotechnol Bioeng 85(2): 177–184.

Furukawa K, Ohsuye K. 1998. Effect of culture temperature on a recombinant CHO cell line producing a C-terminal alpha-amidating enzyme. Cytotechnology 26:153–164.

Furukawa K, Ohsuye K. 1999. Enhancement of productivity of recombinant a-amidating enzyme by low temperature culture. Cytotechnology 31: 85–94.

Fussenegger M, Betenbaugh MJ. 2002. Metabolic engineering II. Eukaryotic systems. Biotechnol Bioeng 79(5):509–531.

Gawlitzek M, Valley U, Nimtz M, Wagner R, Conradt HS. 1995. Characterization of changes in the glycosylation pattern of recombinant proteins from BHK-21 cells due to different culture conditions. J Biotechnol 42(2):117–131.

Jenkins N, Hovey A. 1993. Temperature control of growth and productivity in mutant chinese hamster ovary cells synthesizing a recombinant protein. Biotechnol Bioeng 42(9):1029–1036.

Kaufmann H, Mazur X, Fussenegger M, Bailey JE. 1999. Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells. Biotechnol Bioeng 63(5):573–582.

Kaufmann H, Mazur X, Marone R, Bailey JE, Fussenegger M. 2001. Comparative analysis of two controlled proliferation strategies regarding product quality, influence on tetracycline-regulated gene expression, and productivity. Biotechnol Bioeng 72(6):592–602.

Kunkel JP, Jan DC, Jamieson JC, Butler M. 1998. Dissolved oxygen concentration in serum-free continuous culture affects N-linked glycosylation of a monoclonal antibody. J Biotechnol 62(1):55–71.

Kunkel JP, Jan DC, Butler M, Jamieson JC. 2000. Comparisons of the glycosylation of a monoclonal antibody produced under nominally identical cell culture conditions in two different bioreactors. Biotechnol Prog 16(3):462–470.

Kurano N, Leist C, Messi F, Kurano S, Fiechter A. 1990. Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor: 1. Effects of physical and chemical environments. J Biotechnol 15(1–2):101–111.

Lin A, Kimura R, Miller WM. 1993. Production of tPA in recombinant CHO cells under oxygen-limited conditions. Biotechnol Bioeng 42(3):339–350.

Link T, Backstrom M, Graham R, Essers R, Zorner K, Gatgens J, Burchell J, Taylor-Papadimitriou J, Hansson GC, Noll T. 2004. Bioprocess development for the production of a recombinant MUC1 fusion protein expressed by CHO-K1 cells in protein-free medium. J Biotechnol 110(1):51–62.

Maurer HR. 1992. Towards serum free, chemically defined media for mammalian cell culture. In: Freshney RI, editor. Animal cell culture—A practical approach, 2nd edition, Oxford, UK: IRL Press. pp 15–46.

Miller WM, Blanch HW, Wilke CR. 2000. A kinetic analysis of hybridoma growth and metabolism in batch and continuous suspension culture: Effect of nutrient concentration, dilution rate, and pH. Reprinted from Biotechnology and Bioengineering 32:947–965.

Moore A, Mercer J, Dutina G, Donahue CJ, Bauer KD, Mather JP, Etcheverry T, Ryll T. 1997. Effects of temperature shift on cell culture, apoptosis and nucleotide pools in CHO batch cultures. Cytotechnology 23:47–54.

Mueller PP, Schlenke P, Nimtz M, Conradt HS, Hauser H. 1999. Recombinant glycoprotein product quality in proliferation-controlled BHK-21 cells. Biotechnol Bioeng 65(5):529–536.

Oh HK, So MK, Yang J, Yoon HC, Ahn JS, Lee JM, Kim JT, Yoo JU, Byun TH. 2005. Effect of N-Acetylcystein on butyrate-treated Chinese hamster ovary cells to improve the production of recombinant human interferon-beta-1a. Biotechnol Prog 21(4):1154–1164.

10970290, 2006, 6, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bit.21013 by Tracy Fritz , Wiley Online Library on [20/06/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Ozturk SS, Palsson BO. 1991. Growth, metabolic, and antibody production kinetics of hybridoma cell culture: 2. Effects of serum concentration, dissolved oxygen concentration, and medium pH in a batch reactor. Biotechnol Prog 7(6):481–494.

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(1):22–30.

Roessler B, Luebben H, Kretzmer G. 1996. Temperature: A simple parameter for process optimization in fed-batch cultures of recombinant Chinese hamster ovary cells. Enzyme Microb Technol 18:423–427.

Santell L, Ryll T, Etcheverry T, Santoris M, Dutina G, Wang A, Gunson J, Warner TG. 1999. Aberrant metabolic sialylation of recombinant proteins expressed in Chinese hamster ovary cells in high productivity cultures. Biochem Biophys Res Commun 258(1):132–137.

Schatz SM, Kerschbaumer RJ, Gerstenbauer G, Kral M, Dorner F, Scheiflinger F. 2003. Higher expression of Fab antibody fragments in a CHO cell line at reduced temperature. Biotechnol Bioeng 84(4):433–438.

Schriebl K, Trummer E, Lattenmayer C, Weik R, Kunert R, Müller D, Katinger H, Vorauer-Uhl K. 2006. A novel strategy for quantitative isoform detection directly performed from the culture supernatant. J Pharm Biomed Anal, in press.

Shelikoff M, Sinskey AJ, Stephanopoulos G. 1994. The effect of protein synthesis inhibitors on the glycosylation site occupancy of recombinant human prolactin. Cytotechnology 15(1–3):195–208.

Sung YH, Song YJ, Lim SW, Chung JY, Lee GM. 2004. Effect of sodium butyrate on the production, heterogeneity and biological activity of human thrombopoietin by recombinant Chinese hamster ovary cells. J Biotechnol 112(3):323–335.

Sureshkumar GK, Mutharasan R. 1991. The influence of temperature on a mouse-mouse hybridoma growth and monoclonal antibody production. Biotechnol Bioeng 37:292–295.

Weidemann R, Ludwig A, Kretzmer G. 1994. Low temperature cultivation–a step towards process optimization. Cytotechnology 15(1–3):111–116.

Yang M, Butler M. 2000. Effect of ammonia on the glycosylation of human recombinant erythropoietin in culture. Biotechnol Prog 16(5):751–759.

Yang M, Butler M. 2002. Effects of ammonia and glucosamine on the heterogeneity of erythropoietin glycoforms. Biotechnol Prog 18(1): 129–138.

Yoon SK, Kim SH, Lee GM. 2003a. Effect of low culture temperature on specific productivity and transcription level of anti-4-1BB antibody in recombinant Chinese hamster ovary cells. Biotechnol Prog 19(4):1383–1386.

Yoon SK, Song JY, Lee GM. 2003b. Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. Biotechnol Bioeng 82(3):289–298.

Yoon SK, Hwang SO, Lee GM. 2004. Enhancing effect of low culture temperature on specific antibody productivity of recombinant Chinese hamster ovary cells: Clonal variation. Biotechnol Prog 20(6):1683–1688.

Yoon SK, Choi SL, Song JY, Lee GM. 2005. Effect of culture pH on erythropoietin production by Chinese hamster ovary cells grown in suspension at 32.5 and 37.0 degrees C. Biotechnol Bioeng 89(3):345–356.

Zanghi JA, Fussenegger M, Bailey JE. 1999a. Serum protects protein-free competent Chinese hamster ovary cells against apoptosis induced by nutrient deprivation in batch culture. Biotechnol Bioeng 64(1):108–119.

Zanghi JA, Schmelzer AE, Mendoza TP, Knop RH, Miller WM. 1999b. Bicarbonate concentration and osmolality are key determinants in the inhibition of CHO cell polysialylation under elevated pCO_2 or pH. Biotechnol Bioeng 65(2):182–191.

DOI 10.1002/bit

10970290, 2006, 6, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bit.21013 by Tracy Fritz , Wiley Online Library on [20/06/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License