Effect of Culture pH on Erythropoietin Production by Chinese Hamster Ovary Cells Grown in Suspension at 32.5 and 37.0°C

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Received 11 May 2004; accepted 29 September 2004

Published online 29 December 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.20353

Abstract: To investigate the effect of culture pH in the range of 6.85–7.80 on cell growth and erythropoietin (EPO) production at 32.5 and 37.0°C, serum-free suspension cultures of recombinant CHO cells (rCHO) were performed in a bioreactor with pH control. Lowering culture temperature from 37.0 to 32.5°C suppressed cell growth, but cell viability remained high for a longer culture period. Regardless of culture temperature, the highest specific growth rate (µ) and maximum viable cell concentration were obtained at pH values of 7.00 and 7.20, respectively. Like µ, the specific consumption rates of glucose and glutamine decreased at 32.5°C compared to 37.0°C. In addition, they increased with increasing culture pH. Culture pH at 32.5°C affected specific EPO productivity (qEPO) in a different fashion from that at 37°C. At 37°C, the qEPO was fairly constant in the pH range of 6.85–7.80, while at 32.5°C, the qEPO was significantly influenced by culture pH. The highest qEPO was obtained at pH 7.00 and 32.5°C, and its value was approximately 1.5-fold higher than that at pH 7.00 and 37.0°C. The proportion of acidic EPO isoforms, which is a critical factor for high in vivo biological activity of EPO, was highest in the stationary phase of growth, regardless of culture temperature and pH. Although cell viability rapidly decreased in death phase at both 32.5 and 37.0°C, the significant degradation of produced EPO, probably by the action of proteases released from lysed cells, was observed only at 37.0°C. Taken together, through the optimization of culture temperature and pH, a 3-fold increase in maximum EPO concentration and a 1.4-fold increase in volumetric productivity were obtained at pH 7.00 and 32.5°C when compared with those at 37.0°C. These results demonstrate the importance of optimization of culture temperature and pH for enhancing EPO production in serum-free, suspension culture of rCHO cells. © 2004 Wiley Periodicals, Inc.

Keywords: CHO cells; culture pH; culture temperature; erythropoietin (EPO); heterogeneity of EPO glycoforms; serum-free medium; specific EPO productivity

INTRODUCTION

For the production of therapeutic proteins, recombinant Chinese hamster ovary (rCHO) cells have been most widely used. The popularity of rCHO cells is likely to persist as the demand for therapeutic proteins continues to increase. For large-scale production of therapeutic proteins in rCHO cells, either suspension or microcarrier culture is preferred to monolayer culture. To maximize the productivity in a suspension culture of rCHO cells, key environmental factors such as pH and temperature, which affect yield and quality of recombinant protein, need to be optimized. Despite the popularity of serum-free suspension culture of rCHO cells in industry, it is surprising that there are, to date, few studies on the effect of environmental factors on rCHO cells in suspension culture.

Culture pH is an important environmental factor that should be precisely controlled to ensure the quality of a desired product. Its importance has been demonstrated in T-flask cultures without pH control. Borys et al. (1993) found that culture pH significantly affected the expression rates and glycosylation of recombinant mouse placental lactogen-I (mPL-I) in CHO cells. Zanghi et al. (1999) demonstrated the importance of culture pH and pCO2 interactions which can alter glycosylation of recombinant protein in CHO cells.

Culture temperature is another important environmental factor that should be considered for process optimization. Compared with culture pH, the effect of culture temperature on rCHO cells has been relatively well characterized. However, most of the studies except for a few (Furukawa and Ohsuye, 1999; Ryll et al., 2000) were performed using T flasks or shake flasks without pH control. Like most
mammalian cells, the optimal temperature of CHO cells for growth is around 37.0°C. Lowering the culture temperature from 37.0 to 30–33°C suppresses cell growth (Furukawa and Ohsuye, 1999; Hendrick et al., 2001; Kaufmann et al., 1999; Yoon et al., 2003a). The beneficial effect of low culture temperature on specific productivity \( q_p \) appears to be cell-line-specific. There have been several reports showing decreased or unchanged \( q_p \) (Ryll et al., 2000; Yoon et al., 2003b) and increased \( q_p \) at low culture temperature (Furukawa and Ohsuye, 1999; Hendrick et al., 2001; Kaufmann et al., 1999; Schatz et al., 2003; Yoon et al., 2003a).

Erythropoietin (EPO), a glycoprotein produced from CHO cells, is one of the most important biopharmaceuticals. The carbohydrate moiety of EPO has been shown to be important for the secretion, biosynthesis and biological activity of EPO (Dube et al., 1988). Especially, high sialylation of EPO offers a further enhancement of in vivo biological activity (Takeuchi et al., 1989; Yamaguchi et al., 1991). Accordingly, proper glycosylation of EPO must be considered as one of the critical factors for the optimization of the EPO production.

Previously, we investigated the effect of culture temperature on rCHO cell growth and its EPO production and found the beneficial effect of lowering culture temperature on EPO production (Yoon et al., 2003a). Here, we studied serum-free suspension cultures of rCHO cells in a bioreactor with pH control to investigate the effect of culture pH on cell growth and EPO production at two different culture temperatures, 32.5 and 37.0°C. Furthermore, after the EPO that was secreted under different culture temperatures and pH was purified with reverse-phase HPLC, its heterogeneity was evaluated by isoelectric focusing (IEF) analysis.

**MATERIALS AND METHODS**

**Cell Line and Culture Medium**

The rCHO cells producing human EPO (LGE10-9-27) were used in this study (Yoon et al., 2003a). They were established by transfection of a vector containing the dihydrofolate reductase (dhfr) and human EPO genes into dhfr-deficient CHO cells (DUXK-B11, ATCC CRL-9096) and subsequent DHFR/methotrexate (MTX)-mediated gene amplification. The stable rCHO cells (LGE10-9-27) were selected at 5 μM MTX.

The proprietary, protein-free medium, LG-PF5 (LG Life Science, Daejon, Korea), was used for suspension culture of rCHO cells. The LG-PF5 medium was based on Iscove’s modified Dulbecco’s medium (IMDM), and its glucose and glutamine concentrations were 5 g/L and 8 mM, respectively. The rCHO cells were adapted to LG-PF5 medium with 5 μM MTX using spinner flasks (Bellco Glass, Vineland, NJ) for 2 weeks. Cells were maintained as suspension cultures in 125- or 500-mL spinner flasks on a magnetic stirrer plate (Bellco Glass) at 60 rpm in a 5% CO\textsubscript{2}/air mixture, humidified at 37.0°C.

**Batch Cultures**

Batch cultures were carried out in a 3-L BIOFLO 110 bioreactor (New Brunswick Scientific, Edison, NJ). Exponentially growing cells were inoculated at 2 × 10\textsuperscript{5} cells/mL into a bioreactor with a working volume of 1.8 L. The agitation speed was 50 rpm, and the dissolved oxygen concentration was controlled at 50% of air saturation. The culture temperature was controlled at 32.5 and 37.0°C within ±0.1°C, respectively. The culture pH was controlled at various pH (6.85, 7.00, 7.20, 7.40, 7.60, and 7.80, respectively) within ±0.03 by addition of 0.5 N NaOH or CO\textsubscript{2} gas. In all cases, the osmolality of the medium, which was initially 280–290 mOsm/kg, did not exceed 345 mOsm/kg at the end of the cultures. Periodically, approximately 15 mL of culture medium was taken from the bioreactor. Cell concentration was estimated using a hemacytometer, and viable cells were distinguished from dead cells using trypan blue dye-exclusion method. Culture supernatants, after centrifugation, were aliquoted and kept frozen at −70°C for later analyses.

**EPO, Glucose, Lactate, and Ammonia Assays**

The secreted EPO concentration was quantified by an enzyme-linked immunosorbent assay (ELISA) as described previously (Yoon et al., 2003a). In brief, 96 well-plates (Nalge Nunc Intl., Rochester, NY) were coated with monoclonal mouse anti-human EPO antibody (R&D Systems, Minneapolis, MN) and blocked with bovine serum albumin (BSA) and Tween 20. For the internal EPO standard, culture supernatants and/or cell lysates diluted with blocking buffer were loaded on wells and treated with polyclonal rabbit anti-human EPO antibody (R&D Systems) in diluent solution. The horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (ICN, Costa Mesa, CA) was used as an enzyme-antibody conjugate. EPO assay was repeated three times for each sample to ensure a reproducibility of assay results.

Glucose and lactate concentrations were measured using a glucose/lactate analyzer (Model YSI 2700, Yellow Springs Instrument Co., Yellow Springs, OH). Ammonia concentration was determined using an ammonia detection kit (Roche, Mannheim, Germany) according to the protocol provided by the manufacturer.

**Amino Acid Analysis**

The concentrations of amino acids except for tryptophan were measured by HPLC (Waters Delta Prep 4000, Waters, Milford, MA) with a reverse-phase column (AccQTag, 3.9 × 150 mm, Waters) and a guard column (Sentry® Nova-Pak® C18, Waters) according to Waters’ protocol for
amino acid analysis (modified chromatography for cell culture analysis, Waters). L-α-Amino-n-butyric acid was used as an internal standard. The samples and amino acid standards (Pierce, Rockford, IL) were derivatized using an AccQFluor® reagent kit (Waters). The derivatives were monitored with a fluorescence detector, and the peaks were identified on the basis of retention time. Samples were run in duplicate, and the measurement error was ±5%.

**Evaluation of Specific Growth Rate, Specific EPO Productivity, and Specific Consumption or Production Rates**

The specific growth rate (μ) was calculated by plotting the logarithm of viable cell concentration versus culture time during the exponential growth phase. The specific EPO productivity (qEPO), specific consumption or production rates of glucose (qGlc), lactate, and amino acids except for glutamine were based on the data collected during the growth phase and were evaluated from a plot of the substrate and product concentrations against the time integral values of the growth curve (Renard et al., 1988).

Glutamine spontaneously decomposes following first-order kinetics to ammonia and pyrrolidone carboxylate as a function of temperature and pH (Ozturk and Palsson, 1990; Tritsch and Moore, 1962). The specific glutamine consumption rate (qGln) and specific ammonia production rate (qAmm) were determined by accounting for the degradation of glutamine with the values of the first-order rate constant, k, as obtained previously (Ozturk and Palsson, 1990; Yoon et al., 2003a).

**Evaluation of Volumetric EPO Productivity**

Because the basic proportion of EPO isoforms is removed through the purification process, only the acidic portion of

![Figure 1](image_url)
EPO isoforms is considered for calculation of volumetric productivity as follows:

\[
V_{\text{EPO}}(\mu g/mL/h) = C_{\text{EPO}} \times R_{\text{ACID}}/T_F,
\]

where \( C_{\text{EPO}} \) is an EPO concentration in the medium, \( R_{\text{ACID}} \) is an acidic proportion of EPO isoforms (isoforms 4–8), and \( T_F \) is a culture time, respectively.

**IEF Analysis of Purified EPO**

IEF analysis was based on the method described in European Pharmacopoeia (European Pharmacopoeia Monograph for Concentrated Solution of EPO, 2001). The secreted EPO from CHO cells was purified by reverse-phase chromatography. Culture supernatant samples containing EPO were loaded onto a reverse-phase column (RESOURC\textsuperscript{E}® RPC 3 mL, Amersham Pharmacia Biotech AB, Uppsala, Sweden). The EPO was separated using gradient elution with two solutions: eluent A (10 mM Tris, pH 7.0) and eluent B (10 mM Tris, pH 7.0, 80% ethanol). The volume of purified EPO was reduced by vacuum evaporation and then dialyzed with deionized water by centrifugal filter device (Microcon, Millipore, Bedford, MA). After dialysis, it was stored at 4°C for later analyses.

IEF was performed on a thin-layer polyacrylamide gel in the presence of urea using Servalyte 3-5 ampholines mixed with Servalyte 3-10 (Serva, Heidelberg, Germany). The detailed procedure was described previously (Yoon et al., 2003a).

**RESULTS**

To investigate the effect of culture pH on cell growth and erythropoietin (EPO) production at two different culture temperatures (32.5 and 37.0°C), serum-free suspension cultures of rCHO cells were performed in a bioreactor with pH control. Culture pH was controlled at 6.85, 7.00, 7.20, 7.40, 7.60, and 7.80, respectively. Batch cultures were performed twice at separate times.

**Cell Growth**

Figure 1 shows typical cell growth and viability profiles during batch cultures. Culture pH significantly affected cell growth and viability at both 32.5 and 37.0°C. At 37.0°C, cells started to grow exponentially without a lag period regardless of the culture pH. However, \( \mu \) and maximum cell growth were significantly influenced by culture pH (Fig. 1A). In addition, culture longevity, based on cell viability, appeared to increase as the culture pH decreased (Fig. 1B). At 32.5°C, cells grew more slowly than those at 37.0°C, but they also died more slowly (Fig. 1C,D).

Figure 2 shows \( \mu \) and maximum viable cell concentration during batch cultures shown in Fig. 1. At 37.0°C, \( \mu \) was highest (0.029 ± 0.002 h\(^{-1}\)) at pH 7.20 and lowest (0.016 ± 0.002 h\(^{-1}\)) at pH 6.85. However, despite the relatively low \( \mu \) (0.022 ± 0.001 h\(^{-1}\)) at pH 7.00, the maximum viable cell concentration of \((4.18 ± 0.17) \times 10^6\) cells/mL was obtained. Similarly, the highest \( \mu \) and maximum viable cell concentration at 32.5°C were obtained at pH 7.20 and 7.0, respectively. At 32.5°C, \( \mu \) at pH 7.20 was 0.010 ± 0.0013 h\(^{-1}\), which is approximately one-third of \( \mu \) obtained at 37.0°C. The maximum viable cell concentration at

![Figure 2](image-url)
pH 7.00 and 32.5°C was $(2.98 \pm 0.25) \times 10^6$ cells/mL. At pH 7.60 and 7.80, cells still could grow, but the maximum viable cell concentrations obtained were only $(0.48 \pm 0.07) \times 10^6$ and $(0.33 \pm 0.05) \times 10^6$ cells/mL, respectively.

**Cell Metabolism**

Glucose, lactate, glutamine, and ammonia concentrations were measured during the growth phase. Regardless of culture temperature and pH, glucose utilization was accompanied by a corresponding accumulation of lactate, while glutamine utilization was accompanied by a corresponding accumulation of ammonia (data not shown). When the maximum viable cell concentration was achieved, glucose was depleted completely in most conditions except for pH 7.60 and 7.80 at 32.5°C, indicating that glucose is a limiting nutrient.

As shown in Figure 3A and B, $q_{\text{Glc}}$ and $q_{\text{Lac}}$ were significantly influenced by culture temperature and pH. They decreased by lowering culture temperature and pH. In addition, the effect of culture pH on $q_{\text{Glc}}$ and $q_{\text{Lac}}$ was more significant at 37.0°C than at 32.5°C. The ratio of lactate produced to glucose consumed, $Y_{\text{Lac/Glc}}$, also decreased at low pH. By lowering culture pH from 7.80 to 6.85, $Y_{\text{Lac/Glc}}$ decreased from $1.78 \pm 0.06$ mol/mol to $1.10 \pm 0.09$ mol/mol at 32.5°C, and it decreased from $1.53 \pm 0.03$ mol/mol to $1.14 \pm 0.13$ mol/mol at 37.0°C.

The $q_{\text{Gln}}$ and $q_{\text{Amm}}$ were calculated by considering the spontaneous decomposition of glutamine to avoid the overestimation of their values. Like $q_{\text{Glc}}$ and $q_{\text{Lac}}$, $q_{\text{Gln}}$ and $q_{\text{Amm}}$ also decreased at 32.5°C and increased at pH 7.60–7.80 (Fig. 3C,D). However, the ratio of ammonia produced to glutamine consumed, $Y_{\text{Amm/Gln}}$, was little affected by culture temperature. At 32.5°C, $Y_{\text{Amm/Gln}}$, was $2.12 \pm 0.24$ mol/mol at pH 6.85 and $1.78 \pm 0.28$ mol/mol at pH 7.80. At 37.0°C, it was $1.25 \pm 0.11$ mol/mol at pH 6.85 and $1.38 \pm 0.03$ mol/mol at pH 7.80. The $Y_{\text{Lac/Glc}}$ and $Y_{\text{Amm/Gln}}$ at various culture conditions are summarized in Table I.

![Figure 3](image_url)

*Figure 3. Metabolic rates in batch cultures at various culture temperatures and pH. (A) $q_{\text{Glc}}$ at 37.0°C (○) and 32.5°C (●). (B) $q_{\text{Lac}}$ at 37.0°C (○) and 32.5°C (●). (C) $q_{\text{Gln}}$ at 37.0°C (○) and 32.5°C (●). (D) $q_{\text{Amm}}$ at 37.0°C (○) and at 32.5°C (●). The error bars represent the standard deviations calculated from the data obtained in two independent experiments.*
Table I. $Y_{\text{Lac}/\text{Glc}}$ and $Y_{\text{Amm}/\text{Gln}}$ in batch cultures at various culture temperatures and pH.\textsuperscript{a}

<table>
<thead>
<tr>
<th>pH</th>
<th>32.5°C</th>
<th>37°C</th>
<th>32.5°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.85</td>
<td>1.10 ± 0.09</td>
<td>1.14 ± 0.13</td>
<td>2.12 ± 0.24</td>
<td>1.25 ± 0.11</td>
</tr>
<tr>
<td>7.00</td>
<td>0.96 ± 0.09</td>
<td>1.36 ± 0.09</td>
<td>1.84 ± 0.01</td>
<td>0.91 ± 0.01</td>
</tr>
<tr>
<td>7.20</td>
<td>1.00 ± 0.21</td>
<td>1.53 ± 0.04</td>
<td>1.87 ± 0.34</td>
<td>1.07 ± 0.17</td>
</tr>
<tr>
<td>7.40</td>
<td>1.52 ± 0.03</td>
<td>1.61 ± 0.02</td>
<td>3.17 ± 0.14</td>
<td>1.56 ± 0.26</td>
</tr>
<tr>
<td>7.60</td>
<td>1.58 ± 0.16</td>
<td>1.47 ± 0.02</td>
<td>2.48 ± 0.58</td>
<td>1.05 ± 0.01</td>
</tr>
<tr>
<td>7.80</td>
<td>1.78 ± 0.06</td>
<td>1.53 ± 0.03</td>
<td>1.78 ± 0.28</td>
<td>1.38 ± 0.03</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are means of two independent experiments.

To determine whether amino acids were depleted at the end of the growth phase, we analyzed the concentrations of amino acids in the culture medium. None of them, including glutamine, was completely depleted when the maximum viable cell concentration was achieved. Accordingly, the initiation of the stationary phase was not due to depletion of amino acids.

As summarized in Table II, the specific consumption or production rates of amino acids were significantly influenced by culture temperature and pH. In general, the specific consumption rates of most amino acids except for cysteine (Cys) at pH 7.60–7.80 were lower at 32.5 than at 37.0°C. However, the specific production rates were not always lower at 32.5 than at 37.0°C in this culture pH condition. The specific production rates of some amino acids, for example, such as asparagine (Asn) and glutamate (Glu), at 32.5°C were higher than those at 37.0°C. Meanwhile, the specific consumption rates of serine (Ser) and Cys were significantly higher at pH 7.60–7.80 than those at pH 6.85–7.40, regardless of culture temperature. Likewise, an increase in specific production rates of Glu, glycine (Gly), threonine (Thr), and alanine (Ala) were observed at pH 7.60–7.80, regardless of culture temperature. It was also noted that tyrosine (Tyr) was produced at pH 7.80 and 32.5°C.

EPO Production and Specific EPO Productivity

Figure 4 shows a typical EPO concentration profile during batch cultures shown in Figure 1. Culture pH significantly affected EPO production at both 32.5 and 37.0°C. At 37.0°C, the EPO concentration increased until the stationary phase of growth and thereafter decreased. This result indicates that EPO in the culture medium was degraded in the later phase of culture. The optimal pH for EPO production was different from that for cell growth. The maximum EPO concentrations of 57.2 ± 8.8 μg/mL was obtained at pH 6.85, mainly due to the prolonged culture longevity at pH 6.85.

In contrast, the EPO concentration at 32.5°C continued to increase even in the death phase of growth. In addition, despite the lower μ, the maximum EPO concentration at 32.5°C, which was obtained at pH 7.00, was significantly higher than that at 37.0°C. It was 152.6 ± 1.9 μg/mL, which is 2.7-fold higher than that at 37.0°C and pH 6.85. The high maximum EPO concentration at 32.5°C and pH 7.00 was not just due to the prolonged culture longevity.

Table II. Specific amino acid consumption and production rates in batch cultures at various culture temperatures and pH.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>pH at 32.5°C</th>
<th>6.85</th>
<th>7.00</th>
<th>7.20</th>
<th>7.40</th>
<th>7.60</th>
<th>7.80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>0.91</td>
<td>0.88</td>
<td>0.82</td>
<td>0.74</td>
<td>2.88</td>
<td>2.78</td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>3.68</td>
<td>4.09</td>
<td>5.47</td>
<td>6.23</td>
<td>16.56</td>
<td>26.30</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>-2.85</td>
<td>-1.91</td>
<td>-2.05</td>
<td>-3.21</td>
<td>-4.19</td>
<td>-5.81</td>
<td></td>
</tr>
<tr>
<td>Asn</td>
<td>-0.96</td>
<td>-0.70</td>
<td>-0.76</td>
<td>-1.14</td>
<td>-1.57</td>
<td>-2.23</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>2.32</td>
<td>1.10</td>
<td>1.27</td>
<td>2.32</td>
<td>10.62</td>
<td>14.57</td>
<td></td>
</tr>
<tr>
<td>Gln</td>
<td>-7.16</td>
<td>-8.22</td>
<td>-6.61</td>
<td>-5.36</td>
<td>-7.39</td>
<td>-14.03</td>
<td></td>
</tr>
<tr>
<td>His</td>
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<td>-0.52</td>
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<td>-0.24</td>
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<td>Thr</td>
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<td>1.00</td>
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<td>1.44</td>
<td>5.93</td>
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<tr>
<td>Arg</td>
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<td>-1.47</td>
<td>-2.06</td>
<td>-2.32</td>
<td>-1.22</td>
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</tr>
<tr>
<td>Ala</td>
<td>3.25</td>
<td>5.59</td>
<td>8.96</td>
<td>11.06</td>
<td>19.53</td>
<td>23.78</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>0.70</td>
<td>2.02</td>
<td>2.42</td>
<td>1.35</td>
<td>1.67</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>-0.75</td>
<td>-0.62</td>
<td>-0.65</td>
<td>-0.86</td>
<td>-0.43</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>-3.70</td>
<td>-3.16</td>
<td>-3.59</td>
<td>-5.49</td>
<td>-9.89</td>
<td>-20.09</td>
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<tr>
<td>Val</td>
<td>-1.33</td>
<td>-1.32</td>
<td>-1.41</td>
<td>-1.54</td>
<td>-0.55</td>
<td>0.97</td>
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</tr>
<tr>
<td>Met</td>
<td>-1.70</td>
<td>-1.02</td>
<td>-1.13</td>
<td>-1.67</td>
<td>-1.41</td>
<td>-1.51</td>
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<tr>
<td>Ile</td>
<td>-1.75</td>
<td>-1.52</td>
<td>-1.62</td>
<td>-1.93</td>
<td>-0.46</td>
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<tr>
<td>Leu</td>
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<td>-2.99</td>
<td>-3.33</td>
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<tr>
<td>Lys</td>
<td>-2.41</td>
<td>-1.40</td>
<td>-1.61</td>
<td>-2.46</td>
<td>-1.54</td>
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<tr>
<td>Phe</td>
<td>-0.80</td>
<td>-0.77</td>
<td>-0.88</td>
<td>-1.14</td>
<td>-0.65</td>
<td>-0.76</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are means of two independent experiments.
Enhanced \( q_{EPO} \) at 32.5°C and pH 7.00 also contributed to achieving this high maximum EPO concentration.

At 37.0°C, the culture pH did not influence \( q_{EPO} \) (Fig. 5A). The \( q_{EPO} \) was fairly constant in the range of 0.19–0.23 μg/10⁶ cells/h. On the other hand, \( q_{EPO} \) at 32.5°C was significantly influenced by the culture pH. At 32.5°C, \( q_{EPO} \) was highest at pH 7.00, and its value of 0.31 ± 0.02 μg/10⁶ cells/h was approximately 1.8-fold higher than that at pH 6.85, 7.60, and 7.80. However, \( q_{EPO} \) at 32.5°C was not always higher than that at 37.0°C. The \( q_{EPO} \) at 32.5°C was 1.3- to 1.5-fold higher than that at 37.0°C only in the pH range of 7.00–7.40. Accordingly, in the pH range of 7.60–7.80, the maximum EPO concentration at 32.5°C was lower than that at 37.0°C (Fig. 5B).

Isoform Distributions of Purified EPO

To determine the effect of culture pH on the heterogeneity of EPO secreted to the medium at 32.5 and 37.0°C, we purified EPO in the culture medium sampled at the different phases of growth and performed IEF with purified EPO.

Figure 6 show isoforms of EPO produced at various culture conditions (pH, temperature, and growth phase). EPO
has three N-glycosylation sites and one O-glycosylation site. Theoretically, an EPO protein can hold up to 14 terminal sialic acid molecules, so 14 different EPO isoforms can be secreted. EPO isoforms with less than seven sialic acids are probably too small to be detected by Coomassie staining. Regardless of culture conditions, seven or eight distinct EPO isoforms were most often observed, indicating that the charge heterogeneity of EPO was not significantly influenced by pH, temperature, or growth phase. However, some EPO isoforms appeared to depend on the culture conditions. On the basis of the band intensity of isoforms shown in Figure 6, their proportions were analyzed and plotted (Fig. 7). Indeed, culture conditions such as pH, temperature, and growth phase could affect some EPO isoforms.

At 37.0°C, the acidic proportion of EPO isoforms (isoforms 4–8) was highest at the stationary phase of growth in the pH range of 6.85–7.20 and was 64–67% (Fig. 7A). It decreased to 39% as culture pH increased to pH 7.80. In addition, it decreased significantly in the death phase in the pH range 6.85–7.20, indicating that the sialic acid content of EPO is decreased. Furthermore, in the exponential growth phase, the acidic proportion of EPO isoforms was also lower than that in stationary growth phase, indicating that the acidic isoforms depend not only on the environmental conditions (culture temperature and pH) but also on the growth state of the cells. In contrast, the acidic proportion of EPO isoforms at pH 7.80 remained almost constant throughout the culture.

As observed at 37.0°C, the acidic proportion of EPO isoforms at 32.5°C was also highest at the stationary phase of growth in the pH range of 6.85–7.20 and was 61–66% (Fig. 7B). It also decreased in the death phase but to a much lesser extent than that at 37.0°C. In the pH range of 6.85–7.20, the acidic proportion of EPO in the death phase was 55–62%, which is 0–10% lower than that in the stationary phase of growth.

Figure 6. Isoelectric focusing analysis of EPO secreted at various culture temperatures and pH. The EPO samples were taken out in mid-exponential phase (E), stationary phase (S), and death phase (D), respectively, and were purified. After purification, samples containing 15 μg of EPO were applied to IEF polyacrylamide gel and analyzed as described in Materials and Methods. Arrow indicates the isoform distribution of EPO that was purified with an ion exchange chromatography. (A) IEF gel of EPO at 37.0°C and various pH. (B) IEF gel of EPO at 32.5°C and various pH.

Volumetric Productivity

A maximization of volumetric productivity is one of the key objectives in the development of culture process. Vol-
Volumetric productivity is calculated by dividing the maximum product concentration by the operation time. However, in the case of EPO, the basic portion of EPO isoforms was removed through purification process (Fig. 6), so we considered only the acidic proportion of EPO isoforms for the calculation of volumetric productivity, as described in Materials and Methods. Data for $R_{\text{ACID}}$ and $T_E$ are adopted from Figure 7.

Figure 8 shows volumetric productivities at various culture conditions. In terms of growth phases, the highest volumetric productivity was obtained in the stationary phase of growth, regardless of culture temperature and pH. At 32.5°C and pH 7.00, the highest volumetric productivity of 0.234 µg/mL/h was achieved in the stationary phase of growth, although the EPO concentration continued to increase in the death phase. The volumetric productivity decreased in the death phase (0.194 µg/mL/h at 32.5°C and pH 7.00) because the culture time increased from 319 to 416 h, and the acidic portion of EPO isoforms did not increase significantly.

Due to the extended culture time, the low-temperature culture did not yield a significant enhancement in volumetric productivity. At pH 7.00 in the stationary phase of growth, the acidic EPO isoform concentration at 32.5°C (80.9 µg/mL) was approximately 3-fold higher than that at 37.0°C, but the volumetric productivity at 32.5°C was only 1.4-fold higher than that at 37.0°C.

DISCUSSION

For large-scale commercial production of therapeutic proteins, serum-free suspension culture of rCHO cells is most widely used in industry. To overcome the relatively low yields of therapeutic proteins in rCHO cells, environmental factors such as pH and temperature, which affect the production and quality of therapeutic proteins, need to be optimized. This is done industrially but is usually not published. In an effort to increase EPO production, we
instituted the effect of pH and temperature on cell growth and EPO production in serum-free suspension culture of rCHO cells (LGE10-9-27) using a bioreactor with pH and temperature control. In our previous experiments using T flasks without pH control, we observed that the detrimental effect of lowering culture temperature to 30°C on cell growth outweighed its beneficial effect on $q_{EPO}$ (Yoon et al., 2003a). So, two different culture temperatures, 32.5 and 37.0°C, were used in this study. Because CHO cells are usually cultivated at pH 7.0–7.2 (Borys et al., 1993; Curling et al., 1990), a pH range from 6.85 to 7.80 was used in this study.

Regardless of culture temperature, cells were able to proliferate in a pH range from 6.85 to 7.80 and showed the highest $\mu$ at pH 7.20. However, the influence of culture pH on cell growth at 32.5°C was more significant than that at 37.0°C, suggesting that cells at low culture temperature are more susceptible to the pH changes. At 32.5°C, cells did not grow as much at pH 7.60–7.80, where the maximum cell density of $(2.0-2.7) \times 10^6$ cells/mL was achieved at 37.0°C. For CHO-DUKXB1 cells, the highest $\mu$ at 37.0°C was observed at pH 7.60 (Kurano et al., 1990), suggesting that the optimal pH for cell growth varies among CHO cell lines.

By lowering culture temperature from 37.0 to 32.5°C, cell growth was suppressed regardless of culture pH. As observed previously with many different CHO cell lines (Fox et al., 2004; Furukawa and Ohsuye, 1999; Kaufmann et al., 1999; Yoon et al., 2003a, 2003b), growth suppression of CHO cells at low culture temperature, which may be attributed to the expression of cold-inducible RNA binding protein (Nishiyama et al., 1997), appears to be a universal phenomenon.

Cell metabolism was also affected by culture temperature and pH. Lowering culture temperature from 37.0 to 32.5°C decreased $q_{Glc}$ and $q_{Lac}$, which is in good agreement with previous observations made with other CHO cell lines (Fogolin et al., 2004; Furukawa and Ohsuye, 1998). Regardless of culture temperature, $q_{Glc}$ and $q_{Lac}$ increased at high pH in this study. This increased $q_{Glc}$ and $q_{Lac}$ at high pH is a well-known phenomenon for hybridoma cells (Miller et al., 1988; Ozturk and Palsson, 1991; Schmid et al., 1990). At high pH, the activity of glycolytic enzymes could be increased or the membrane potential could be changed, allowing more glucose to be transported (Ozturk and Palsson, 1991). However, the molecular mechanism underlying the increased $q_{Glc}$ and $q_{Lac}$ at high pH in CHO cells needs to elucidated.

The utilization pattern of amino acids was not affected significantly by both culture temperature and pH, but their consumption and production rates were significantly affected. Like $q_{Glc}$, the consumption rates of most amino acids, including glutamine, were reduced at low culture temperature. However, none of them, including glutamine, was completely depleted when the maximum viable cell concentration was achieved. Accordingly, the reduced glucose consumption rate is likely to be more responsible for the extended culture longevity at 32.5°C rather than the reduced amino acid consumption rate. Like $q_{Glc}$ and $q_{Lac}$, the consumption and production rates of many amino acids such as Glu, Ser, Asn, Gly, Gln, Thr, Ala, and Cys were significantly elevated at high pH.

EPO production was also affected significantly by culture temperature and pH. Culture pH at 32.5°C affected $q_{EPO}$ in a different fashion from that at 37°C. At 37°C, the $q_{EPO}$ was fairly constant at the pH range of 6.85–7.80, while the $q_{EPO}$ at 32.5°C was significantly influenced by culture pH. The highest $q_{EPO}$ was obtained at pH 7.00 and 32.5°C, and its value was approximately 1.5-fold higher than that at pH 7.00 and 37.0°C. This enhancement of $q_{EPO}$ at pH 7.00 and 32.5°C was not as significant as that observed in our previous report, where an approximately 3-fold increase of $q_{EPO}$ was obtained in T-flask culture by lowering the culture temperature from 37 to 33°C (Yoon et al., 2003a). In this study, we used the protein-free medium, which is different from the serum-free medium containing insulin used in our previous T-flask experiments. Accordingly, the medium as well as cultivation method affects the response of cells to culture pH and temperature regarding $q$. Schatz et al. (2003) also made a similar observation with CHO cells producing Fab antibody fragments. By lowering the culture temperature from 37 to 28°C, they obtained a 20-fold increase in average $q$ by using serum-containing medium and 47-fold increase by using serum-free medium.

Unlike CHO cells (LGE10-9-27) used in this study, Borys et al. (1993) observed that $q$ of mPL-I producing CHO cells at 37.0°C varied significantly over the pH range of 6.10–8.70, and found that the maximum $q$ occurred between pH 7.60 and 8.00. Although they conducted the experiments using T flasks with confluent cells, their results suggest that the effect of culture pH on $q$ depends on rCHO cell lines and therefore the culture pH needs to be optimized for an individual CHO cell line.

The highest EPO concentration (>150 μg/mL) was obtained at pH 7.00 and 32.5°C because of the highest $q_{EPO}$ and extended culture longevity. Reduced activities of proteases in culture medium are also likely to contribute to the achievement of the highest EPO concentration at pH 7.00 and 32.5°C. Interestingly, although cell viability rapidly decreased in death phase at both 32.5 and 37.0°C, significant degradation of produced EPO was only observed at 37.0°C (Fig. 4). Because cell concentration at 32.5°C is lower than that at 37.0°C, the amount of proteases released from lysed cells as well as their activities are likely to be lower at 32.5°C, resulting in reduced degradation of EPO at 32.5°C.

A higher sialylation of EPO is critical because asialoerythropoietin, which lacks sialic acid, is rapidly cleared from the blood circulation by asialoglycoprotein receptors in the liver (Fukuda et al., 1989). Therefore, the sialic acid content of EPO must be considered for the optimization of EPO production. Most of the basic isoforms, which have relatively lower numbers of sialic acids, are removed
through the purification process and the proportion of acidic isoforms is positively correlated with in vivo biological activity of EPO (data not shown). Accordingly, we analyzed the proportion of acidic isoforms as an indicator of in vivo biological activity.

An IEF gel analysis showed that the proportion of acidic EPO isoforms was affected more significantly by growth phase and culture pH, rather than the culture temperature. The proportion of acidic isoforms is higher at the stationary phase of growth than at the mid-exponential phase of growth, regardless of culture temperature and pH, suggesting that the sialylation of EPO would occur actively in slowly growing cells. In addition, it was observed that the proportion of acidic isoforms decreased in the death phase, more severely at 37.0 than at 32.5°C. This phenomenon is probably due to the action of glucosidase resulting from secretion and/or cell lysis in death phase. It has been reported that the glucosidases are released by secretion or cell lysis (Gramer and Goochee, 1993), and they play an important role in degradation of carbohydrate moiety (Munzert et al., 1996). The activity of sialidase at 32°C is approximately 70% of its activity at 37.0°C (Gramer and Goochee, 1993). Accordingly, the activities of glucosidases are probably higher at 37.0 than at 32.5°C, resulting in a severe reduction in the proportion of acidic isoforms in the death phase at 37.0°C.

As observed in CHO cells producing mPL-I (Borys et al., 1993), culture pH affected the glycosylation of EPO. The proportion of acidic isoforms increased with decreasing the culture pH, suggesting that the pH range of 6.85–7.20 would be favorable for sialylation of EPO.

From the industrial point of view, volumetric productivity may be a more important parameter than maximum product concentration. In a batch culture, suppressed cell growth at low culture temperature, which gives low μ and maximal cell concentration, yields the low volumetric productivity. In this study, although the maximum EPO concentrations in the pH range of 7.00–7.40 at 32.5°C was 2.2- to 3.2-fold higher than those at 37.0°C, the volumetric productivity at 32.5°C showed only a 1.4-fold increase, compared to that at 37.0°C, because the culture longevity at 32.5°C was extended. If downtime for harvest and preparation of a new batch is included in the calculation of volumetric productivity, the volumetric productivity at 32.5°C increases but still does not increase as much as that in the maximum EPO concentration. To further enhance the volumetric productivity of EPO, a biphasic culture that consists of the growth and production phases would be a good strategy, as suggested with other rCHO cell lines (Fox et al., 2004; Furukawa and Ohsuve, 1999; Kaufmann et al., 1999; Kim et al., 2002; Schatz et al., 2003). For example, cells are cultivated at 37.0°C and pH 7.20 in order to achieve reasonably high cell density, and then the temperature and pH are shifted to 32.5°C and pH 7.00 to achieve high q and improved EPO quality. The feasibility study of biphasic culture using bioreactor is on the way.

In conclusion, we found that culture temperature and pH significantly affected cell growth, and EPO production. By optimizing culture temperature and pH, a more than 2.7-fold increase in maximum EPO concentration was obtained at pH 7.00 and 32.5°C, compared with that at 37.0°C. On the other hand, the proportion of acidic isoforms, regardless of culture temperature and pH, was the highest in the stationary phase, indicating that the stationary phase is the optimal harvest time. Taken together, the results obtained here indicate that culture temperature and pH are important environmental factors that should be optimized for enhancing EPO production in serum-free, suspension culture of rCHO cells.

References


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