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ORIGINAL RESEARCH PAPER

Hyperosmolarity enhances transient recombinant protein yield in Chinese hamster ovary cells

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Abstract The effect of hyperosmolarity on transient recombinant protein production in Chinese hamster ovary (CHO) cells was investigated. Addition of 90 mM NaCl to the production medium ProCHO5 increased the volumetric yield of recombinant antibody up to 4-fold relative to transfection in ProCHO5 alone. Volumetric yields up to 50 mg l^{-1} were achieved in a 6 day batch culture of 3 l. In addition, hyperosmolarity reduced cell growth and increased cell size. The addition of salt to cultures of transiently transfected CHO cells is a simple and cost-effective method to increase TGE yields in this host.

Keywords CHO cells · Transfection · Hyperosmolarity · Polyethylenimine · Orbital shaken bioreactor

Introduction

Large-scale transient gene expression (TGE) for recombinant protein production has been performed mainly with human embryo kidney (HEK 293) and Chinese hamster ovary (CHO) cells (Pham et al. 2006; Baldi et al. 2007; Geisse 2009). CHO cells have yielded recombinant antibody titers up to 50 mg l^{-1} at large scale (>10 l) and up to 80 mg l^{-1} at smaller scales (Stettler et al. 2007; Wulhfard et al. 2008). By comparison, the highest recombinant antibody yields for TGE in HEK 293 cells have been 1 g l^{-1} (Backliwal et al. 2008). The highest TGE yields to date in CHO cells have been achieved by exposing the transfected cells to hypothermia or histone deacetylase inhibitors such as sodium butyrate or valproic acid (Galbraith et al. 2006; Stettler et al. 2007; Wulhfard et al. 2008; Backliwal et al. 2008). These approaches and others including an increase in medium osmolarity have been used to increase the specific productivity of stable cell lines (Ozturk and Palsson 1991; Oh et al. 1995; Lee and Lee 2000; Ryu et al. 2000; Kim et al. 2002; Lee et al. 2003; Sun et al. 2004; Wu et al. 2004; Shen and Sharfstein 2006). For this reason, we explored hyperosmolarity as a strategy for increasing the specific productivity of transiently transfected CHO cells.

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Materials and methods

Cell culture

Suspension cultures of CHO DG44 cells were routinely maintained in disposable 50-ml TubeSpin

bioreactors (TPP, Trasadingen, Switzerland) in serum-free ProCHO5 medium (Lonza, Verviers, Belgium) as described (De Jesus et al. 2004). The cultures were incubated in an ISF-4-W incubator (Adolf Kühner AG, Birsfelden, Switzerland) in the presence of 5% CO_2 and 85% humidity at 37°C with agitation at 180 rpm. Cells were subcultivated every 3–4 days at a seeding density of 3–5 $\times$ 10^5 cells ml^{-1}. Cell number was determined manually using a Neubauer hemocytometer. Viability was assessed using the Trypan Blue exclusion method. The packed cell volume (PCV) was measured with mini-PCV tubes (TPP) as previously described (Stettler et al. 2006). The osmolarity was determined using a freezing point osmometer (Muti-Osmette 2340; Precision Systems, Natick, MA).

Plasmid DNA

pKML and pKMH carrying the full-length cDNAs of the anti-Rhesus D IgG light and heavy chain genes, respectively, were described previously (Derouazi et al. 2004). pEGFP-N1 was purchased from Clontech (Palo Alto, CA). Plasmid DNA was purified on a Nucleobond AX anion exchange column (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol.

Small-scale transfection

One day before transfection, cells were centrifuged, resuspended in the appropriate volume of ProCHO5 to a cell density of 2–3 $\times$ 10^6 cells ml^{-1} and incubated overnight as described (Muller et al. 2007). The next day the cells were centrifuged and resuspended in ProCHO5 to 10 $\times$ 10^6 cells ml^{-1}, and aliquots of 1 ml were added to each 50 ml TubeSpin bioreactor. Transfections were performed with a mixture of pKML, pKMH, and pEGFP-N1 at 45:45:10 (by wt). Linear 25 kDa polyethylenimine (PEI) (pH 7.0) (Polysciences, Eppenheim, Germany) was prepared in water at 1 mg ml^{-1} and filter-sterilized. For each transfection, 2.5 µg DNA and 10 µl PEI were added separately to 50 and 40 µl 150 mM NaCl, respectively. The PEI was then mixed with the DNA and allowed to stand at room temperature for 10 min. Following addition of the PEI/DNA solution to the culture, the tubes were agitated at 180 rpm in a CO_2- and humidity-controlled atmosphere at 37°C. At 2 h post-transfection (pst) the culture was diluted to 10^6 cells ml^{-1} by addition of 9 ml pre-warmed ProCHO5. At the same time, various amounts of 3 M NaCl were added to the cultures as indicated in the text. The transfected cultures were incubated at 37°C with agitation as before. Recombinant IgG titers were determined by sandwich ELISA as described previously (Meissner et al. 2001).

Three litre transfection

A 10-l square-shaped polycarbonate container (Cellon SA, Bereldange, Luxembourg) fitted with a three-port cap for active headspace aeration was used for TGE as described (Stettler et al. 2007). The transfection was performed in 120 ml ProCHO5 at 12 $\times$ 10^6 cells ml^{-1} in a 250 ml square-shaped glass bottle (Muller et al. 2005). A mixture of 300 µg pKML and pKMH at a 1:1 (w/w) and 1.2 mg PEI were added separately to 6 ml 150 mM NaCl. The PEI and DNA were then mixed as described above and added to the culture. The transfected culture was maintained at 37°C in an ISF-4-W incubator in a CO_2- and humidity-controlled atmosphere with a shaking speed of 110 rpm. At 2 h pst, the culture was transferred into the 10 l container containing 1.5 l pre-warmed ProCHO5 with an additional 90 mM NaCl. The container was agitated at 60 rpm in a CO_2-controlled atmosphere at 37°C. At 2 days pst, another 1.5 l of pre-warmed ProCHO 5 and an additional 90 mM NaCl was fed to the culture to reach a final volume of about 3.2 l. Samples taken from the culture before and after the feed at 2 days pst were transferred into TubeSpin bioreactors and maintained separately with agitation at 180 rpm.

Results

Hyperosmolarity improves transient antibody production in CHO cells

Cells were transfected with vectors for the expression of a monoclonal antibody and EGFP. At 2 h pst, transfected cells were diluted 10-fold and different amounts of 3 M NaCl were added to give 60, 90, 120, and 150 mM. The osmolarity of the cultures increased from a value of 315 mOsm kg^{-1} for unmodified ProCHO5to 430, 490, 550, and

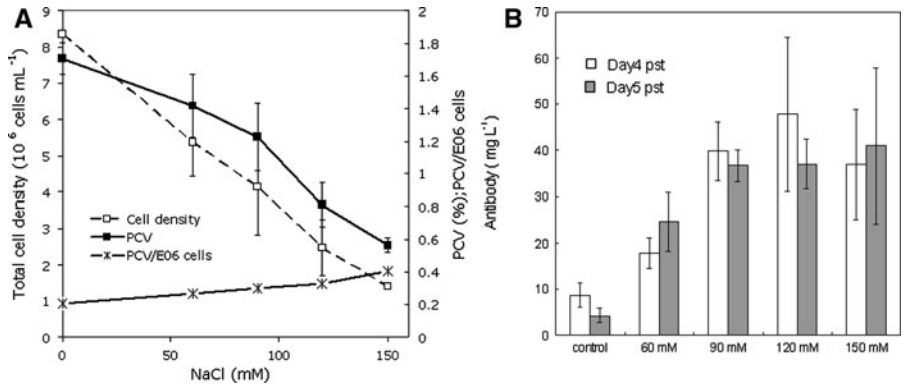

Fig. 1 Effect of hyperosmolarity on transiently transfected CHO cells. Cells were transfected with vectors to express a recombinant antibody and EGFP. **a** The cell density, PCV, and ratio of biomass volume to cell number (per 10^6 cells) were determined on day 4 pst. **b** The antibody concentration was determined on days 4 and 5 pst. All data represents the average of three independent experiments

610 mOsm kg^{-1}, respectively. The addition of NaCl decreased the maximal cell density and the biomass (% PCV) as compared to the transfected control culture (Fig. 1a). An increase in the average cell size was also observed in the presence of additional NaCl as judged by the PCV results and additional measurements with a CASY1 cell counter (Schärfe System GmbH, Reutlingen, Germany) (data not shown). It should be noted, however, that the change in cell size was only observed about 5 h after addition of NaCl. All transfections with additional NaCl yielded higher antibody titers than did the control transfection (Fig. 1b). The cell growth kinetics and cell viability of a culture with the addition of 90 mM NaCl and the control culture are shown in Fig. 2. In the presence of an additional 90 mM NaCl, cells proliferated, albeit with a reduced rate compared to the control. The cell viability of the two cultures was similar (Fig. 2). Transient transfections of CHO cells using other promoters to drive expression of the same antibody gene generated similar results (data not shown).

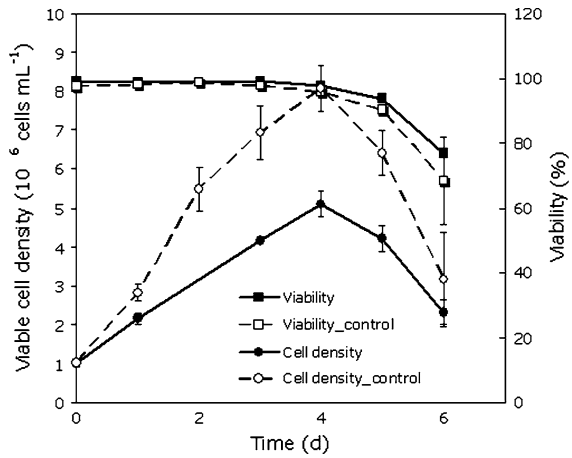

Fig. 2 Effect of hyperosmolarity on transiently transfected CHO cells. The transfected cells were diluted at 2 h pst with ProCHO5 with or without (control) an additional 90 mM NaCl. The cell density and viability of the cultures were determined at the times indicated. The data represent the average of three independent experiments

Development of a feeding strategy based on hyperosmolarity

Cells were transfected as before and at 2 h pst the cultures were diluted 10-, 20-, or 30-fold with ProCHO5 with an additional 90 mM NaCl (termed NaCl–ProCHO5). At 1 or 2 days pst, the cultures that were diluted 10-fold were further diluted with one, two, or three volumes of NaCl–ProCHO5. At day 7 pst, the highest volumetric (Fig. 3a) and total antibody yield (Fig. 3b) was measured in the culture that was diluted 10-fold at 2 h pst and then diluted with one volume of medium on day 1 pst. The maximal integral of viable cells (IVC) was lower for this culture than for the control culture that was only diluted 10-fold immediately after transfection (Fig. 3c).

Three litre transfection

To test the feasibility of the process strategy described above at a larger scale, TGE was performed

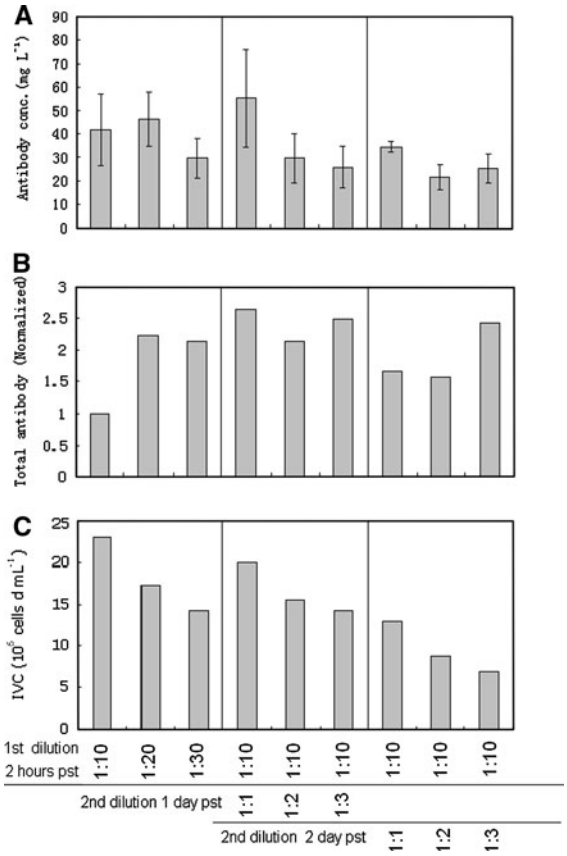

Fig. 3 Preliminary process optimization of transient transfection of CHO cells under hyperosmotic conditions. At 2 h pst the transfected cells were diluted 10-, 20-, or 30-fold in ProCHO5 with an additional 90 mM NaCl. Cells diluted 10-fold after transfected were also diluted with one, two, or three volumes of the same medium either 1 or 2 days pst. The maximal antibody concentration (**a**), total antibody production (**b**), and integrated viable cell density (**c**) were determined at day 7 pst. *Error bars* represent the standard deviation of three independent experiments

in a 10 l square-shaped bottle with a final working volume of about 3 l. The cells were transfected in 120 ml at 12×10^6 cells ml^{-1} and then diluted to 1×10^6 cells ml^{-1} by the addition of 1.5 l NaCl–ProCHO5 at 2 h pst. In this container, the cells had an extended lag phase as compared to cells cultivated in 50 ml TubeSpin bioreactors (Stettler et al. 2007). For this reason, the second dilution with one volume of medium was performed on day 2 pst. Aliquots of 10 ml were removed before and after the second dilution for the inoculation of satellite cultures in 50-ml Tubespin™ bioreactors. The cells reached a maximum of 4×10^6 cells ml^{-1} in the 10 l container and $4–5 \times 10^6$ cells ml^{-1} for the two satellite cultures (Fig. 4a). A volumetric antibody yield of 50 mg l^{-1} was obtained by 6 days pst for the culture in the 10-l container (Fig. 4b). Satellite cultures in TubeSpin bioreactors taken before and after the second dilution yielded antibody concentrations of 60 and 80 mg l^{-1}, respectively (Fig. 4b).

Discussion

Hyperosmolarity has been shown to be a cost-effective and simple method for enhancing recombinant protein production from CHO- and NS0-derived cell lines and monoclonal antibody production from hybridomas (Ryu et al. 2000; Sun et al. 2005). For bioprocesses using stable cell lines, osmolarities of 450–550 mOsm kg^{-1} have been reported for increasing protein yields (Kim et al. 2002). Here the effect of hyperosmolarity on TGE yields in CHO cells was determined. In the presence of an additional 90 mM NaCl, giving an osmolarity of 490 mOsm kg^{-1}, TGE yields were increased up to 400% relative to the control transfection, allowing volumetric yields up to 50 mg l^{-1} to be achieved for a recombinant antibody. The production medium used here has an osmolarity of 350 mOsm kg^{-1}. Thus, addition of 50–100 mM NaCl results in a similar osmolarity range as that typically used for increasing yields for bioprocesses using stable cell lines.

Here CHO DG44 cells showed an osmolarity-dependent decrease in growth. Nearly complete growth arrest was observed with addition of 150 mM NaCl, corresponding to an osmolarity around 650 mOsm kg^{-1} while the addition of 90 mM NaCl did not completely block cell division. Under conditions of high osmolarity the transfected cells were substantially larger than the control cells as has been reported previously (Ozturk and Palsson 1991). Increased cell size was also observed following the introduction of other stress condition like hypothermia (Wulhfard et al. 2008) and nocodazole treatment (Tait et al. 2004).

Most transiently transfected cells cease to produce recombinant protein within 5–14 days after transfection depending on the culture conditions, most likely due to a combination of gene silencing and physical loss of the plasmid DNA (Carpentier et al. 2007; Sun et al. 2006). This limits production options to rather short batch or fed-batch processes (Sun et al. 2006). It

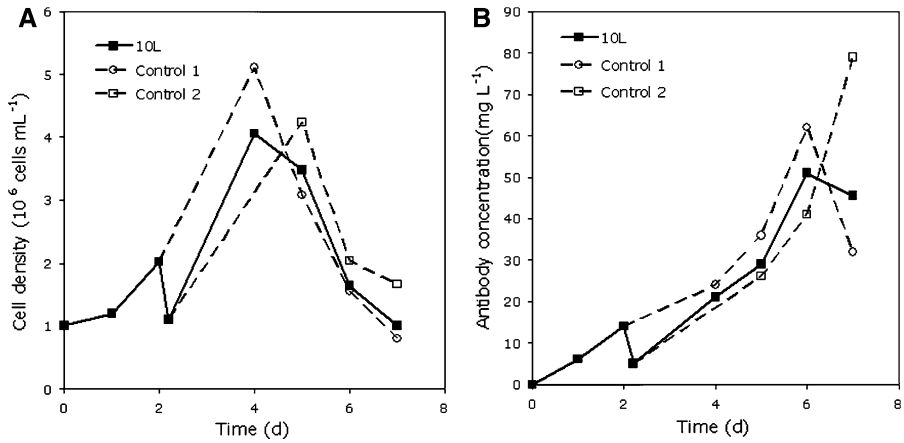

Fig. 4 Transient expression of a recombinant antibody in a 3-l culture. Transfected cells were diluted 10-fold at 2 h pst with ProCHO5 with an additional 90 mM NaCl at 2 h pst. One volume of the same medium was added at 2 days pst. The 10-ml control cultures were removed from the main culture before (control 1) or after (control 2) dilution of the culture at day 2 pst and maintained in TubeSpin bioreactors. Cell density (**a**) and antibody concentration (**b**) were determined at the times indicated

also implies that a high cell growth rate will result in a rapid loss of expression capacity. The transfection strategy developed in this study resulted in an improved specific productivity while allowing only a slight increase of the total cell number over time.

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