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Differences in Optimal pH and Temperature for Cell Growth and Antibody Production Between Two Chinese Hamster Ovary Clones Derived from the Same Parental Clone

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Abstract To investigate clonal variations of recombinant Chinese hamster ovary (rCHO) clones in response to culture pH and temperature, serum-free suspension cultures of two antibody-producing CHO clones (clones A and B), which were isolated from the same parental clone by the limiting dilution method, were performed in a bioreactor at pH values in the range of 6.8–7.6, and two different temperatures, 33°C and 37°C. In regard to cell growth, clone A and clone B displayed similar responses to temperature, although their degree of response differed. In contrast, clones A and B displayed different responses to temperature in regard to antibody production. In the case of clone A, no significant increase in maximum antibody concentration was achieved by lowering the culture temperature. The maximum antibody concentration obtained at 33°C (pH 7.4) and 37°C (pH 7.0) were 82.0±2.6 and 73.2±4.1 μg/ml, respectively. On the other hand, in the case of clone B, an approximately 2.5-fold increase in maximum antibody concentration was achieved by lowering the culture temperature. The enhanced maximum antibody concentration of clone B at 33°C (132.6±14.9 μg/ml at pH 7.2) was due to not only enhanced specific antibody productivity but also to prolonged culture longevity. At 33°C, the culture longevity of clone A also improved, but not as much as that of clone B. Taken together, CHO clones derived from the same parental clone displayed quite different responses to culture temperature and pH with regards antibody production, suggesting that environmental parameters such as temperature and pH should be optimized for each CHO clone.

Keywords: Antibody production, CHO cells, clonal variation, culture pH, culture temperature

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Recombinant Chinese hamster ovary (rCHO) cells are most widely used for the commercial production of therapeutic important proteins. As the demand for therapeutically proteins continues to increase, the popularity of rCHO cells is likely to persist [8, 12, 14, 16, 21]. To maximize the foreign protein productivity in rCHO cell cultures, key environmental factors such as temperature and pH need to be optimized [2, 22]. Despite the popularity of rCHO cells in industry, it is surprising that there are, to date, only a few studies on controlled environmental factors on rCHO cells in suspension culture [21].

Culture temperature is an important environmental parameter that affects the cell growth and foreign protein production of rCHO cells. Like most mammalian cells, the optimal temperature for CHO cell growth is around 37°C. Although lowering the culture temperature below 37°C decreases specific growth rate (μ), it maintains high viability for a longer period [5–7, 9]. Increase in specific productivity (q) is another consequence attainable by lowering the culture temperature. However, this effect on q, unlike μ, is variable among different rCHO clones, suggesting that this particular result of low culture temperature depends on the sites of integration of the foreign gene [22].

Culture pH is another important environmental parameter that affects the cell growth and foreign protein production of rCHO cells [1, 21]. In CHO cells, integration of a transfected foreign gene into the chromosomes occurs almost exclusively through nonhomologous recombination, resulting in randomly distributed integration sites [10]. Because of this fact, the expression of the foreign gene is unpredictable and position-dependent. In addition, the position of the foreign gene in the chromosome may be further altered during methotrexate (MTX)-mediated gene amplification, which results in an increase in the clonal variability of the

rCHO cell population [3, 4, 13]. As observed at low culture temperatures, the effect of culture pH with regards q may also be variable among different rCHO clones.

In this study, we investigated CHO clonal variation while considering the optimal culture pH and temperature for cell growth and antibody production. To accomplish this, two CHO clones, which were isolated from the same parental clone by the limiting dilution method, were cultivated in a bioreactor at various pH levels in the range of 6.8 and 7.6, and two different temperatures, 33°C and 37°C.

MATERIALS AND METHODS

Cell Lines

rCHO cell clones producing a recombinant antibody (clone A and clone B) were used in this study and their establishment is schematically represented in Fig. 1. Parental CHO cells were made by transfection of a vector containing the dihydrofolate reductase (*dhfr*) gene and genes of heavy and light chains into *dhfr*-deficient CHO cells (DG44). Drug selection was carried out by seeding 2×10^3 cells per well in 96-well tissue culture plates (Nunc, Roskilde, Denmark), containing Iscove's modified Dulbecco's medium (IMDM, Invitrogen, Grand Island, NY, U.S.A.) supplemented with 10% dialyzed fetal bovine serum (dFBS, Invitrogen) and 500 μg/ml of G418 (Invitrogen). G418 was used only in the first selection. Among G418 resistant clones isolated in 96-well tissue plates, 29 parental clones based on their antibody concentration were selected for gene amplification and were subjected to increasing levels of MTX (0.02 and 0.08 μM). The highest producing clone at the 80 nM MTX level (C11-0.08) was selected and subjected to subcloning using a limiting dilution method [13]. After subcloning, two clones (clone A and clone B), based on their antibody concentration, were finally selected for this study.

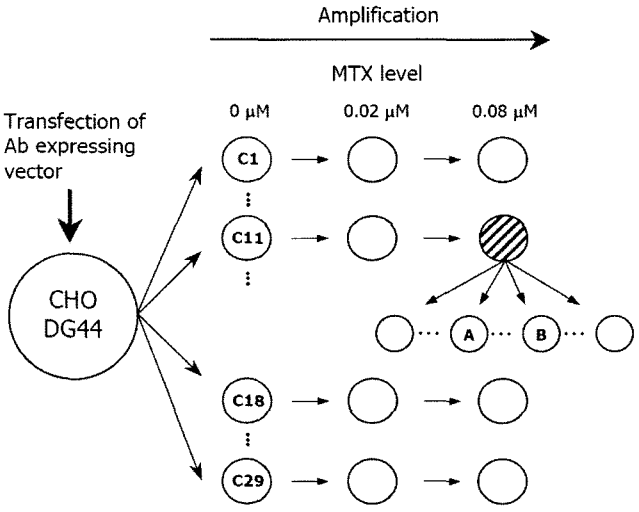

Fig. 1. Schematic representation of the procedure for establishment of clones producing a recombinant antibody.
A clone used for subcloning (C11-0.08) was shaded.

Medium and Culture Maintenance

Proprietary protein-free (PF) medium was used for the suspension culture of rCHO clones. The PF medium was based on IMDM, and its glucose and glutamine concentrations were 4.5 g/l and 5 mM, respectively. rCHO cells were adapted to PF medium containing 0.08 mM MTX, using spinner flasks (Bellco Glass, Vineland, NJ, U.S.A.), for 2 weeks. Cells were maintained as suspension cultures in 125-ml or 500-ml spinner flasks on a magnetic stirrer plate (Bellco Glass) at 80 rpm in a 5%CO_2/air mixture, humidified at 37°C.

Batch Culture

Batch cultures were carried out in a 3-l BIOFLO 110 bioreactor (New Brunswick Scientific, Edison, NJ, U.S.A.). Exponentially growing cells were inoculated at 2×10^5 cells/ml into the bioreactor with a working volume of 1.8 l. The agitation speed was set at 50 rpm, and the dissolved oxygen concentration was controlled to be equivalent to 50% of the air saturation. The culture temperature was controlled at 33°C and 37°C within ±0.1°C, respectively. The culture pH was controlled at various pH values (6.8, 7.0, 7.2, 7.4, and 7.6, respectively) within ±0.02 by addition of 1 M $NaHCO_3$ or CO_2 gas. In all cases, the osmolality of the medium, which was initially 290–300 mOsm/kg, did not exceed 345 mOsm/kg at the end of the cultures. Periodically, approximately 15 ml of the culture medium was taken from the bioreactor. Cell concentration was estimated using the trypan blue dye exclusion method. Culture supernatants, after centrifugation, were aliquoted and kept frozen at −70°C for later analyses.

Analytical Methods

The secreted antibody concentration was quantified by enzyme-linked immunosorbent assay as described previously [11, 13]. Briefly, 96-well plates (Nunc) were coated with anti-human IgG (Sigma) and blocked with bovine serum albumin and Tween 20. The human IgG standard (Sigma) and culture supernatants diluted with blocking buffer were loaded on wells, and treated with horseradish peroxidase-conjugated goat anti-human IgG (Sigma) in diluent solution.

Glucose, lactate, glutamine, and ammonia concentrations were measured using a YSI 7100 multiparameter bioanalytical system (Yellow Spring Instrument, Yellow Springs, OH, U.S.A.).

Evaluation of Specific Growth Rate, Specific Antibody Productivity, Specific Consumption or Production Rates, and Volumetric Productivity

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 antibody productivity (q_{Ab}), and specific consumption or production rates of glucose and lactate 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 [17].

Glutamine spontaneously decomposes, following first-order kinetics, to ammonia and pyrrolidone carboxylate as a function of temperature and pH [15, 19]. The specific glutamine consumption rate (q_{Gln}) and specific ammonia production rate (q_{Amm}) were determined by accounting for the degradation of glutamine with the values of the first-order rate constant k at 33°C (0.0024 h^{-1}) and 37°C (0.0031 h^{-1}), as measured experimentally [15].

Volumetric productivity was calculated by dividing the maximum antibody concentration by the culture time.

RESULTS

To investigate the clonal variation of CHO clones in regard to culture pH and temperature for cell growth and antibody production, serum-free suspension cultures of two CHO clones were performed twice at separate times in a bioreactor with pH and temperature control.

Cell Growth

Fig. 2 shows the typical cell growth and viability profiles during batch cultures of clones A and B. Culture pH significantly affected cell growth and viability at both 33°C and 37°C. At 37°C, both clones started to grow exponentially without a lag phase, regardless of the culture pH, except for pH 6.8. Both clone A and clone B did not grow over 5×10^5 cells/ml at a pH of 6.8, regardless of the culture temperature. At 33°C, both clones grew more slowly than those at 37°C, but they also died more slowly. In general, the culture longevity of clone B, based on cell viability, was longer than clone A.

Fig. 3 shows the μ and maximum viable cell concentration during the batch cultures shown in Fig. 1. In the case of clone A, the highest μ and maximum viable cell concentration

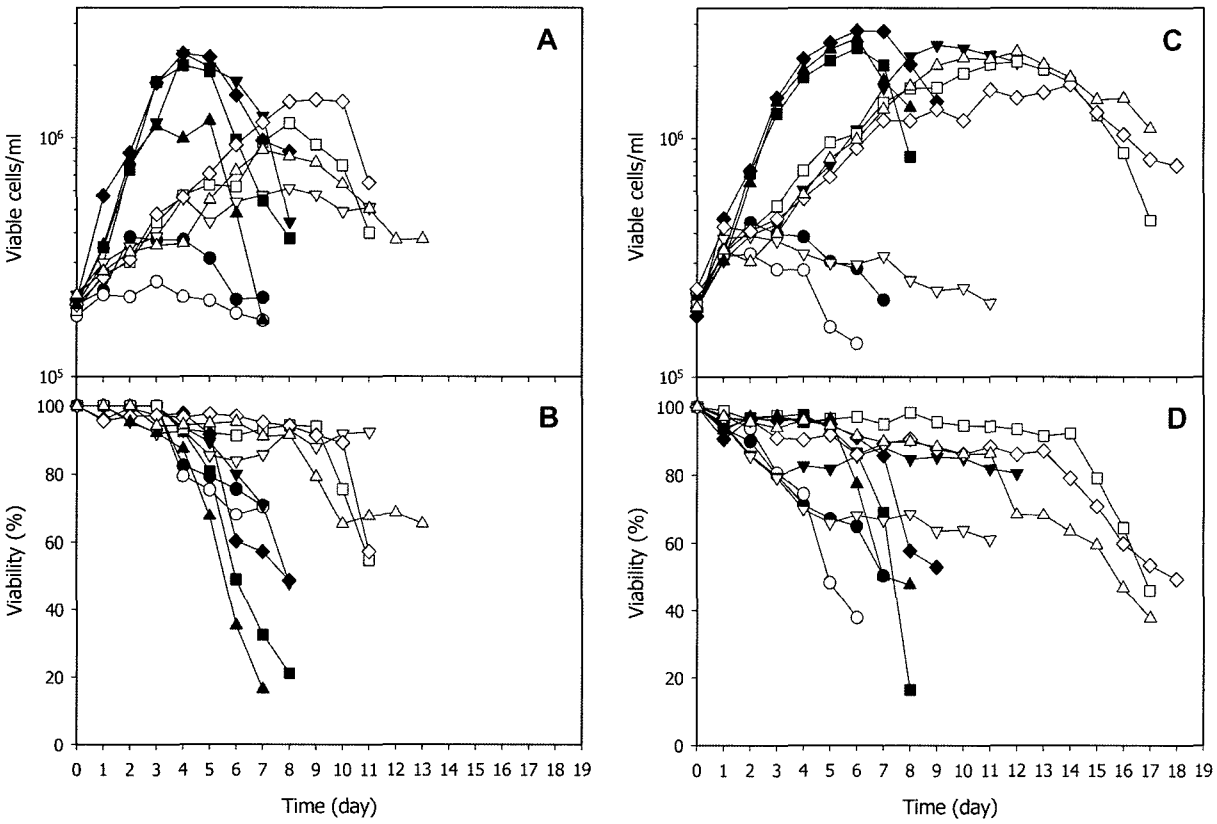

Fig. 2. Cell growth and viability profiles during batch cultures at various culture temperatures and pH (●: pH 6.8, 37°C; ▼: pH 7.0, 37°C; ■: pH 7.2, 37°C; ◆: pH 7.4, 37°C; ▲: pH 7.6, 37°C; ○: pH 6.8, 33°C; ▽: pH 7.0, 33°C; □: pH 7.2, 33°C; ◇: pH 7.4, 33°C; △: pH 7.6, 33°C).

A. Viable cell concentration of clone A. **B.** Cell viability of clone A. **C.** Viable cell concentration of clone B. **D.** Cell viability of clone B.

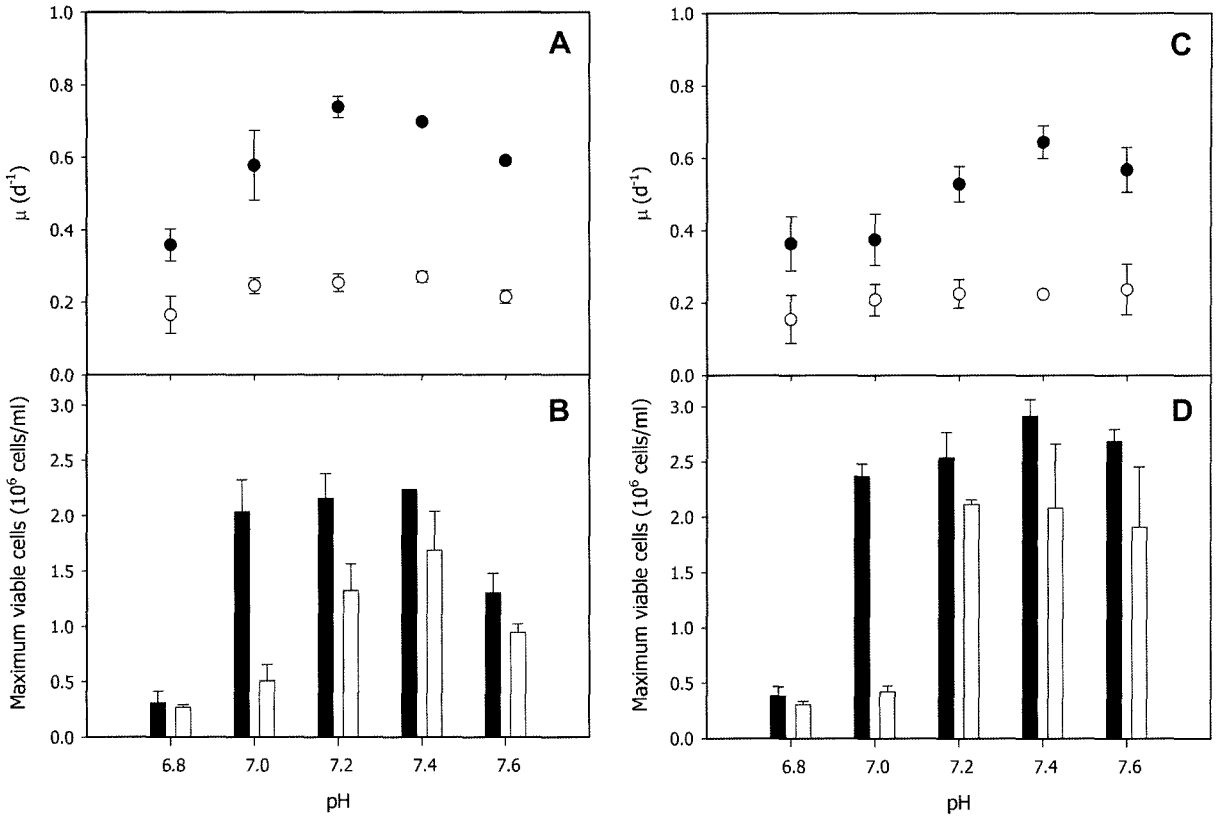

Fig. 3. Specific growth rate and maximum viable cell density in batch cultures at various culture temperatures and pH (●: 37°C; ○: 33°C; black bar: 37°C; gray bar: 33°C).
A. Specific growth rate of clone A. **B**. Maximum viable cell density of clone A. **C**. Specific growth rate of clone B. **D**. Maximum viable cell density of clone B. The error bars represent the standard deviations calculated from the data obtained in two independent experiments.

at 37°C were obtained at pH 7.2 and pH 7.4, respectively. The μ values at 33°C were significantly lower than those at 37°C and almost constant regardless of the culture pH, whereas the maximum viable cell concentration was affected by the culture pH. In the case of clone B, μ and maximum viable cell concentration at 37°C were higher at pH 7.4 than those at the other pH values. Despite the relatively low μ (0.375±0.071 d^{-1}) at pH 7.0, the

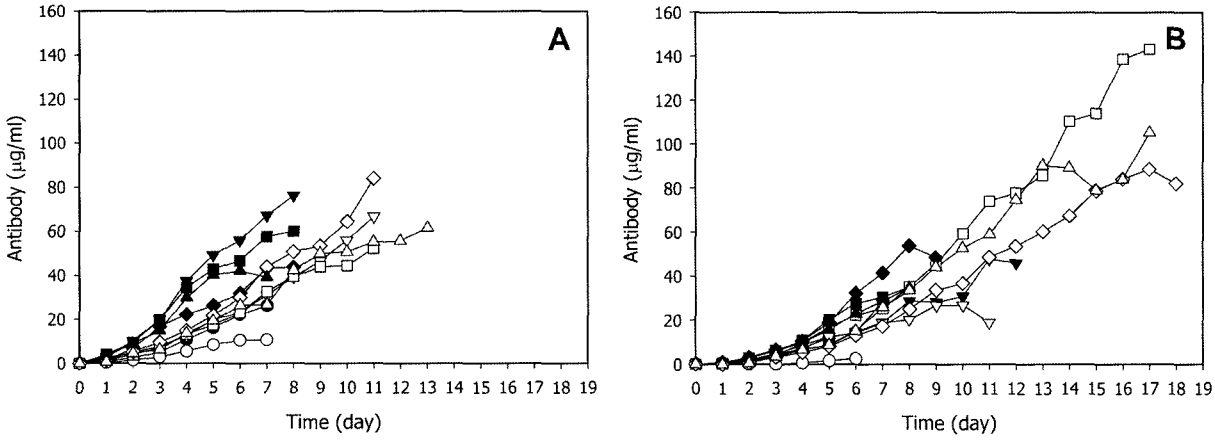

Fig. 4. Antibody production profiles during batch cultures at various culture temperatures and pH (●: pH 6.8, 37°C; ▼: pH 7.0, 37°C; ■: pH 7.2, 37°C; ◆: pH 7.4, 37°C; ▲: pH 7.6, 37°C; ○: pH 6.8, 33°C; ▽: pH 7.0, 33°C; □: pH 7.2, 33°C; ◇: pH 7.4, 33°C; △: pH 7.6, 33°C).
A. Clone A. **B**. Clone B.

maximum viable cell density of (2.37±0.11)×10^6 cells/ml was obtained. As with clone A, μ values at 33°C of clone B were significantly lower than those at 37°C and almost constant regardless of the culture pH, whereas the maximum viable cell density was affected by the culture pH. Clone B showed higher maximum viable cell concentrations than clone A at various culture conditions except for pH 6.8.

Antibody Production

Fig. 4 shows the typical antibody concentration profiles during the batch cultures shown in Fig. 2. Regardless of culture temperature, the culture pH significantly affected antibody production of both clones. The optimal pH for antibody production was different from that for cell growth. At 37°C, the highest maximum antibody concentration of clone A obtained at pH 7.0 (73.2±4.1 μg/ml) was higher than that of clone B (53.2±7.8 μg/ml) obtained at pH 7.0. However, at 33°C, the highest maximum antibody concentration of clone B obtained at pH 7.2 (132.6±14.9 μg/ml) was higher than that of clone A (82.0±2.6 μg/ml) at pH 7.4. Unlike clone A, clone B showed an enhanced antibody production at low culture temperature, which was due to not only the prolonged culture longevity but also to the elevated q_{Ab}. In the case of clone A, q_{Ab} at 33°C was not higher than q_{Ab} at 37°C except for pH 7.4, whereas q_{Ab} of clone B at 33°C was higher than that at 37°C except for pH 6.8 (Figs. 5A and 5C). As a result, in the case of clone B, an approximately 2.5-fold increase in maximum antibody concentration was achieved by lowering the culture temperature, whereas no significant increase in maximum antibody concentration was achieved in the case of clone A (Figs. 5B and 5D).

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, whereas glutamine uptake was accompanied by a corresponding accumulation of ammonia (data not shown).

As shown in Fig. 6, q_{Glc} and q_{Lac} of clones A and B were significantly influenced in a similar fashion, but to a different degree, by culture temperature and pH. They decreased by lowering the culture temperature and pH, and the effect of culture pH on q_{Glc} and q_{Lac} was more significant

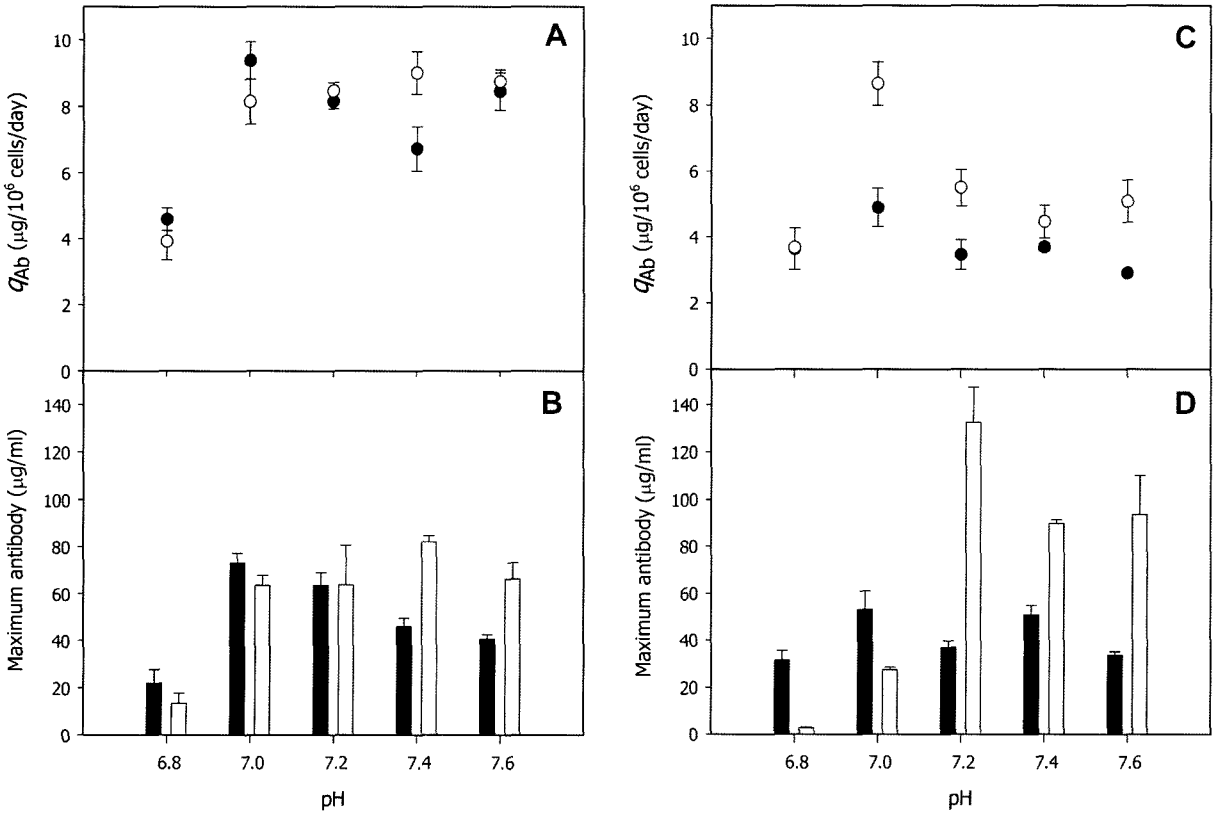

Fig. 5. Specific antibody productivity and maximum antibody concentration in batch cultures at various culture temperatures and pH (●: 37°C; ○: 33°C; black bar: 37°C; gray bar: 33°C).

A. q_{Ab} of clone A. **B.** Maximum antibody concentration of clone A. **C.** q_{Ab} of clone B. **D.** Maximum antibody concentration of clone B. The error bars represent the standard deviations calculated from the data obtained in two independent experiments.

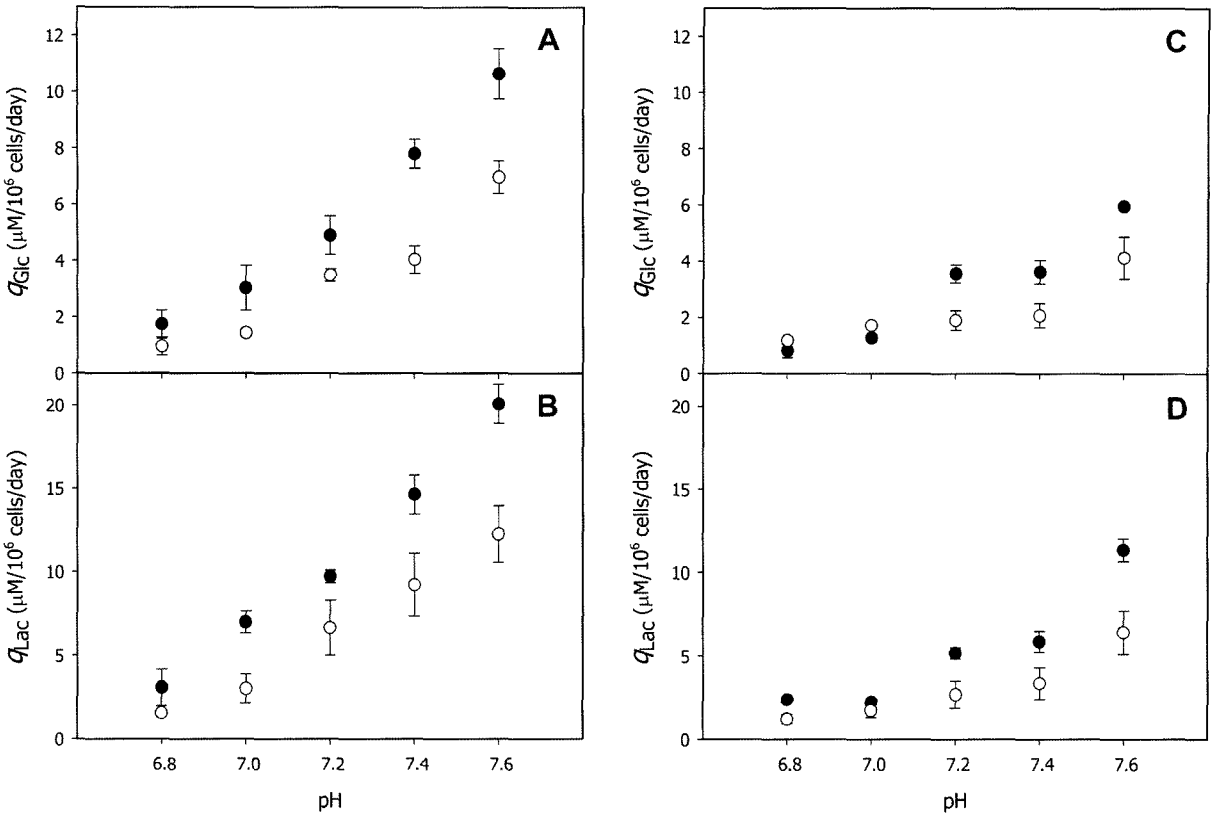

Fig. 6. Specific glucose consumption rate and specific lactate production rate in batch cultures at various culture temperatures and pH (●: 37°C; ○: 33°C)

A. q_{Glc} of clone A. **B.** q_{Lac} of clone A. **C.** q_{Glc} of clone B. **D.** q_{Lac} of clone B. The error bars represent the standard deviations calculated from the data obtained in two independent experiments.

at 37°C than at 33°C. In addition, q_{Glc} of clone A was, in general, higher than that of clone B. The q_{Glc} of clone A at pH 7.2–7.6 and 37°C was approximately 1.8-fold higher than that of clone B.

Like q_{Glc}, the q_{Lac}, q_{Gln}, and q_{Amm} of both clones appeared to decrease by lowering the culture temperature and pH, and the effect of culture pH on q_{Gln} was also more significant at 37°C than that at 33°C. In addition, the q_{Gln} of clone A was higher than that of clone B. The q_{Gln} of clone A at pH 7.2–7.6 and 37°C was approximately 1.9-fold higher than that of clone B.

Volumetric Productivity

A maximization of volumetric productivity is one of the key objectives when developing a culture process. Volumetric productivity was calculated by dividing the maximum antibody concentration by the culture time.

Fig. 8 shows the volumetric productivities at various culture conditions. In the case of clone A, the highest volumetric productivity (8.3±1.8 μg/ml/day) was achieved at 37°C and pH 7.0. The volumetric productivity was not enhanced by lowering temperature, owing to the extended culture time. In contrast, clone B had the highest volumetric productivity at 33°C and pH 7.2 (7.3±1.6 μg/ml/day), which is approximately 1.7-fold higher than that at 37°C and pH 7.2.

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 need to be optimized. Since CHO clones may have different optimal environmental conditions, the optimization process is performed for each clone. However, it takes easily several months because of slow cell growth. The understanding of the clonal variability of rCHO cells in response to environmental parameters such as pH and temperature, which has not been reported yet, may facilitate this optimization process.

To accomplish this, serum-free suspension cultures of two CHO clones were performed in a bioreactor with pH and temperature control. Since these two clones were isolated from the same parental clone, their response to

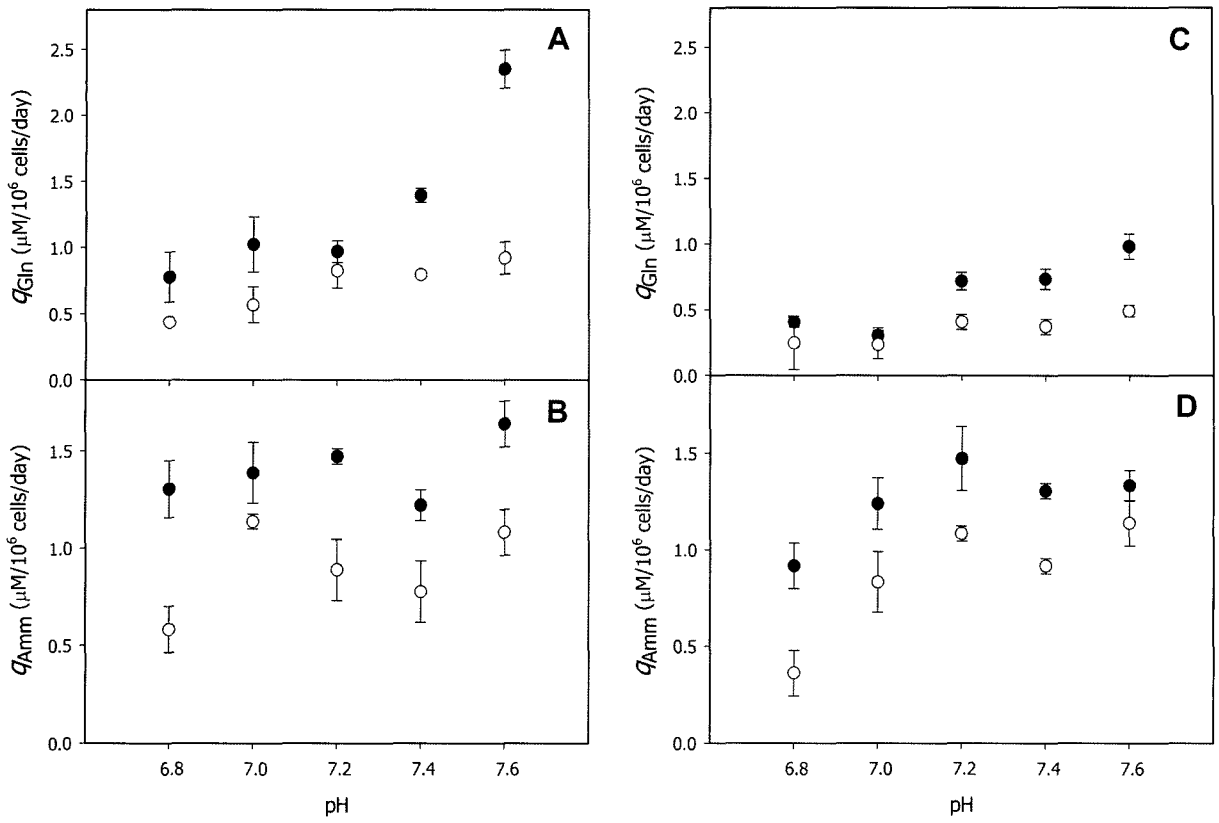

Fig. 7. Specific glutamine consumption rate and specific ammonia production rate in batch cultures at various culture temperatures and pH (●: 37°C; ○: 33°C).
A. q_{Gln} of clone A. **B.** q_{Amm} of clone A. **C.** q_{Gln} of clone B. **D.** q_{Amm} of clone B. The error bars represent the standard deviations calculated from the data obtained in two independent experiments.

environmental parameters was expected to be more or less similar.

With regard to cell growth, clone A and clone B displayed a similar response to temperature, although their degree of response differed. When the culture temperature was lowered from 37°C to 33°C, the cell growth was depressed, cell culture longevity improved, and the effect of pH on μ became less significant. In addition, the culture pH resulting in the highest maximum viable cell concentration shifted from 7.0–7.4 to 7.4 for clone A and from 7.0–7.6 to 7.2–7.6 for clone B.

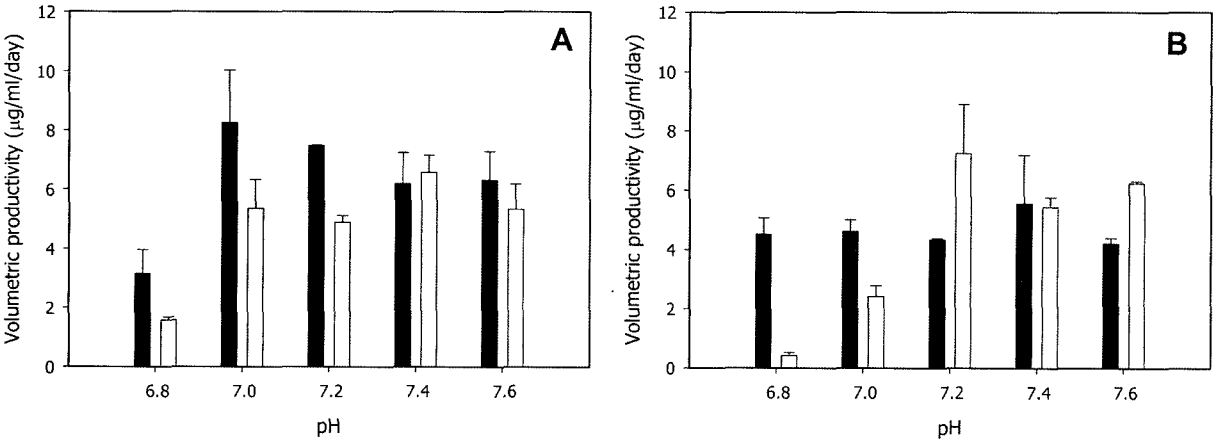

Fig. 8. Volumetric productivity in batch cultures at various culture temperatures and pH (black bar: 37°C; gray bar: 33°C).
A. Clone A. **B.** Clone B. The error bars represent the standard deviations calculated from the data obtained in two independent experiments.

Unexpectedly, clones A and B displayed different responses to temperature in regard to antibody production. In the case of clone B, an approximately 2.5-fold increase in maximum antibody concentration was achieved by lowering the culture temperature, whereas no significant increase in maximum antibody concentration was achieved in the case of clone A. The enhanced maximum antibody concentration of clone B at 33°C was due to not only enhanced q_{Ab} but also to prolonged culture longevity. At 33°C, the culture longevity of clone A also improved, but not as much as that of clone B.

A time integral of viable cells in batch culture is a parameter used for quantification of cell growth and culture longevity [17, 18]. When the time integral of viable cells was calculated at the cell viability of less than 50%, its value of clone B at 33°C and pH 7.2 was approximately 1.7-fold higher that that of clone A at 33°C and pH 7.4. Thereby, the highest maximum antibody concentration of clone B obtained at 33°C and pH 7.2 (132.6±14.9 μg/ml) was higher than that of clone A obtained at 33°C and pH 7.4 (82.0±2.6 μg/ml). In addition, the highest volumetric productivity of clone B obtained at 33°C and pH 7.2 was compatible to that of clone A obtained at 37°C and pH 7.0.

A high time integral value of viable cells of clone B is likely owing their efficient metabolism. In most culture conditions, the metabolic rates (q_{Glc}, q_{Lac}, q_{Gln}, and q_{Amm}) of clone B were significantly lower than those of clone A, suggesting that clone B consumed glucose and glutamine as an energy source more slowly and efficiently than clone A. Thus, these metabolic rates should also be considered as one of the important criteria for CHO clone selection.

In conclusion, two CHO clones, although isolated from the same parental clone, displayed quite different responses to culture temperature with regards antibody production. Their metabolic rates were also different. Thus, for maximization of antibody production, environmental parameters such as temperature and pH need to be optimized for each CHO clone.

Acknowledgments

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