Cytotechnology **30:** 95–105, 1999.

Influence of bcl-2 on antibody productivity in high cell density perfusion cultures of hybridoma

D. Fassnacht[1], S. Rössing[1], R. P. Singh[2], M. Al-Rubeai[2] & R. Pörtner[1]
[1] *Technische Universität Hamburg-Harburg, Bioprozeß- und Bioverfahrenstechnik, Denickestraße 15, D-21071 Hamburg, Germany*
[2] *Centre for Bioprocess Engineering, School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham, B15 2TT, U.K.*

Received 7 April 1998; accepted 25 November 1998

Key words: apoptosis, Bcl-2, fixed-bed, hollow fibre, hybridoma, perfusion, protein-free medium

Abstract

Apoptosis is an active, genetically determined death mechanism which can be induced by a wide range of physiological factors and by mild stress. It is the predominant form of cell death during the production of antibodies from murine hybridoma cell lines. A number of studies have now demonstrated that the suppression of this death pathway, by means of over-expression of survival genes such as bcl-2, results in improved cellular robustness and antibody productivity during batch culture. In the present study, the influence of bcl-2 expression on hybridoma productivity in two high density perfusion bioreactor systems was investigated. In the first system, a fixed-bed reactor, the DNA content in the spent medium was 25% higher in the control (TB/C3-pEF) culture than that found in the bcl-2 transfected (TB/C3-bcl2) cultures at all perfusion rates. This is indicative of a higher level of cell death in the control cell line. The average antibody concentration for the TB/C3-pEF cell line was 14.9 mg L^{-1} at perfusion rates of 2.6 and 5.2 d^{-1}. However, for the TB/C3-bcl2 cell line it was 33 mg L^{-1} at dilution rates of 2 and 4 d^{-1}. A substantial increase in antibody concentration was also found in the Integra Tecnomouse hollow fibre reactor. The antibody titre in the TB/C3-bcl2 cassette was nearly 100% higher than that in the TB/C3-pEF cassette during the cultivation period which lasted 6 weeks. Clearly, these results demonstrate the positive impact of bcl-2 over-expression on production of antibody in hybridoma perfusion cultures.

Introduction

Suspension batch and fed batch cultures in stirred tank bioreactors are the preferred choice for the commercial production of biopharmaceuticals from mammalian cell lines at industrial scale. Increased production may be achieved by increasing the scale of operation, which invariably increases the capital cost of the process. Alternatively, productivity may be raised by means of culture intensification. This approach utilises a variety of techniques which allow retention of cells in the bioreactor, whilst the culture is perfused with fresh medium. Waste medium is removed at the same rate, thus maintaining a constant low culture volume. As a result very high cell densities and product titres can be achieved in a relatively small bioreactor. A wide variety of intensive culture systems have been developed, including spin-filter perfusion bioreactors, dialysis systems, hollow fibre devices and fixed-bed reactors (Himmelfarb, 1969; Tharakan and Chau, 1986; Comer et al., 1990; Ong et al., 1994; Jan et al., 1992; Amos et al., 1994; Emery et al., 1995; Pörtner et al., 1997; Fassnacht et al., 1998b).

One of the characteristic features of these systems is the presence of large numbers of dead cells, which limits culture performance in two ways. Obviously, there will be a need for high rates of cell division in order to replace lost cells, which invariable reduces the specific protein productivity (Al-Rubeai and Emery, 1990; Al-Rubeai et al., 1992). Secondly, release of proteases, DNA and other cellular components will lead to biopharmaceutical degradation and

will complicate down-stream processing.

A number of studies have now shown that most of the stresses that the cell encounters in the bioreactor environment lead to the induction of the active, genetically determined mechanism of death called apoptosis. These stresses include deprivation of glucose, serum, glutamine, essential amino acids and exposure to toxic metabolites such as ammonia (Al-Rubeai et al., 1990; Franěk and Dolníková, 1991; Kerr et al., 1972; Mercille and Massie, 1994; Singh et al., 1994; Simpson et al., 1998; Westlund and Haggstrom, 1998; reviewed by Singh et al., 1996b). In response to these stresses, the cell engages a biochemical pathway comprising of a protease cascade, which leads to the induction of a range of enzymes which are responsible for its death and destruction (reviewed by Cohen, 1997). As a result of the controlled nature of the death, the dead cell retains a high degree of structural integrity, which is essential when apoptosis occurs in-vivo, thus providing phagocytic cells with the opportunity to engulf the dead cell before lysis and leakage of contents onto surrounding cells occurs. As a result, the inflammatory response associated with necrotic death (which is induced by overwhelming damage) is avoided.

Because of its key importance in embryonic development in numerous disease states, the study of apoptosis has attracted considerable attention in the last few years. As a consequence, rapid progress has been made in our understanding of the regulation and expression of this form of death, and a growing number of workers have applied this knowledge to the control of apoptosis during the cultivation of industrially important cell lines (Itoh et al., 1995; Singh et al., 1996; Simpson et al., 1997; Suzuki et al., 1997; Mitchell-Logean and Murhammer, 1997; Fassnacht et al., 1998a; Kim et al., 1998) The majority of these studies have investigated the influence of one of the most widely studied anti-apoptosis genes, namely bcl-2 (Tsujimoto et al., 1985). This 27 kDa membrane associated protein has been reported to suppress the induction of the apoptosis induced in a wide of cell types in response to numerous agents (Yang et al., 1996), although its activity is by no means universal. In the present study, we consider the influence of the bcl-2 on hybridoma culture viability and antibody productivity in two intensive culture systems, a fixed-bed bioreactor (Meredos, Germany) and the Integra Tecnomouse hollow fibre system.

Materials and methods

Cell lines

NS1-derived mouse hybridoma TB/C3 was transfected either with the control vector pEF-MClneopA to give the control cell line TB/C3-pEF, or with the bcl-2 expression vector pEF bcl2-MClneopA to give the bcl-2 positive cell line TB/C3-bcl2. For a description of transfection and selection protocols, see Simpson et al. (1997). Both lines produce IgG antibodies against the hapten Cγ2 domain in the Fc region of human IgG. Expression of bcl-2 was monitored regularly by immuno-staining and flow cytometry (Simpson et al., 1997).

Culture media

Fixed-bed reactor
1:1 mixture of Iscove's MDM and Ham's F12 supplemented with 2 mmol L^{-1} L-glutamine, 2 g L^{-1} $NaHCO_3$, 0.01% PEG and 50 mmol L^{-1} ethanolamine was used as a basal medium. This medium was supplemented with 1% (v/w) of a protein-free iron-rich supplement (IR) containing 20 mmol L^{-1} ferric citrate (Franěk and Dolníková, 1991). The total amounts of glutamine and glucose in both media were 3 mmol l^{-1} and 15 mmol L^{-1}, respectively. Cells from 175 cm^2 stationary flasks were used to inoculate the fixed-bed reactor.

Hollow fibre system
RPMI-1640 medium was used as a basal medium (Gibco BRL, Scotland, U.K.) and supplemented with 5% foetal calf serum (Advanced Protein Products Ltd., West Midlands, U.K.), penicillin at 100 UI mL^{-1}, and streptomycin at 100 μg mL^{-1} (Gibco BRL, Scotland, U.K.).

Fixed-bed reactor

The fixed-bed reactor (Meredos, Germany) used in this study consisted of a 100 mL fixed-bed in which the cells were immobilised on porous carriers (Figure 1). During operation, the medium was constantly pumped from a conditioning vessel to the fixed-bed. Bubble aeration took place in the conditioning vessel to supply the cells with oxygen. The exhausted gas was cooled before leaving the system to retain moisture. Dissolved oxygen was measured (Ingold, Germany) in the conditioning vessel as well as at the outlet of the fixed-bed to determine the oxygen consumption

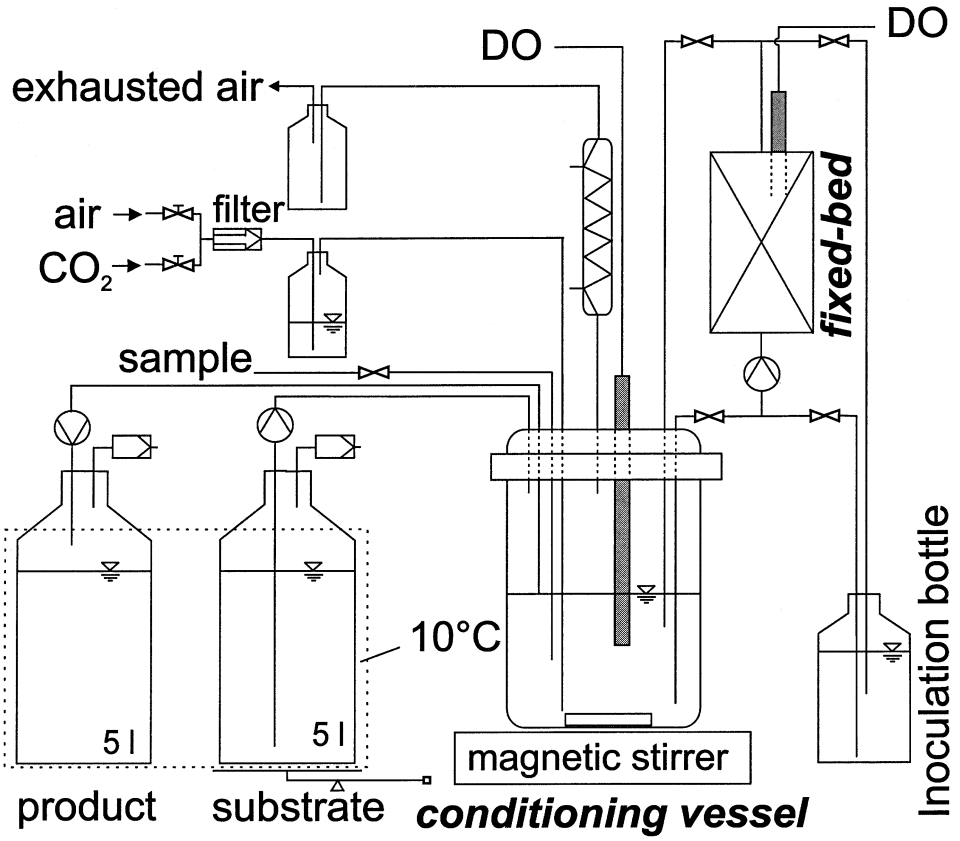

Figure 1. Experimental set-up of the fixed-bed reactor system. The system consists of a perfusion loop of fresh substrate and product, a fixed-bed loop of the medium circulating from the conditioning vessel through the fixed-bed and back, and an inoculation circuit to immobilise the cells at the beginning of the experiment.

rate of the cells. The dissolved oxygen at the outlet was controlled to 40% of air saturation by regulating the pump rate through the fixed-bed. The maximum superficial flow velocity in the fixed-bed was kept below 1.1 mm s^{-1} in order to prevent cells from being washed out of the fixed-bed. The superficial flow velocity was determined by dividing the volumetric flow rate by the cross sectional area of the fixed-bed. The pH was measured (electrode CPS11-1DA4GSA, Endress + Hauser, Germany) and controlled automatically by addition of CO_2. The medium in the conditioning vessel was agitated (approx. 250 rpm) by a conventional magnetic stirrer to prevent protein sedimentation and heated to 37 °C by an electrical heater. No separate heating was required for the fixed-bed because it was integrated into the conditioning vessel (Figure 2). Sampling was achieved from the conditioning vessel and the medium exchange during continuous operation.

The carriers used were porous glass beads with a diameter of 3 to 5 mm (Siran, Schott, Germany) in which the cells were immobilised. This carrier type proved to be especially suitable for the cultivation of hybridoma cell lines.

During inoculation the circulation between the conditioning vessel and the fixed-bed was closed. The suspended cells were filled into a bottle which was connected to the fixed-bed in a separate circulation. The cells were then slowly pumped for approximately 2 hr through the fixed-bed until the inoculation bottle turned clear. The inoculation circuit was then closed and the circulation between the conditioning vessel and the fixed-bed reopened. The fixed-bed was inoculated with 1×10^6 cells per mL of fixed-bed volume which corresponded to a cell density of 1×10^5 cells per mL of medium.

Glucose and lactate were automatically determined using YSI Analyser 2700. Glutamine and ammonia were analysed enzymatically. Monoclonal antibody was determined by a sandwich enzyme-linked im-

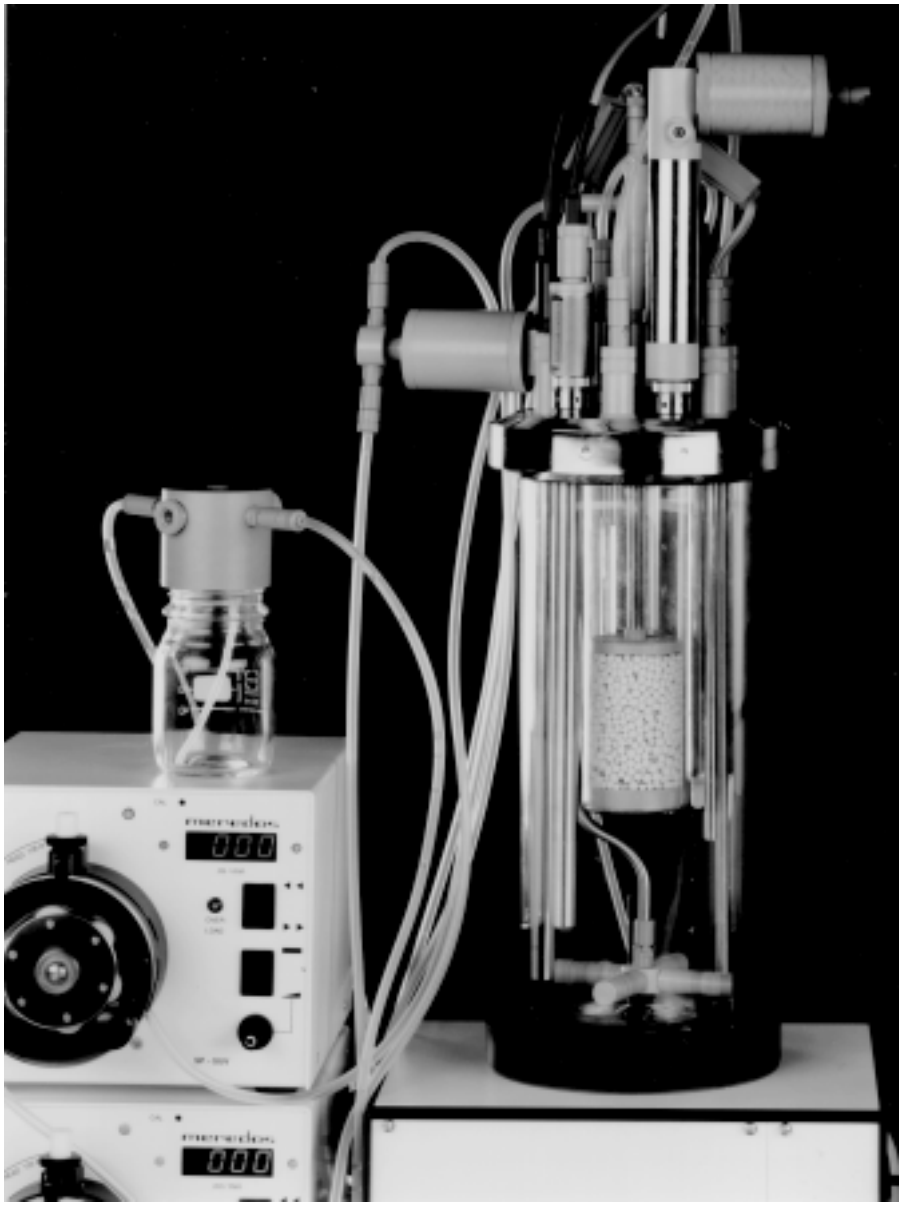

Figure 2. Photograph of the fixed-bed reactor (meredos, Germany). The fixed-bed (100 mL) is integrated into the conditioning vessel (1 L) for easy temperature control of the fixed-bed and convenient handling of the whole system. The inoculation bottle is shown on the left.

munosorbent assay (ELISA) using human IgG coated plates and sheep anti-mouse IgG peroxidase conjugate as the second antibody. The optical density at 490 nm was recorded by an ELISA reader after treating the plates with the peroxidase substrate, o-phenylenediamine dihydrochloride. All chemicals were obtained from Boehringer Mannheim, Germany. The total DNA content in the supernatant was determined with Hoechst-33258 (Sigma, Germany) staining. 100 μg of H-33258 was dissolved in 1 mL of Ca^{2+}- and Mg^{2+}-free PBS. 13 μL of this solution was then added to 250 μL of the sample and 1050 μL of a Tris-buffer (250 mmol L^{-1} Tris, pH adjusted to 8.0 with phosphoric acid). The fluorescence of the DNA-flourochrome complex, excited at 353 nm, was measured at 460 nm on a spectrofluorometer (Shimadzu, RF-540). The amount of DNA was evaluated using a calibration curve. The total cell number and

membrane intact index (percentage of membrane intact cells) were evaluated using a haemocytometer and trypan blue exclusion.

The following equations were applied for the fixed-bed reactor:

D is the dilution rate of medium in the conditioning vessel, c_i is the substrate or product concentration, c_{i0} is the substrate or product feed concentration and q_i^* is the substrate uptake or metabolite production rate per volume conditioning vessel.

$$\frac{\partial c_i}{\partial t} = D \cdot (c_{i0} - c_i) \pm q_i^* \qquad (1)$$

The dilution rate and the substrate consumption or metabolite production rates per volume fixed-bed are defined in Equation (2). V_C is the volume of the conditioning vessel and V_{FB} is the volume of the fixed-bed.

$$D_{FB} = \frac{V_C}{V_{FB}} \cdot D \qquad q_{i,FB}^* = \frac{V_C}{V_{FB}} \cdot q_i^* \qquad (2)$$

Using Equations (1) and (2), the following Equation (3) are then obtained for a steady state.

$$\frac{\partial c_i}{\partial t} = 0 \Rightarrow \begin{array}{l} D \cdot (c_{i0} - c_i) = \mp q_i^* \\ D_{FB} \cdot (c_{i0} - c_i) = \mp q_{FB,i}^* \end{array} \qquad (3)$$

Hollow fibre system

Hollow fibre studies were carried out using a Tecnomouse bioreactor (Integra Biosciences, U.K.) with two OxyCell hollow fibre cassettes (Figure 3), one for each cell line. Aeration was achieved using a mixture of 5% CO_2/air. Medium was recirculated through the cassette at a flow rate of 100 mL hr^{-1} from a 2 L reservoir bottle. Samples were harvested from the extra-capillary space at weekly intervals and cell number, viability and antibody titre assayed as described above. Glucose levels were determined using a Reflolux II® system (Boeringer, Germany). The medium bottle was replaced at regular intervals such that the glucose concentration remained above 3 mM.

Results

Cell culture in the fixed-bed reactor

The cell lines TB/C3-pEF and TB/C3-bcl2 were compared in high cell-density fixed-bed reactors in order to assess the potential of bcl-2 during sub-optimal conditions. The experiments were run in continuous operation at low dilution rates to put further stress on the cells. As emphasis was put on accurate steady-states, each dilution rate was carried on for at least 20 days. Figure 4 shows the glucose and lactate concentration over the time the experiment with the control cell line TB/C3-pEF (top diagram) and the bcl-2 over-expressing cell line TB/C3-bcl2 (bottom diagram).

Following inoculation, the fixed-bed reactor was run for both cell lines for 4 days in batch mode until glucose was almost exhausted, at which time continuous operation was initiated. For the TB/C3-pEF cell line a first steady-state at the dilution rate of 3.9 d^{-1} was achieved after 24 days. The dilution rate was then reduced to 2.6 d^{-1} and a new steady-state was reached on the 37th day. At this point the recirculation pump malfunctioned and was non operational for two days. As shown in Figure 4 (TB/C3-pEF), the resultant hypoxic conditions led to the production of a high level of lactate. Consequently, the culture took in excess of 20 days to return to the steady-state. The dilution rate was then changed to 5.2 d^{-1} and a new steady-state was quickly reached. In the experiment using the TB/C3-bcl2 cell line the first steady-state was obtained after 27 days at a dilution rate of 4 d^{-1}. On the 19th day air was pumped through the fixed-bed due to excessive agitation and aeration which resulted in increased cell death. The problem was immediately rectified but it took 8 days for the system to recover. On the 27th day the dilution rate was set at 2 d^{-1} and a steady-state was reached on the 63rd day.

Cells were removed from both experiments at the end of the cultivation, and the bcl-2 content was analysed by flow cytometry (FITC, data not shown). Both cell lines revealed the same concentration as at the beginning of the each experiment which proved the stable transfection of the cells.

As an indicator for the cell activity, the glucose consumption rates $q_{FB,GLC}^*$ at the steady-states were compared (Figure 5). For both cell lines the values increased comparable with increasing dilution rates, and the glucose consumption of both cell lines were almost identical at the lowest dilution rate D_{FB}. If bcl-2 had a striking effect on the cell activity, it would have been especially evident on such an unfavourable condition.

The glutamine concentrations reached a level between 0.5 and 1 mmol L^{-1} at all steady-states of both cell lines, while ammonia reached non-inhibiting con-

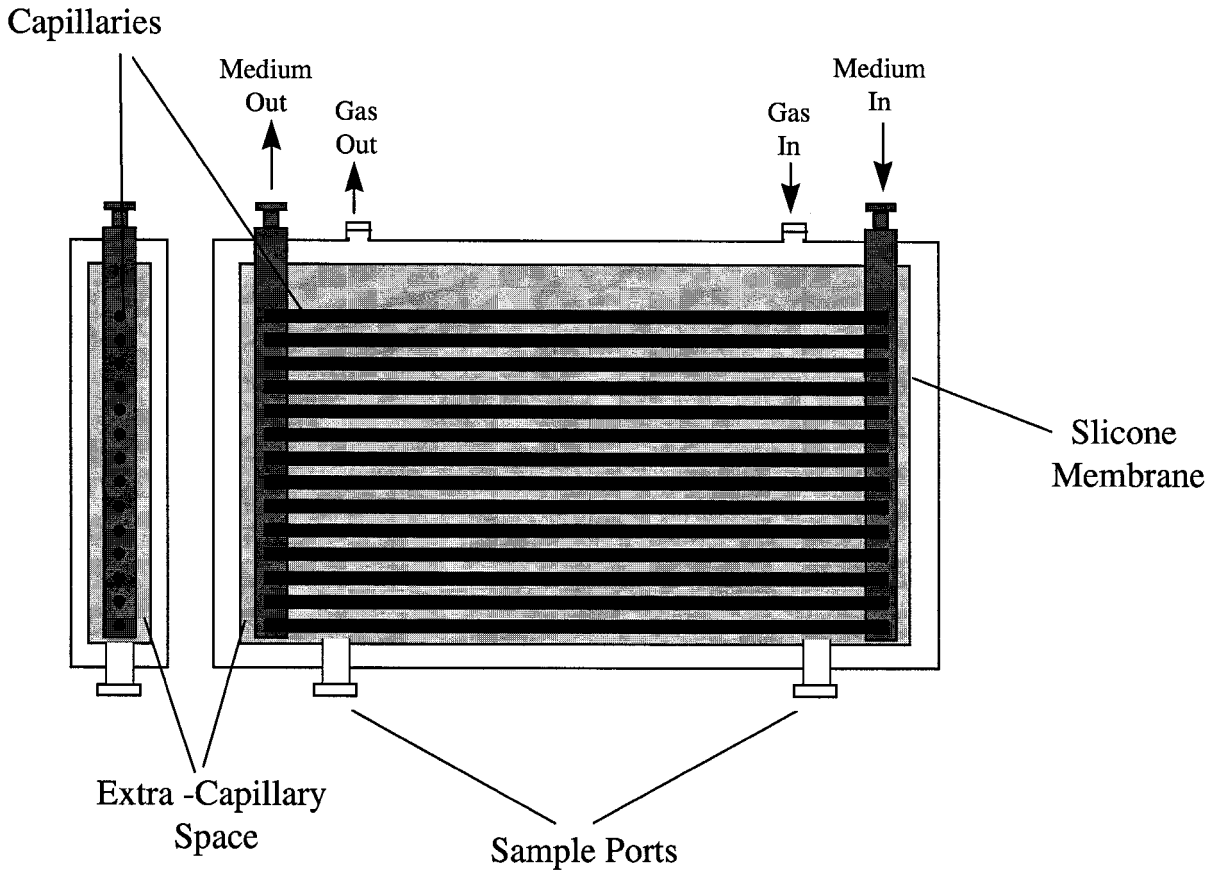

Figure 3. Schematic diagram of the Integra Technomouse hollow fibre culture cassette.

centrations of between 2.5 and 3.5 mmol L^{-1} at all steady-states (data not shown).

The amount of DNA released by damaged cells into the culture medium was used to estimate cell death (comparable to the LDH release assay). Figure 6 shows the DNA concentration in the supernatant of both cell lines as a function of the dilution rate. For each cell line the DNA concentration remained constant with increasing dilution rates which corresponds to a linear increase of the DNA release rate ($q^*_{FB,DNA}$). It can be assumed that this increase is due to an increasing cell density within the fixed-bed. Comparing both cell lines revealed that the DNA concentration of the control cell line TB/C3-pEF was approximately 25% higher than that of the TB/C3-bcl2 cells, indicating significantly higher levels of cell death in the former.

The antibody concentration at the steady-states provided the most striking difference between the two cell lines in the fixed-bed reactors (Figure 7). At all steady-states the antibody titre during the run with TB/C3-bcl2 cells was nearly double that obtained in the control cell line culture.

Cell cultivation in the hollow fibre reactor

Productivity of the cell lines was also assessed at very high cell density in the Tecnomouse Hollow Fibre System (Nagel et al., 1994). As shown in Figure 3, this system allows direct aeration of the cells across the silicone membrane of the reactor, and thereby separates the aeration from the medium supply.

When the hybridoma cell lines were cultured in the system the non-attached viable cell number remained rather low and erratic (data not shown). This may reflect a characteristic of the cell line under study, which appears to attach particularly well to the silicone membrane used to supply oxygen to the cells in the cassette. Flourescence microscopic analysis of nuclear morphology revealed the presence of apoptotic cells and large numbers of chromatin free 'ghosts' thus

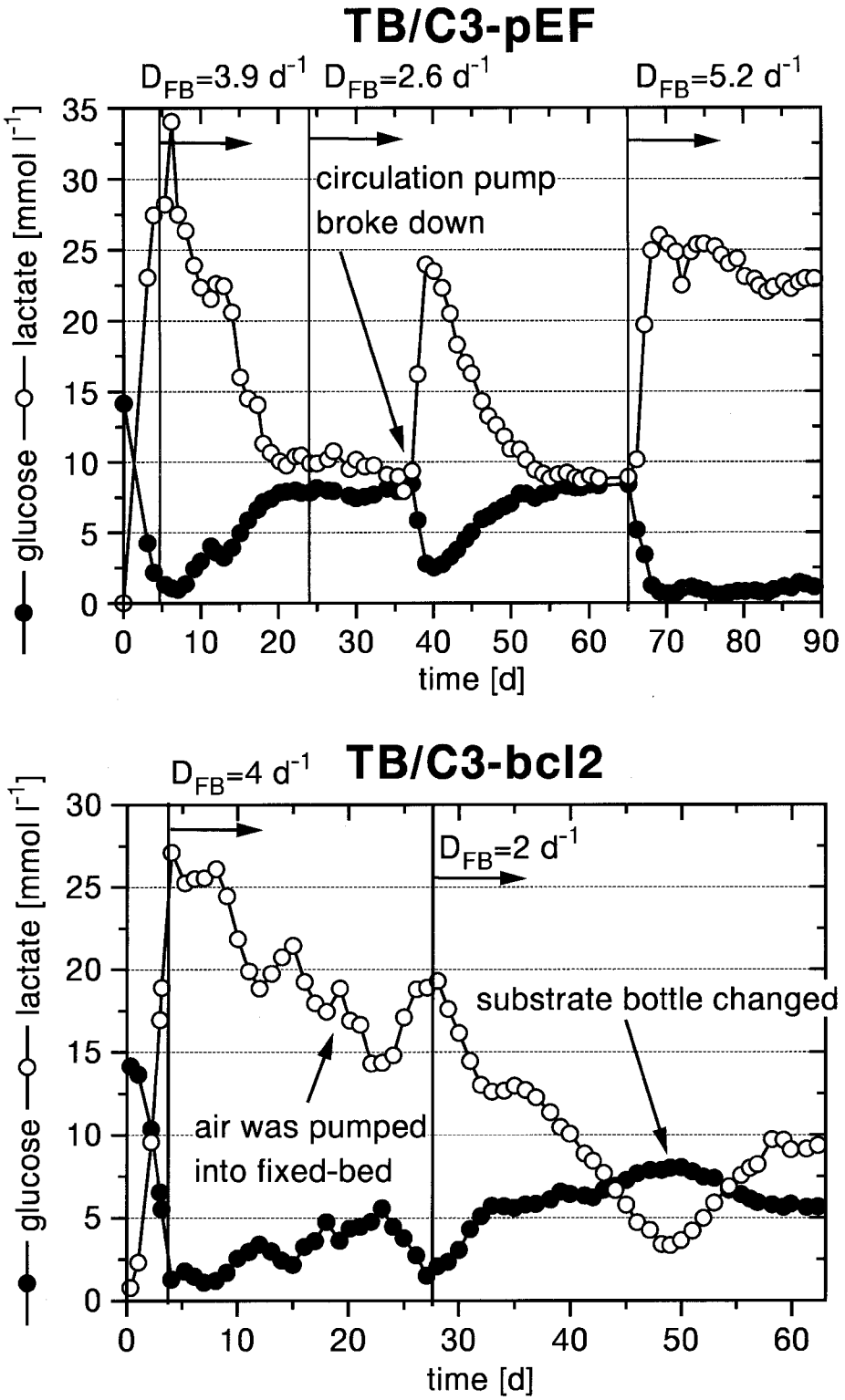

Figure 4. Fixed-bed cultures in continuous operation. Glucose (closed circles) and lactate (open circles) concentrations for the cell lines TB/C3-pEF (top) and TB/C3-bcl2 (bottom). Steady-states were obtained for several dilution rates D_{FB}.

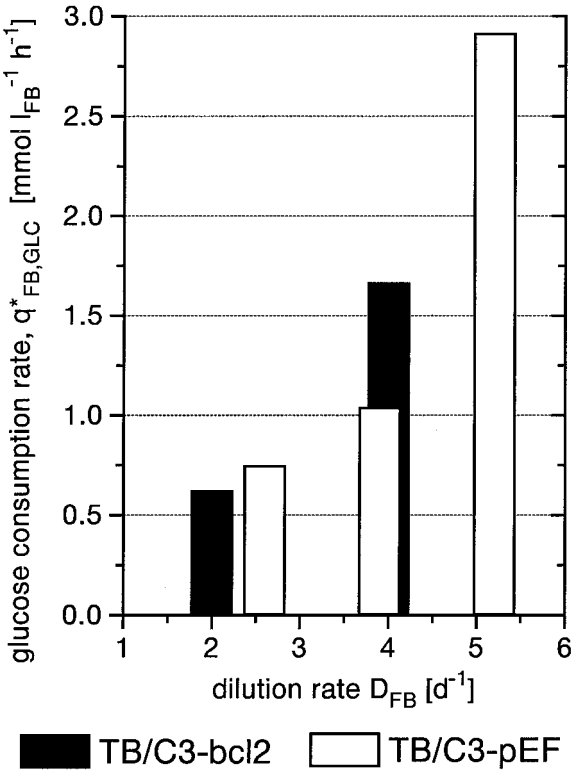

Figure 5. The glucose consumption rate $q^*_{GLC,FB}$ (Equation 2) at each steady-state of the fixed-bed reactor for both cell lines at various perfusion rates D_{FB}.

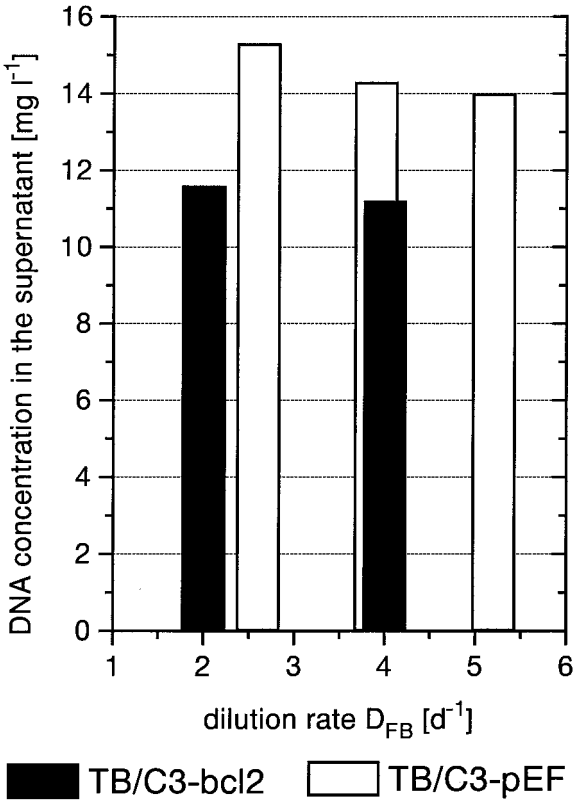

Figure 6. DNA-content in the supernatant at each steady-state in the fixed-bed reactor for both cell lines at various perfusion rates D_{FB}.

confirming that apoptosis was the major form of cell death in the system.

The culture cassettes were perfused with basal medium recirculated from a 2 L reservoir bottle. The glucose level in the medium was monitored regularly, and the reservoir bottle changed such that the glucose level remained above 3 mM. Antibody titre in the harvests is shown in Figure 8. At the end of the run, the bcl-2 cassette harvest antibody titre was about twice that of the control cultures, a result which is consistent with the findings in the fixed-bed reactor.

Discussion

The over-expression of the bcl-2 gene has been shown to increase the robustness of a range of cell lines under culture conditions relevant to large scale protein production. These include Burkit's Lymphoma (Singh et al., 1997), hybridoma (Simpson et al., 1997; Fassnacht et al., 1998a; Itoh et al., 1995; Suzuki et al., 1997), myeloma and COS-1 cells (Suzuki et al., 1997).

In the case of the hybridoma, myeloma and COS-1 cells, all studies have reported that enhanced survival leads to the increased production of the protein product. However, studies have been limited to cells grown in suspension batch cultures at relatively low cell densities. In high cell density perfusion cultures, such as those studied here, local limitations would be significant. Although the internal environment of the hollow fibre system cannot be monitored it was clear that Technomouse is capable of providing adequate oxygen supply independently of medium supply. Direct and effective evaluation of culture viability and identification of the mechanism of cell death also proved difficult during the operation of the fixed-bed system. However, the use of the fluorometric assay for analysis of DNA content provided evidence of a higher release of dead cells in the control cell line culture. This indirectly indicates that the primary mechanism of cell death must have been apoptosis. The presence of large numbers of ghosts in the harvests taken from the hollow fibre reactor provides further support for this conclusion.

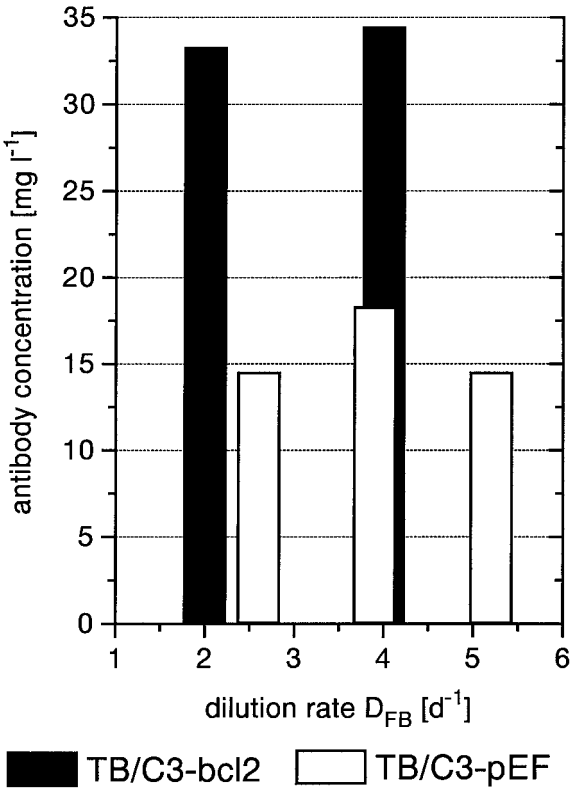

Figure 7. The antibody concentrations of both cell lines in the fixed-bed reactor at various perfusion rates D_{FB}.

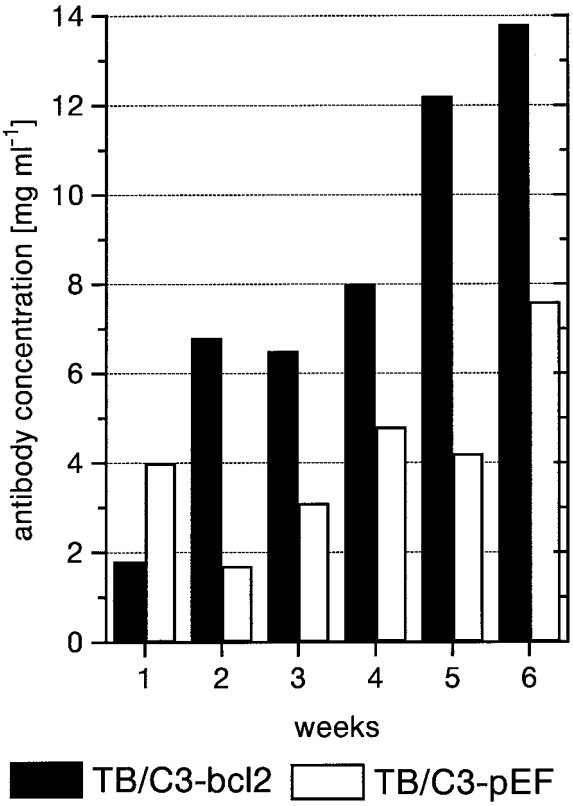

Figure 8. Antibody titre in the weekly harvests from the hollow fibre bioreactor.

Due to the difficulties of determining exact cell densities in fixed-bed carriers, other parameters such as the substrate consumption or metabolite production rates have to be applied when comparing cell lines in the fixed-bed system. There was no significant difference in the glutamine consumption as well as in lactate and ammonia production rates (identical concentrations) when comparing the two transfected cell lines TB/C3-pEF and TB/C3-bcl2 at all dilution rates, and the glucose consumption rates of both cell lines (Figure 5) was also identical at low dilution rates (D_{FB} < 3 d^{-1}). This corresponds to the results from batch experiments (Fassnacht et al., 1998a) where the cell specific consumption and production rates of both cell lines were nearly identical. The reason for the difference of the glucose consumption rates (Figure 5) between both cell lines at high dilution rates (D_{FB} > 3 d^{-1}) is not clear, but may be partly explained by the difficulty to determine the steady-state value of the TB/C3-bcl2 cell line at the dilution rate D_{FB} of 4 d^{-1} because of the disturbance of the run on the 19th day (Figure 4) caused by air which was pumped through the fixed-bed resulting in cell death. It can be assumed that the viable cell density of both cell lines were comparable in the fixed-bed system at low dilution rates, and the higher release of dead cells in the control cell line culture was caused by a higher death rate of the cells. As the fixed-bed was at steady-state, the higher death rate must be counter balanced by a higher rate of proliferation. This may, in part, explain the more than twofold increase of the antibody productivity of the TB/C3-bcl2 cell line in both systems, as hybridoma cells are known to exhibit a negative correlation between growth rate and antibody productivity (Fazekas, 1983; Boraston et al., 1984; Reuveny et al., 1986; Al-Rubeai and Emery, 1990; Al-Rubeai et al.,1992). Moreover, under stressful culture conditions, such as growth factor deprivation, bcl-2 over-expressing cells become quiescent (Vaux et al., 1988) and re-enter the cell cycle more slowly when stimulated with mitogens (Linette et al., 1996; Mazel et al., 1996; O'Reilly et al., 1996, 1997a,b; Huang et al., 1997). Over-expression of bcl-2 in hybridoma cells in continuous culture appears to mediate cell cycle ar-

rest under glutamine limited conditions (Simpson et al., 1998). Thus, under the stressful conditions of the fixed-bed and hollow fibre reactors studied here, this characteristic of bcl-2 may have partly contributed to the increased antibody production.

One of the basic concerns when cultivating cells over a long period is the stability of the antibody production and the transfection. The transfection stability was confirmed by flow cytometry. The bcl-2 content of cells removed at the end of the both fixed-bed cultures corresponded to the initial amount of each cell line at the beginning of the fixed-bed experiments. The cell line TB/C3 also proved to be extremely stable in respect to the antibody production over extended periods of culture. This was recently verified for both transfected cell line TB/C3-pEF and TB/C3-bcl2 where the antibody productivity remained stable in chemostat cultures over a period of 3 month (unpublished data).

In conclusion, this study represents the first demonstration that over-expression of bcl-2 results in improved cellular robustness and antibody productivity in intensive culture processes.

Acknowledgments

This project is partly funded by the EC Framework IV project 'Increasing cell culture productivity by control of programmed cell death (apoptosis)' (Contract No.: 950207) and the Volkswagen Stiftung (Grant No.: I/71230). The SIRAN carriers were kindly donated by Schott, Germany.

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Address for correspondence: R. Pörtner, Technische Universität Hamburg-Harburg, Bioprozeß- und Bioverfahrenstechnik, Denickestraße 15, D-21071 Hamburg, Germany.
Phone: +49-40-7718-2886;
Fax: +49-40-7718-2909;
E-mail: poertner@tu-harburg.de