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The "Push-to-Low" Approach for Optimization of High-Density Perfusion Cultures of Animal Cells

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Abstract High product titer is considered a strategic advantage of fed-batch over perfusion cultivation mode. The titer difference has been experimentally demonstrated and reported in the literature. However, the related theoretical aspects and strategies for optimization of perfusion processes with respect to their fed-batch counterparts have not

been thoroughly explored. The present paper introduces a unified framework for comparison of fed-batch and perfusion cultures, and proposes directions for improvement of the latter. The comparison is based on the concept of "equivalent specific perfusion rate", a variable that conveniently bridges various cultivation modes. The analysis shows that development of economically competitive perfusion processes for production of stable proteins depends on our ability to dramatically reduce the dilution rate while keeping high cell density, i.e., operating at low specific perfusion rates. Under these conditions, titer increases significantly, approaching the range of fed-batch titers. However, as dilution rate is decreased, a limit is reached below which performance declines due to poor growth and viability, specific productivity, or product instability. To overcome these limitations, a strategy referred to as "push-to-low" optimization has been developed. This approach involves an iterative stepwise decrease of the specific perfusion rate, and is most suitable for production of stable proteins where increased residence time does not compromise apparent specific productivity or product quality. The push-to-low approach was successfully applied to the production of monoclonal antibody against tumor necrosis factor (TNF). The experimental results followed closely the theoretical prediction, providing a multifold increase in titer. Despite the medium improvement, reduction of the specific growth rate along with increased apoptosis was observed at low specific perfusion rates. This phenomenon could not be explained with limitation or inhibition by the known nutrients and metabolites. Even further improvement would be possible if the cause of apoptosis were understood.

In general, a strategic target in the optimization of perfusion processes should be the decrease of the cell-specific perfusion rate to below 0.05 nL/cell/day, resulting in high, batch-like titers. The potential for high titer, combined with high volumetric productivity, stable performance over many months, and superior product/harvest quality, make perfusion processes an attractive alternative to fed-batch production, even in the case of stable proteins.

Keywords Animal cell culture · Antibody production · Media development · Perfusion process optimization

Abbreviations

CSPR	Cell specific perfusion rate (nL/cell/day)
D	Dilution rate (fermentor volumes/day)
OP	Operating point
OTR	Oxygen transfer rate (mM/L/day)
OUR	Oxygen uptake rate (mM/L/day)
QP	Specific production rate (pg/cell/day)
RT	Residence time (h)
SGR	Specific growth rate (1/day)
t	Time
V	Fermentor volume (L)
VP	Volumetric productivity (mg/L/day)
X	Cell concentration in fermentor (cells/mL)
X_H	Cell concentration in harvest (cells/mL)

1 Introduction

Over the last several years, it has become evident that the success of perfusion technology depends to a great extent on our ability to dramatically reduce the volumetric perfusion rate. Ideally, the perfusion rate would be around 1 volume/day, resulting in a high, batch-like titer and low liquid throughput. In combination with high cell densities of $20–60 \times 10^6$ cells/mL and superior product quality, this would significantly enhance the economic potential of perfusion technology.

However, the reduction of perfusion rate depends on multiple factors, including the relationship between specific productivity and specific perfusion rate, the medium formulation and cost, the half life of the product, and the dependence of product quality on fermentor residence time. As the perfusion rate is decreased, a limit is reached below which cultivation is impossible due to poor growth, decline in specific productivity, product degradation, or compromised product quality. The main directions in research to overcome these problems are: (1) development of media with enhanced "depth"; (2) systematic evaluation of the effect of ultralow perfusion rates on cell physiology and productivity; (3) protection of the product from degradation.

In the case of a stable protein, the concern about product degradation is minimal. The optimization objective is simplified to the development of a medium and a feeding strategy that enables operation at low perfusion rate while maintaining good cell growth, viability, and specific productivity. To this end, the "push-to-low" optimization technique has been developed and successfully applied. This approach involves an iterative stepwise decrease of the specific perfusion rate in highly instrumented, computer controlled fermentors. The cell density is maintained constant, at a maximum level. At each optimization step, a steady metabolic state is established, and the performance of the cell culture is evaluated. This involves monitoring of key physiological variables, including growth rate, cell death, specific production rate, as well as the concentration of selected nutrients and inhibitory metabolites. Based on this analysis, a decision on whether and how to perform another push towards lower perfusion rate is made. If necessary, the medium formulation is "in-process" modified at each step, so that medium depth progressively increases over the course of the optimization. The process continues until the lowest possible perfusion rate is reached.

The push-to-low technique was used in the optimization of a murine hybridoma perfusion process for production of antibody against TNF. Starting from standard conditions and medium, the perfusion rate was successfully decreased several fold. This resulted in a significant increase in antibody titer, while maintaining good growth and viability. A substantial improvement of the process was achieved, positively impacting the up- and downstream manufacturing steps. In general, our results suggest that for the production of

stable proteins, the operation of perfusion cultures at low feed rate is physiologically possible, economically feasible, and should be considered as a major direction for perfusion culture optimization.

2 Materials and Methods

2.1 Cell Line, Medium, and Fermentation System

Mouse-mouse hybridoma cells producing a monoclonal antibody against TNF were cultured in a proprietary medium buffered with 2.0 g/L $NaHCO_3$, and supplemented with glucose and glutamine. All experiments were conducted in 15 L fermentors equipped with external cell retention devices (Fig. 1). DO was maintained at 50% air saturation by diffusing oxygen through silicone tubing. The agitation speed was kept constant at 80 rpm and pH was controlled at 6.8 by automatic addition of 0.3 M NaOH or CO_2. The fermentors were inoculated at an initial cell density of approximately 1.0×10^6 cells/mL. Cell density was maintained at a set point of 20×10^6 cells/mL according to the control logic described below [1].

2.2 On-Line Measurements and Off-Line Analyses

DO and pH were monitored by retractable Ingold electrodes (Ingold Electrodes, MA). The accuracy of the on-line measurements of DO and pH was confirmed off-line using a NOVA blood gas analyzer (NOVA Biomedical, MA). The same instrument was used to quantify the dissolved CO_2 concentration. Cell density was monitored by a retractable optical density probe (Aquasant Messtechnik, Switzerland) calibrated to display the cell number. Calibration was checked daily and recalibration was performed when deviation from the off-line cell counts was detected. Generally, the probe performed reliably, requiring only infrequent, minor adjustments.

The fermentor and the harvest were sampled on a daily basis. The cell concentration was determined by averaging several hemacytometer counts. Cell viability was estimated via trypan blue exclusion. Cell size was determined by an electronic particle counter CASY (Scharfe Systems, Germany). The glucose and lactate concentrations were measured off-line using a YSI Model 2700 analyzer (Yellow Springs Instruments, OH). A modification of the same instrument, equipped with appropriate enzymatic membranes and software, was used for glutamine and glutamate assay. Ammonia was measured by Ektachem DT60 analyzer (Eastman Kodak, NY). Apoptosis was quantified following the standard An-

nexin V and Apo 2.7 (Clontech, CA) procedures provided by the indicator dye manufacturer.

Product concentration was determined by a nephelometric assay. To quantify and compare product quality (integrity and glycosylation) under different conditions, fermentor harvest was collected during steady state fermentation periods. Before purification, the harvest was passed through a cell separation filter, and concentrated by ultrafiltration.

2.3 Control of Cell Density

A prerequisite for the success of the perfusion culture optimization experiments is reliable long-term monitoring and control of cell concentration. Stable control cannot be achieved if the perfusion system relies on its "natural", chemostat-like equilibrium between growth and washed out cells. The drifts in the specific growth rate and in the harvest cell density often result in large fluctuations of fermentor cell density even if the perfusion rate remains unchanged. To enable robust control, an additional factor referred to as "cell discard rate", *CDR* (measured in L/day), needs to be introduced as described by the following equation:

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \mu \cdot X - D \cdot X_{\mathrm{H}} - \frac{CDR}{V} \cdot X\,. \tag{1a}$$

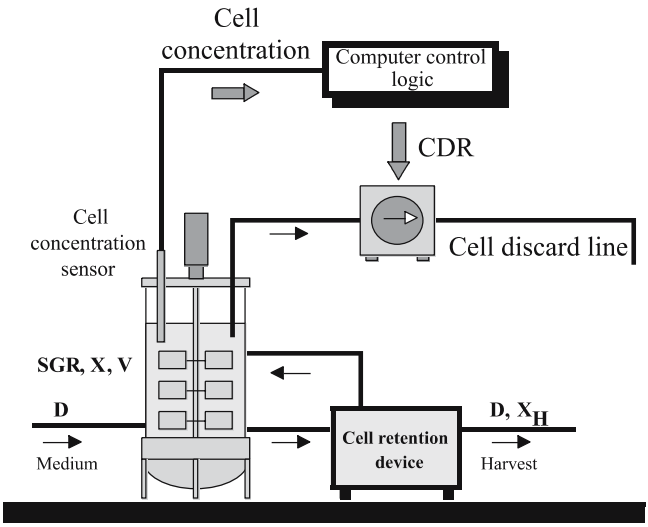

Fig. 1 Scheme of the 15 L perfusion fermentor system equipped with an external cell retention device and CDR-based cell density control

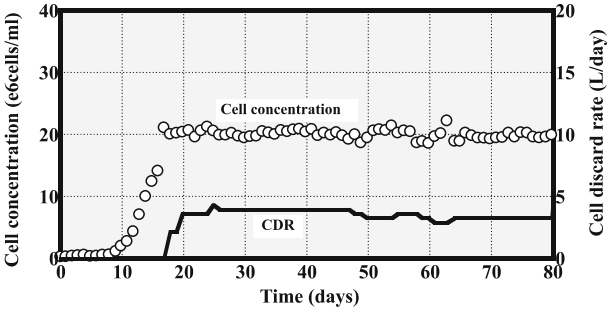

Fig. 2 Reliable CDR-based control of cell density in a perfusion animal cell culture over a period of 80 days

Assuming steady state, the expression is simplified to:

$$X = \frac{D}{\mu - \frac{CDR}{V}} X_{\mathrm{H}} \,, \tag{1b}$$

where μ is the apparent specific growth rate, X and X_{H} are the fermentor and harvest cell density, respectively, V is fermentor volume, and D is the perfusion rate (note that the term "perfusion rate" used in this paper is equivalent to "dilution rate"). The scheme of the CDR-based cell density control system is shown in Fig. 1. Cell concentration is computer controlled in a closed loop at the desired set point below the natural equilibrium by automatic removal of the extra cells from the fermentor. Excellent control can be achieved using this scheme, which guarantees long-term stable operation and high quality optimization data (Fig. 2).

2.4 Specific Perfusion Rate and Medium Depth

The cell-specific perfusion rate (*CSPR*) is a composite variable routinely used in monitoring and control of Bayer perfusion processes [2]. Its calculation is simple and requires only D and X (monitored either off-line or on-line):

$$CSPR\,(\mathrm{nL/cell/day}) = \frac{D(\mathrm{L/L} \times \mathrm{day})}{X(10^6\ \mathrm{cells/mL})} \,. \tag{2}$$

CSPR represents the volume of medium given to one cell in one day. Depending on the process, *CSPR* may vary widely, typically in the range 0.05–0.5 nL/cell/day. *CSPR* does not provide direct information about cell metabolic activity. Therefore, perfusion control based on *CSPR* remains fundamentally open loop with respect to cell physiology. The underlying assumption of the *CSPR*-based feed control is that cells are always in the same physiological state, disregarding possible metabolic changes that may occur during the process [3]. Despite its limitations, however, *CSPR* is indispensable

in quantifying and controlling perfusion cultures, conveniently "packaging" all medium components into a single entity. In comparison, other strategies, such as the glucose-based perfusion control [4], rely on a single medium component, assuming one-to-one relationship between glucose uptake and the overall cellular metabolism.

Another advantage of *CSPR* is that it links key perfusion process variables, such as titer, specific productivity (QP), cell density, and volumetric productivity (VP):

$$TITER = \frac{QP}{CSPR} \tag{3}$$

$$VP = TITER \times D = X \times QP\,. \tag{4}$$

CSPR is also closely related to the term "medium depth", which is often referred to in this paper. The medium depth is the reciprocal of the lowest possible *CSPR* ($CSPR_{\min}$):

$$MEDIUM\ DEPTH = \frac{1}{CSPR_{\min}} = \frac{X_{\max}}{D} \tag{5}$$

and represents the maximum number of cells that can be supported by 1 mL of medium in 1 day. For example, if $CSPR_{\min} = 0.1$ nL/cell/day, then medium depth is 10×10^6 cells/mL/day.

3
Conceptual Framework for Optimization of Perfusion Cultures

Before discussing the experimental data, it will be useful to outline the conceptual framework of our study. This focuses on some general aspects of fed-batch and perfusion cultivation modes. Although the issue is not new, the publications are still controversial [5, 6]. Our goal is to interpret the subject in view of some emerging trends in perfusion technology.

3.1
Application of Fed-Batch and Perfusion

Numerous publications dealing with the choice of cultivation method give the impression that one of the existing approaches – batch or perfusion – is clearly superior [7–13]. It is the authors' opinion that the question "which process is better – batch or perfusion?" is conceptually wrong, and that the right question asks when to use batch and when perfusion. At the present state of development of fermentation technology, it is unreasonable to look for a single universal answer.

There are several "easy" cases in which it is relatively straightforward to select the optimal process mode. In general, products prone to degradation require perfusion. So does a cell line that produces only in an active growth stage, the situation known as "growth-associated" production kinetics. On the other hand, a fed-batch approach may be favored in the case of high medium costs, where titer significantly affects the cost-of-goods. Fed-batch would also be the method of choice when cells secrete product in a non-proliferative state, or if the cell line is unstable, so that the production time horizon is limited. Unfortunately, many real situations fall in the gray zone between these "easy" cases, and the batch-or-perfusion decision can be difficult. The choice is often based on company tradition, existing facilities, infrastructure, and experience. Nevertheless, there is a growing interest in high-density perfusion culture, rationalized by some of the advantages of perfusion technology. These include superior product quality, steady state operation, excellent culture control, and high culture viability. Further development of perfusion technology is likely to result in more efficient processes operating at high cell densities in the range $40–80 \times 10^6$ cells/mL (providing high volumetric productivity) and ultralow specific perfusion rates below 0.05 nL/cell/day (providing batch-like titer).

3.2 Four Limiting Factors in Perfusion Culture: Determination of the Optimization Space

Perfusion culture is limited by several factors that reflect the physical characteristics of the perfusion system and the properties of the cell culture and the product. The intersection of these factors defines the process optimization space. The four most important are:

1. Maximum allowable residence time (RT_{max}) in the fermentor, defined by product stability. This corresponds to the minimum perfusion rate ($D_{min} = 1/RT_{max}$).
2. Maximum perfusion rate (D_{max}). Typically, D_{max} reflects the volumetric capacity of the cell retention device.
3. Maximum cell density (X_{max}). In most cases, X_{max} is defined by the maximum O_2 transfer rate (OTR) of the fermentor. The OTR limitation reflects the physical characteristics of the fermentor system, as well as the shear sensitivity of the cell culture.
4. Minimum cell-specific perfusion rate ($CSPR_{min}$) defined by the nutritional depth of the medium (Eq. 5).

Figure 3 illustrates the relationship between these factors. This simplified description enables one to define the zone of high D and low X (high $CSPR$, low titer, and low RT) and the zone of low D and high X (low specific perfusion rate, high titer, and high RT). These "natural" limitations are usually not

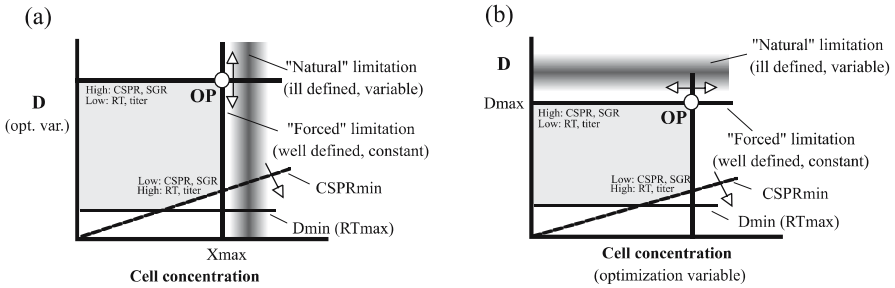

Fig. 3 Limiting factors in perfusion culture: **a** cell density limited culture, and **b** dilution rate limited culture. The optimization subspace is defined by the *gray polygon* (D_{min}, D_{max}, X_{max}, $CSPR_{min}$)

crisp. If the process is left to be controlled by them, large fluctuations would occur. For example, the volumetric capacity of the cell retention device may change over time due to various reasons, such as cell aggregation, fouling, etc. (Fig. 3b). If the perfusion rate is controlled to equilibrate the current cell retention capacity, the fermentor throughput will fluctuate. Similarly, the OTR capacity of the fermentor is likely to change over time due to antifoam addition, fouling of the silicone tubing in case of membrane oxygenation, change in the specific OUR of the cells, etc. If cell density is controlled to match the maximum OTR, then cell density will drift (Fig. 3a).

To provide stable control, the process should not be left to operate at its maximum OTR or D defined by the "natural" limiting (equilibrium) point. Instead, an artificial, "forced" limitation that will keep the process close to, but below, the natural equilibrium shell should be introduced. An example of a forced limitation is the above-described cell discard rate control (Eq. 1). In this case, the fermentation can run for many months at a stable operation point (OP). In this sense, the optimization of the perfusion process can be viewed as an upward or downward sliding of OP on the forced limitation line (Fig. 3), so that a particular optimization criteria is met. In the case of OTR limited culture, cell density will be controlled at a constant level, and *D* will be the optimization variable (OP will slide vertically). If *D* is limiting, perfusion rate will be kept constant below the natural limitation zone, and cell density will be the optimization variable (OP will slide horizontally).

3.3 Product Stability

The first critical task that has to be completed before initiating the series of optimization experiments is to determine the long-term stability of the product under real fermentor conditions. The results can force process development in one or another direction. In terms of stability, the spectra of

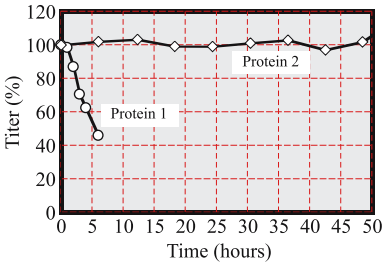

Fig. 4 Degradation of stable and unstable recombinant proteins produced in cell culture. The tests were conducted in supernatant under conditions equivalent to those in a fermentation run. *Protein 1* degrades quickly, while *Protein 2* remains stable for many days

biotechnology derived proteins is broad, ranging from stable to extremely labile molecules that degrade within hours. For example, monoclonal antibodies are usually stable, while large, heavily glycosylated molecules, such as FVIII [14] and ATIII [15], are very labile. Two examples from the authors' laboratory are shown in Fig. 4. While one of the proteins degrades quickly in a matter of hours (half life of about 5 h), the other remains stable for days. Obviously, these two molecules would require different production strategies. Often the degradation depends not only on the protein, but on the cell line itself. Degradation rates of the same protein may vary widely in different cultures [16], most likely due to proteolysis.

To quantify the degradation, a family of product concentration/quality time profiles measured in supernatants from several specific perfusion rates has to be generated. The collected data will enable the determination of the maximum allowable residence time, RT_{max}, possibly as a function of the cell-specific perfusion rate. RT_{max} defines the lowest limit of the process optimization space on the D axis (Fig. 3). In the context of perfusion technology, RT_{max} longer than 24 h defines the product as stable (RT_{max} of 1–3 weeks will be needed for batch), and opens up the bottom area in Fig. 3 for process development at low D. Then, the minimum perfusion rate from a product stability standpoint will be $D_{min} = 1/RT_{max}$.

3.4 Cell Retention

The upper limit of D is typically a result of mechanical limitations. Most often, the bottleneck is the cell retention device, which is characterized by its maximum volumetric throughput rate. In other cases, the limiting factor may be the upstream or downstream operation capacity (medium production or purification). The outcome is that D cannot increase above a certain limit D_{max}, which defines the upper end of the process optimization window for the perfusion rate (D_{min}, D_{max}).

3.5
Maximum Cell Density with Respect to O_2 Transfer Rate

The third key limitation in perfusion culture is the maximum cell density X_{max} that can be supported with respect to the O_2 transfer rate. This restriction depends on the fermentor hardware and the characteristics of the cell line (specific OUR, shear sensitivity), and graphically represents the right-side border of the optimization space (Fig. 3). Assuming growth-independent production kinetics, the volumetric productivity will be proportional to the cell density (see the antibody example below), and for optimal performance the OTR-limited bioreactor should be operated at X_{max}. Then, the key optimization parameter is the dilution rate D, which should be adjusted in the range (D_{min}, D_{max}). In general, one should try to slide the operation point OP on the X_{max} line, so that certain performance criterion is maximized. This optimization strategy, tuned up for the case of stable products, is the main focus of the present paper.

3.6
Minimum Cell-Specific Perfusion Rate (*CSPR*)

The *CSPR* (Eq. 2) cannot be reduced below a certain minimum, $CSPR_{min}$, determined by the nutritional depth of the medium (Eq. 5). Graphically, this limitation is represented by the inclined $CSPR_{min}$ line in Fig. 3. In some cases, this line may cross the $D_{min} = 1/RT_{max}$ line, and become the dominant limitation of D in the area of high cell density. Medium improvement would result in downward rotation of the $CSPR_{min}$ line, and would relax the *CSPR* limitation.

3.7
Optimization Space

The area between the D_{min}, D_{max}, X_{max}, and $CSPR_{min}$ lines defines the process optimization space (Fig. 3) for a given cell line, fermentor hardware, and medium formulation. Of practical interest is the "high X, low D" area, where perfusion culture is most productive. Therefore, in cases of stable product, the optimization will likely result in shifting OP towards the bottom right corner of the polygon.

3.8
Types of Perfusion Optimization Experiments

Table 1 outlines the four types of perfusion optimization experiments. The independent (manipulated) variables are two: the cell concentration (X) that can be easily varied using the control logic described earlier, and the per-

Table 1 Four types of perfusion optimization experiments

	Type I		Type II		Type III		Type IV	
	X	*D*	*X*	*D*	*X*	*D*	*X*	*D*
	const	const	var	const	var	var	const	var
CSPR	constant		variable		constant		variable	
RT	constant		constant		variable		variable	
Goal	Long-term stability evaluation		CSPR optimization (any product)		RT optimization (unstable product)		CSPR optimization (stable product)	

fusion rate (*D*). The dependent variables are also two: the residence time (*RT*) and the cell specific perfusion rate (*CSPR*). Clearly, these should not be perceived as output variables of the process. Instead, *RT* and *CSPR* are two factors in the beginning of the complex cause–effect cascade. Their consideration as dependent variables is practical because they represent two different aspects of the process: nutrition (*CSPR*) and degradation (*RT*). The four optimization experiments discussed below enable decoupling of the phenomena that may be taking place in the perfusion system: growth limitation due to nutrient deprivation (at low *CSPR*), and product degradation due to high exposure time to potentially proteolytic environment (at high *RT*).

Experiment Type I: This is the simplest case, when all variables are kept constant, providing a steady environment for the cells and the product. Such an experiment is most appropriate during the advanced development phase when the optimal *X* and *D* (and also *CSPR* and *RT*) have already been already determined, and the goal is to demonstrate long-term stability. The corresponding time profiles are shown in Fig. 5a.

Experiment Type II: This case is applicable to processes with stable product, not degrading up to time RT_{max}. Then, *RT* is fixed at that set point, and *CSPR* is independently optimized. The latter is varied by changing the cell concentration, as illustrated in Fig. 5b.

To achieve a reasonable variation of *CSPR*, one should target an order-of-magnitude change in cell density. The highest cell density, X_{max}, will correspond to the maximum OTR; the lowest should be in the $X_{max}/10$ range. For example, if X_{max} is 50×10^6 cells/mL, then X_{min} can be in the 5×10^6 cells/mL range. Then, if *D* is fixed at 2.5 volumes/day (*RT* = 9.6 h), *CSPR* would range from 0.05 nL/cell/day (at 50×10^6 cells/mL) to 0.5 nL/cell/day (at 5×10^6 cells/mL).

The goal of this experiment is to quantify the dependence of several metabolic rates, including specific productivity and specific growth rate on *CSPR*. This helps identify the type of production kinetics (growth-associated,

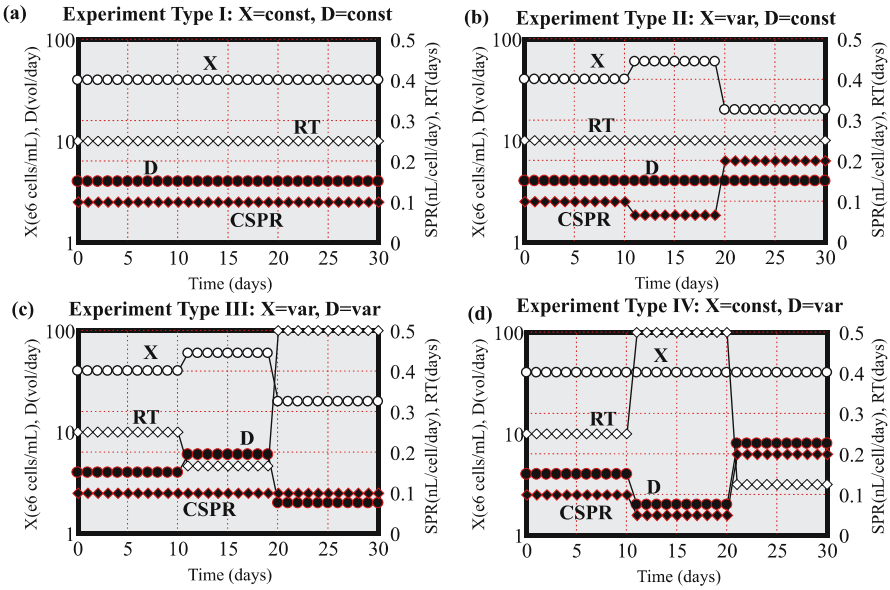

Fig. 5 Expected time profiles of *X*, *D*, *RT*, and *CSPR* in optimization experiments: **a** Type I, **b** Type II, **c** Type III, and **d** Type IV (Table 1)

non-growth-associated, inversely growth-associated). Among these, the non-growth-associated kinetics is preferred for process optimization [17].

Experiment Type III: This experiment is suitable for labile molecules, when the RT effect must be accurately determined. To decouple RT from the nutritional effect, *CSPR* is kept constant and the only variable that changes is *RT*. This is achieved by simultaneous and proportional manipulation of *X* and *D*, so that their ratio, *CSPR*, remains steady while *RT* changes as a function of *D*. It is appropriate to vary *RT* in the range 2–24 h. The left and right limits are difficult to expand. On the low end, *RT* is hard to reduce below 2 h (D_{max} = 12 volumes/day) because of the cell retention capacity (Fig. 3a). On the high side, it is usually impractical to target *RT* > 24 h (D_{min} = 1 volume/day) because the cell density must be decreased significantly to maintain realistic *CSPR*. The corresponding time profiles are shown in Fig. 5c. The key deliverable is the dependence of the apparent specific productivity and product quality on *RT*.

Experiment Type IV: This experiment can be considered when there is no concern about product degradation, so that *RT* can be disregarded as an optimization factor. *X* is maintained at the desired set point, and *D* is changed; *CSPR* and *RT* are not decoupled, and will both vary (Fig. 5d). Since cell density might be more difficult to control at various set points than *D*, the single but significant advantage of this experiment is conve-

nience. It is appropriate to keep cell density around 20×10^6 cells/mL and change *D* in the range 1–10 volumes/day, yielding *CSPR* values between 0.05 and 0.5 nL/cell/day.

3.9
Bridging of Fed-Batch and Perfusion Processes with Stable Products

Figure 6a shows the simulated time profiles of cell concentration and titer of a well developed fed-batch process. The assumptions are constant specific production rate of 20 pg/cell/day, non-growth associated production kinetics, peak cell density of 10×10^6 cells/mL, process length of 10 days, 20% increase of fermentor volume due to feeding, and stable product. Under these conditions, final titer in the fed-batch fermentor is approximately 740 mg/L.

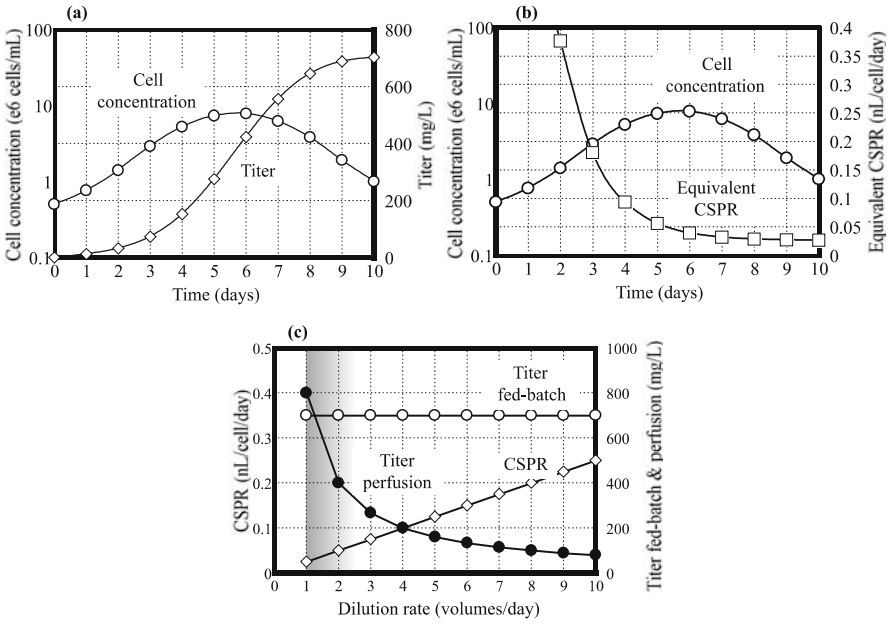

Fig. 6 Profiles of a simulated fed-batch process, and comparison with a perfusion process: **a** fed-batch cell density and titer; **b** fed-batch cell density and equivalent $CSPR_{eq}$; **c** comparison of the end-point fed-batch titer to the titer in a perfusion process run at constant cell density of 40e6 cells/mL and varying dilution rate. The *shadowed zone* indicates the area where fed-batch and perfusion titers are similar

3.9.1 The Concept of the "Equivalent Specific Perfusion Rate" in Fed-Batch Culture

While the concept of *CSPR* was originally developed for perfusion culture, it is possible to introduce a similar variable referred to as equivalent *CSPR* ($CSPR_{eq}$) for fed-batch culture:

$$CSPR_{eq}(t) = \frac{V(t)}{\int_0^t V(t) \cdot X(t)\,dt}, \tag{6}$$

where t is time and $V(t)$ is fermentor volume. This formula is more general than Eq. 2 because it accounts for the dynamic change of V and X. Equation 2 can be derived from Eq. 6 after considering $V(t)$ and $X(t)$ as constant, which is the case in perfusion culture. Equation 6 enables direct quantitative comparison of batch and perfusion processes. Note that the "titer" formula Eq. 3 is correct for both static (perfusion) and dynamic (batch) process. In the latter case, the calculation can be carried out by replacing *CSPR* with $CSPR_{eq}(t)$.

Figure 6b shows the time-profile of $CSPR_{eq}$ in the above-discussed fed-batch. $CSPR_{eq}$ gradually decreases down to an extremely low level of approximately 0.027 nL/cell/day, indicating that less and less medium remains "unconsumed" by the cells with the progress of the process. According to Eq. 3, low *CSPR* translates into low product dilution and higher titer. Considering the numerical data in Fig. 6a, the end-point titer can be calculated using Eqs. 3 and Eq. 6:

$$TITER(t_{final}) = \frac{QP}{CSPR_{eq}(t_{final})} = \frac{QP}{\frac{V(t_{final})}{\int_0^{t_{final}} V(t) \times X(t)\,dt}}$$
$$= \frac{20\ \text{pg/cell/day}}{0.027\ \text{nL/cell/day}} = 740\ \text{mg/L}\,.$$

In the context of our discussion, the reason for the high titer of fed-batch processes is the extremely low $CSPR_{eq}$ that can be achieved at the end of the run, significantly lower than the *CSPR* typically maintained in perfusion cultures.

3.9.2 Comparison of Fed-Batch and Perfusion Titers as a Function of *CSPR*

Figure 6c shows a comparison of the described fed-batch process and its perfusion counterpart. The following assumptions are made about the perfusion process: same specific productivity of 20 pg/cell/day, non-growth-associated production kinetics, constant cell density of 40e6 cells/mL. The optimization

variable is *D*, changing in the range 1–10 volumes/day. *CSPR* decreases linearly with *D* from 0.25 nL/cell/day to 0.025 nL/cell/day.

The perfusion process titer increases in a non-linear fashion with the decrease of *D*. At $D = 10$ volumes/day ($CSPR = 0.25$ nL/cell/day), titer is low (80 mg/L), but reaches 200 mg/L at $D = 4$ volumes/day ($CSPR = 0.1$ nL/cell/day). Further decrease of *D* is followed by a steep increase in titer, reaching 400 mg/L at $D = 2$ volumes/day ($CSPR = 0.05$ nL/cell/day) and 800 mg/L at $D = 1$ volume/day ($CSPR = 0.025$ nL/cell/day). At this point, the perfusion titer has surpassed the fed-batch benchmark. This is possible because the *CSPR* of the former is lower than $CSPR_{eq}$ of the latter. In general, it can be expected that if *CSPR* of the perfusion system goes below 0.05 nL/cell/day, perfusion and fed-batch process become comparable in terms of titer.

However, in reality, fed-batch would always offer the potential of lower *CSPR* and higher titer than perfusion. The reason is that in the final phase of the fed-batch process, the cell culture is sacrificed due to severe nutrient limitation. Viability decreases, often down to 0%. This is not an option in the perfusion process where maintenance of high viability is mandatory. This high viability comes at the price of higher *CSPR* and, correspondingly, lower titer. However, as long as the perfusion process is operated in the far left side of Fig. 6c, the titer difference may not be dramatic. This, in combination with the superior product/harvest quality of perfusion culture (low impurities, low residence time), makes the latter an attractive manufacturing option, even in the case of stable proteins.

3.9.3 The Push-to-Low Optimization Approach

Figure 6c shows that high titer perfusion processes can be developed by the substantial decrease of *CSPR* (low *D*, high *X*, or combination of both). A strategic way to accomplish this task is systematic medium improvement, incrementally reducing the nutrient limitation barrier.

Figure 7 illustrates graphically a concept referred to as push-to-low optimization. The name reflects the incremental in-process shifting of the cell culture towards lower *CSPR*s, typically starting at relatively high values. The process either maintains constant cell density, while *D* is incrementally decreased (Fig. 7a), or *D* is kept steady, while cell density is incrementally increased (Fig. 7c). These two options correspond to experiments Type IV and Type II, respectively (see Table 1). Good initial values of cell density and *D* are 20×10^6 cells/mL and 4 volumes/day, corresponding to a *CSPR* of 0.2 nL/cell/day.

The push-to-low optimization consists of several steps, each of which includes a stepwise decrease of *D* (or increase of *X*), establishing a new steady state, and comprehensive in-process analysis of the residual medium components and specific metabolic rates to discover possible limitations. Key

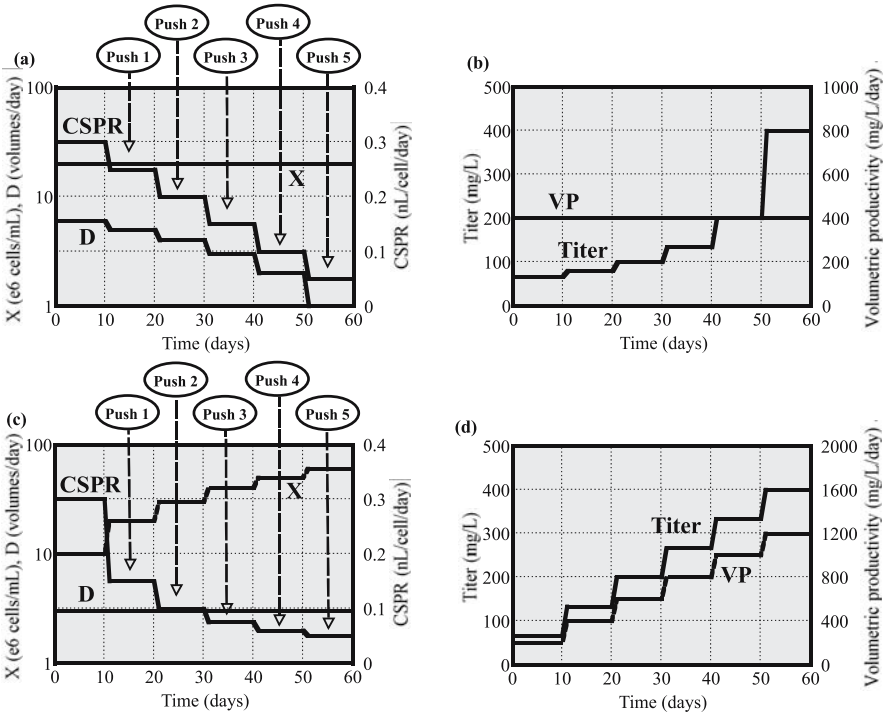

Fig. 7 The push-to-low concept: theoretical profiles of *D*, *X*, *CSPR*, titer and *VP*. **a**,**b** Case when *X* is kept constant, and *CSPR* is "pushed" downwards by decreasing *D*. **c**,**d** Case when *D* is kept constant, and *CSPR* is "pushed" downwards by increasing *X*

physiological state variables, such as specific growth rate, specific O_2 uptake rate, specific glucose uptake rate, and most importantly, the specific production rate, should be closely monitored. If limitation is identified, the medium must be improved before another downward *CSPR* "push" is made. This iterative procedure continues until no further decrease of *CSPR* is possible, and the culture inevitably crashes, even if it appears that all medium components are available. This can be caused by either metabolite inhibition or "hidden" limitation by unknown compounds. The last safe steady state before the crash defines the optimal *CSPR* at which the process should be operated.

4
Results and Discussion

4.1
Push-to-Low Optimization of Hybridoma Culture

The push-to-low optimization approach was applied to a hybridoma culture producing antibody against TNF. The cell concentration was automatically controlled at 20×10^6 cells/mL using the CDR-based control scheme shown in Fig. 1. *CSPR* of 0.3 nL/cell/day ($D = 6$ volumes/day) was considered as stan-

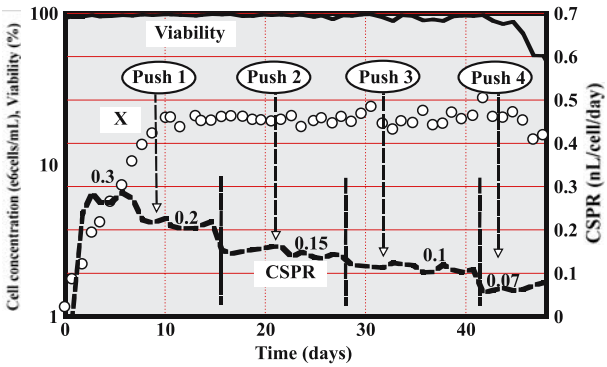

Fig. 8 Time profiles of cell concentration, viability, and *CSPR* in a push-to-low optimization run for production of monoclonal antibody against TNF. *CSPR* was reduced stepwise from 0.3 nL/cell/day down to 0.07 nL/cell/day

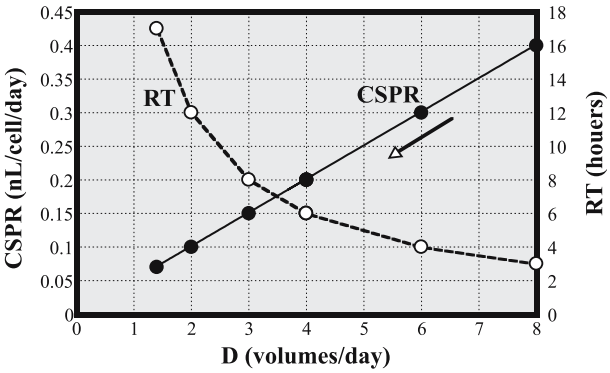

Fig. 9 Dependence of *RT* and *CSPR* on *D* in the push-to-low optimization run for production of monoclonal antibody against TNF. *CSPR* decreased linearly with *D*, which was changed from 8 volumes/day to 1.3 volumes/day. *RT* increased from 3 h to approximately 17 h

dard before the optimization began. This high *CSPR* provided long-term stable operation, but antibody titer was low, and large medium/harvest volumes needed to be stored and processed. The goal of the optimization was to increase titer by stepwise reduction of *CSPR* without compromising volumetric productivity. Figure 8 shows the time profiles of cell concentration, viability, and *CSPR* in one of our experimental runs. During this process, *CSPR* was reduced in several steps: 0.3 – 0.2 – 0.15 – 0.1 – 0.07 nL/cell/day, following experiment Type IV approach (Table 1, Fig. 7a). This was possible because the antibody was found to be stable, and *RT* was not a critical optimization factor. The major optimization variable was *CSPR*, which was manipulated by decreasing *D* from 6 to 1.3 volumes day, corresponding to an increase in *RT* from 3 to 17 h (Fig. 9).

4.2 Dependence of Key Substrates and Metabolites on *CSPR*

Figure 10 shows the dependence of glucose, lactate, glutamine, and ammonia concentration on *CSPR*. Glucose concentration decreased at lower *CSPR*, from ~ 3 g/L at 0.4 nL/cell/day to ~ 1.2 g/L at 0.07 nL/cell/day. Correspondingly, lactate concentration increased from ~ 0.5 g/L at 0.4 nL/cell/day to ~ 1.0 g/L at 0.07 nL/cell day. Neither of these values were significant in terms of substrate limitation or metabolite inhibition.

The profiles of glutamine and ammonia were similar. Ammonia increased from ~ 4 mM to ~ 6 mM with the decrease of *CSPR*, and glutamine decreased from ~ 4 mM to ~ 1 mM. These levels were not in the range where substrate limitation or metabolite inhibition is to be expected.

Steady state amino acid concentrations at *CSPR*s of 0.07, 0.10, 0.15, and 0.20 nL/cell/day are shown in Fig. 11. At each steady state, the amino acids

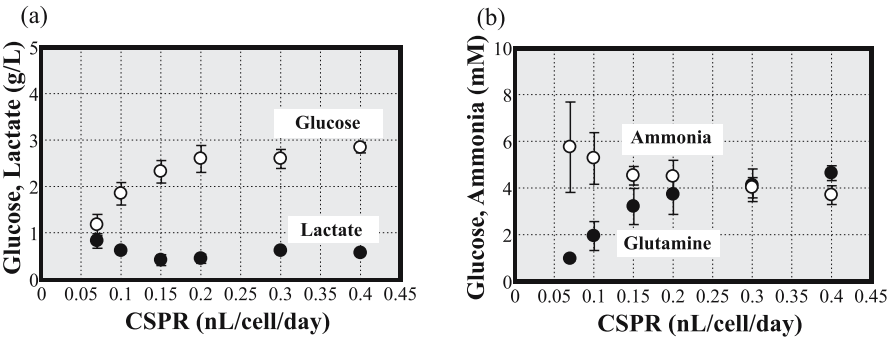

Fig. 10 Dependence of key process substrates and metabolites on *CSPR* in the push-to-low optimization run for production of monoclonal antibody against TNF: **a** glucose and lactate, **b** glutamine and ammonia

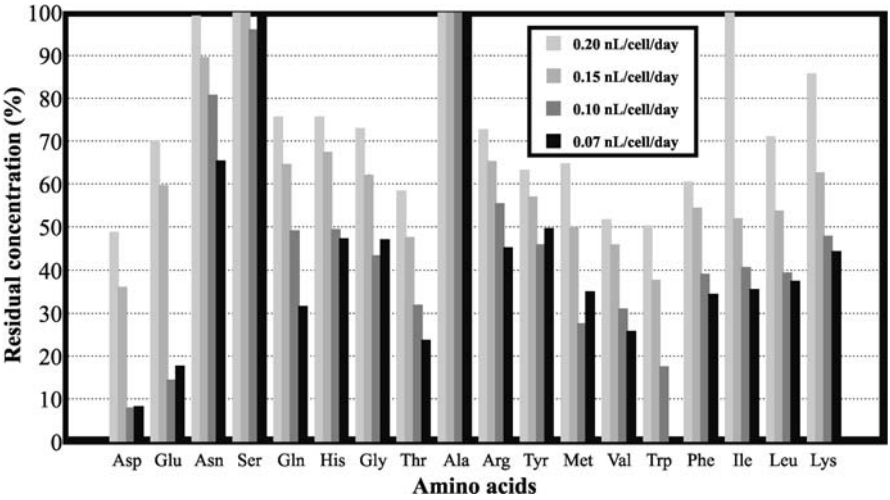

Fig. 11 Dependence of the residual concentrations of amino acids (percent of medium concentration) on *CSPR* in the push-to-low optimization run for production of monoclonal antibody against *TNF*

were analyzed by HPLC, enabling in-process correction of the medium formulation. At all *CSPR*s amino acids were generally above 20% of initial concentration, except for asparagine and tryptophan, which were depleted after the last *CSPR* push.

4.3 Physiological Response to Low *CSPR*

The dependence of the specific growth rate (SGR) on *CSPR* is shown in Fig. 12a. Decrease in *CSPR* slows down cell growth to low levels, reaching practically zero at *CSPR* of 0.07 nL/cell/day. The simplest hypothesis – substrate limitation – was not confirmed by the glucose, glutamine, and amino acids analysis. However, the possibility of a "hidden" limitation by a non-identified medium component cannot be ruled out.

SGR inhibition caused by the accumulation of a toxic metabolite is another plausible hypothesis. Since the well-known metabolites, such as lactate, ammonia, and dissolved CO_2, did not reach toxic levels, their role in inhibition was not obvious. While the possible presence of unknown toxic compounds in the culture supernatant has attracted some attention recently [18–22] only one inhibitor (methylglyoxal) has been well characterized [20]. In any case, an understanding of the low SGR phenomenon is essential for the further development of perfusion technology. Maintenance of an active cell population with good SGR at ultralow *CSPR*s would enable substantial process improvement.

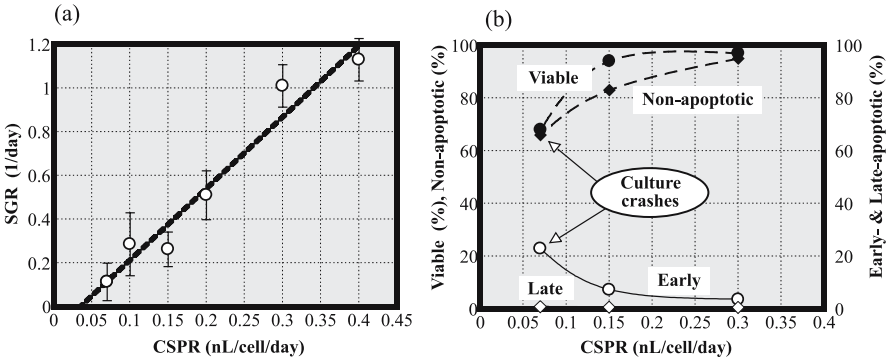

Fig. 12 Dependence of **a** SGR, and **b** concentration of apoptotic cells on *CSPR* in the push-to-low optimization run for production of monoclonal antibody against TNF

Figure 12b shows the effect of *CSPR* on cell viability and on the portion of apoptotic cells. At low *CSPR*, the population of apoptotic cells increases. While it is unclear what the cause of this phenomenon is, the link with the reduction in SGR is obvious. Decrease in apoptosis at low *CSPR* might be achieved by identifying the underlying factor(s), use of apoptosis-resistant cell lines, or application of anti-apoptosis medium additives.

4.4 Dependence of Specific Productivity, Titer, and Volumetric Productivity on *CSPR*

The success of the push-to-low approach depends to a great extent on the type of production kinetics, which varies widely between different cell lines. For example, if specific productivity goes down with *CSPR*, then decrease of the

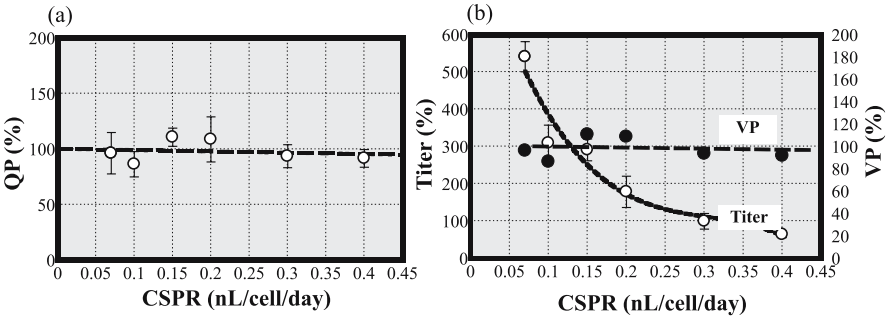

Fig. 13 Dependence of **a** *QP*, and **b** *VP* and titer on *CSPR* in the push-to-low optimization run for production of monoclonal antibody against TNF. Decrease of *CSPR* down to 0.07 nL/cell/day resulted in a ~ 500% increase in titer, while specific productivity, *QP*, and volumetric productivity, *VP*, remained unchanged

latter will likely lower the volumetric productivity. Therefore, it is essential to quantify the relationship between specific productivity and *CSPR*. Figure 13a reveals that in our case specific productivity does not depend on *CSPR*, and cells are producing at the same rate regardless of the *CSPR*-induced changes in their environment. This is the most favorable situation for the application of the push-to-low optimization approach. Since cell concentration was kept constant, decrease of *CSPR* did not change the volumetric productivity (Fig. 13b, Eqs. 3 and 4). However, improvement in titer was significant, closely following the theoretical prediction in Figs. 6c and 7b. Compared to the initial titer obtained in the non-optimized process, the final titer increased approximately 500%, resulting in a simpler process, lower liquid volume to handle, and overall reduction in cost-of-goods.

5 Conclusions

Experimental application of the push-to-low approach for optimization of anti-TNF production can be conveniently illustrated in the context of the *X*–*D* plot introduced earlier (Fig. 3). Figure 14 shows the optimization space of the 15 L development system defined by D_{max} = 10 volumes/day, X_{max} = 20×10^6 cells/mL ("forced" limitation, the "natural" limitation was far beyond 20e6 cells/mL), and initial $CSPR_{min}$ = 0.3 nL/cell/day. Due to the high product stability, there was no restriction on D_{min}. The pre-optimization

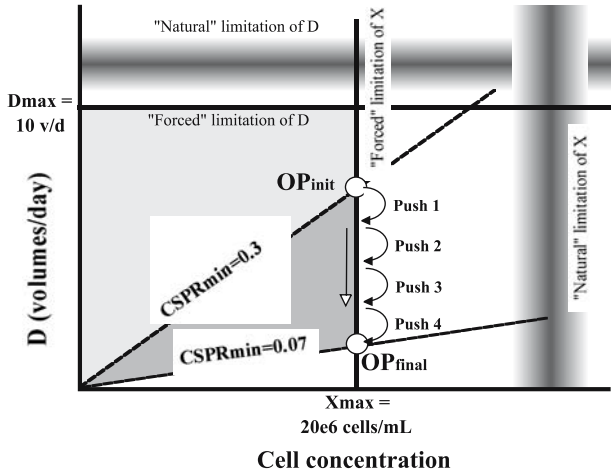

Fig. 14 Representation of the 15 L push-to-low optimization run from Fig. 8: D_{min} not restricted (stable product); D_{max}10 volumes/day ("forced" limitation); $X_{max}20 \times 10^6$ cells /day ("forced" limitation); initial $CSPR_{min}$0.3 nL/cell/day; final $CSPR_{min}$0.07 nL/cell/day

operating point, OP_{init}, was located at the intersection of X_{max} and initial $CSPR_{min}$ = 0.3 nL/cell/day. The push-to-low optimization resulted in the incremental sliding of the operating point down the X_{max} line to the lowest possible location, OP_{final}, defined by the new value of $CSPR_{min}$ = 0.07 nL/cell/day. Once the optimal operating point is determined at a small scale, scale up to production is straightforward, requiring only repositioning of the X_{max} and D_{max} lines to reflect the maximum capacity of the large scale reactors, whose optimal OP will be located at the intersection of $CSRP_{min}$ and X_{max}.

The proposed approach for optimization of perfusion cultures is most suitable to stable products for which residence time is not a critical parameter. If this is not the case, the dilution rate cannot be reduced to low levels. The push-to-low optimization procedure works with cell lines exhibiting either non-growth-associated or inversely growth-associated production kinetics. In these cases, the decrease of *CSPR* to low levels would significantly increase titer, approaching the range of fed-batch titers. This was demonstrated in a perfusion antibody fermentation, where a multifold increase in titer was achieved at constant volumetric production rate. Significant reduction of the specific growth rate accompanied with increased apoptosis at low *CSPR* was observed. This could not be explained with limitation or inhibition by the known medium components and toxic metabolites. Further improvements in titer will be possible if this phenomenon is understood and alleviated.

Successful application of the proposed optimization approach requires reliable cell concentration control, incremental in-process medium improvement, and continuous monitoring of cell physiology and product quality. The targeted *CSPR* in the case of stable product and proper production kinetics should be 0.05 nL/cell/day or lower, which will increase titer to the levels typical for fed-batch processes. The potential for high titer, combined with high volumetric productivity, stable performance over many months, and superior product/harvest quality, render perfusion processes an attractive production technology. To make high titer a standard feature of perfusion processes, further work on medium optimization, cell line improvement (apoptosis-resistant, low auto-inhibitor production), and understanding of cell physiology at ultralow *CSPR* will be necessary.

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