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Development and application of a high-throughput platform for perfusion-based cell culture processes

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ABSTRACT

A high-throughput (HT) cell culture model has been established for the support of perfusion-based cell culture processes operating at high cell densities. To mimic perfusion, the developed platform takes advantage of shake tubes and operates them in a batch-refeed mode with daily medium exchange to supply the cultures with nutrients and remove toxic byproducts. By adjusting the shaking parameters, such as the speed and setting angle, we have adapted the shake tubes to a semi-continuous production of a recombinant enzyme in a perfusion-like mode. We have demonstrated that the developed model can be used to select clones and cell culture media ahead of process optimization studies in bioreactors and confirmed the applicability of shake tubes to a perfusion-like cell culture reaching ~50E6 viable cells/mL. Furthermore, through regular cell mass removal and periodic medium exchange we have successfully maintained satellite cultures of bench-top perfusion bioreactors, achieving a sustainable cell culture performance at ≥30E6 viable cells/mL and viabilities >80% for over 58 days. The established HT model is a unique and powerful tool that can be used for the development and screening of media formulations, or for testing selected process parameters during both process optimization and manufacturing support campaigns.

1. Introduction

Perfusion cultures have been extensively used in the biotechnology industry as an effective means to achieve high yields of unstable recombinant products due to the reduced residence time of the product in the reactor and the decreased product exposure to different degrading enzymes found in the culture (Goudar et al., 2007; Gramer and Goochee, 1993; Kompala and Ozturk, 2006; Shi et al., 1993). Recently, the potential of perfusion cultures has been demonstrated for the manufacture of various kinds of therapeutic proteins, including enzymes and monoclonal antibodies, highlighting additional benefits, such as reduced bioreactor size and applicability to disposable technologies among others (Clincke et al., 2013a; Clincke et al., 2013b; Warikoo et al., 2012). Despite the success of this platform, there has been a general lack of a suitable high-throughput (HT) cell culture model capable of mimicking perfusion. Hence, there has been a longstanding gap for appropriately screening clones, media, and selected process parameters in support of perfusion process optimization. Although several HT systems have been used for fed-batch processes, to our knowledge, no experimental HT model of a perfusion-based cell culture process has been described to date.

To address this gap we have chosen the TubeSpin® bioreactors, here termed "shake tubes", which can successfully increase the throughput of the traditional shake flask-based methods for their ease of handling and the considerable space savings that they offer (De Jesus et al., 2004; Jordan and Jenkins, 2007). Shake tubes have been shown to support CHO cell growth reaching 12–14E6 cells/mL in non-instrumented batch (Jordan et al., 2013; Zagari et al., 2013) and close to 25E6 cells/mL in fed-batch (Rouiller et al., 2013) cultures. However, neither higher cell densities nor application of shake tubes to a perfusion-like process have been reported thus far. In our HT platform, we have modified the traditional shake tube shaking parameters to support cell culture processes operating at viable cell densities reaching 50E6 cells/mL. Following Ozturk, we have calculated the volumetric gas mass transfer coefficient, expressed as $k_L a$, necessary to provide oxygen to 50E6 viable cells/mL consuming 0.05–0.5 pmol O_2/cell-h on average, which is the typical cellular oxygen consumption rate for mammalian cells. Assuming that pure oxygen is sparged to the reactor and the

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dissolved oxygen concentration (DO) in the culture liquid is 50%, the required k_La amounts to 28–280 h^{-1}, based on the cells' oxygen demand (Ozturk, 1996). In shake tubes, however, the oxygen is supplied to the cultures through the vessel's headspace, where the oxygen partial pressure is equal to 0.2095 bars. Therefore, the respective shake tube k_La required by the cells would add up to 23–230 h^{-1}. The k_La of shake tubes at the typical settings (shaking angle equal to 90° from horizontal, rotation rate of 180 rpm, and working volume of 10 mL at an orbital throw of 50 mm) has been reported to be ~24 h^{-1}, at which it supported a recombinant CHO culture at 6E6 viable cells/mL (Xie et al., 2011). Consequently, we suspected that at cell densities around 50E6 viable cells/mL, or even much below, the system would struggle to provide sufficient aeration to the high-density cultures of highly metabolically active cells, thus limiting cell growth. Moreover, product stability could be reduced at such high densities due to the intensified mixing required to fulfill the raised O_2 requirement as well as the increase in concentration of active proteases and glycosidases in the spent medium (Gramer and Goochee, 1993; Thomas and Geer, 2011).

Since it has been previously documented for shake flasks (Klockner and Buchs, 2012), deep-well plates (Duetz et al., 2000), and 5 L cylindrical vessels (Zhang et al., 2009) that the culture's surface to volume ratio has the most impact on the oxygen transfer rate (OTR) from the gas to the liquid phase, we evaluated the shake tubes at a reduced shaking angle and speed relative to the typical settings (Xie et al., 2011) to increase the gas–liquid interface and introduce a different mixing pattern. Lastly, a study carried out by Strnad et al. pointed out the difficulty of developing a small-scale model truly representative of a bioreactor process (Strnad et al., 2010). Using an EPO-producing cell line, the researchers evaluated the shake tubes at varied working volumes and shaking speeds in a batch mode in an attempt to find operating conditions that would lead to productivity and product quality comparable to those obtained in a 5 L stirred-tank bioreactor. While matching product titer was obtained at higher than the typical rpm, a decrease in sialylation was also noted. Conversely, when the shaking speed was reduced, product quality was more similar between platforms but the overall productivity had dropped by a third (Strnad et al., 2010). Therefore, once we had optimized the operating conditions of our perfusion-like model, we assessed its comparability to traditional stirred-tank bioreactor processes by conducting studies in support of clone and media selection and by testing critical product quality attributes in satellite cultures of the perfusion bioreactors.

2. Materials and methods

2.1. Shake tube cell culture

The four cell lines used for the experiments were suspension-adapted Genzyme proprietary CHO clones (*a*, *b*, *c*, and *d*) expressing the same recombinant enzyme (-r-enzyme). Clones *a* and *c* were derived from the same parental pool, while clones *b* and *d* were selected from different pools of transfected cells. Unless otherwise indicated, the cells were cultured in a chemically defined (CD) Medium I supplemented with 4 mM glutamine (Invitrogen, Carlsbad, CA). The start-up cultures for the shake tubes (TubeSpin® Bioreactor 50, TPP Techno Plastic Products AG, Trasadingen, Switzerland) were either extracted from perfusion bioreactors at a given cell density (Sections 2.3 and 3.1), or generated from seed cultures expanded in shake flasks following the vial thaw of a specific research cell bank until inoculation into shake tubes at 5E5 viable cells/mL (Sections 3.2 and 3.3). Cultures were maintained at a constant working volume of 10 mL per tube in a shaker incubator (Multitron Standard, Infors AG, Switzerland) with an orbital throw of 25 mm and a controlled environment of 5% CO_2, 37 °C, and 80% relative humidity. The tubes were agitated at different shaking speeds, reduced in a stepwise manner form 250 to 180 to 160 rpm at 45° shaking angle, or from 250 to 220 rpm at the 90° angle following empirical results. The initial shaking speed of 250 rpm for both shaking angles was approximated based on Eq. (1) and using the settings recommended for agitating the tubes at a 90° angle and a 50 mm orbital throw (www.tpp.ch):

$$\omega_2 = \omega_1 \sqrt{\frac{r_1}{r_2}}, \tag{1}$$

where ω_1 is the recommended shaking speed of 180 rpm, r_1 and r_2 are the shaking diameters of 50 and 25 mm, respectively, and ω_2 is the shaking speed at 25 mm shaking diameter. Noteworthy, Eq. (1) does not take shaking angle into account and was used only as a starting point for the experiments. Cell density and viability were determined by Vi-CELL® Cell Viability Analyzer (Beckmann Coulter Inc., Brea, CA). The cumulative cell time was calculated for each culture following Adams et al. (Adams et al., 2007) and according to:

$$\mathrm{IVCD}_n = \mathrm{IVCD}_{n-1} + \left(\left(\frac{\mathrm{VCD}_n + \mathrm{VCD}_{n-1}}{2} \right) \times (t_n - t_{n-1}) \right) \tag{2}$$

where IVCD_n and $\mathrm{IVCD}_{n\text{-}1}$ are the integrated viable cell densities calculated for culture time t_n and $t_{n\text{-}1}$, respectively, while VCD_n and $\mathrm{VCD}_{n\text{-}1}$ the viable cell densities measured at time t_n and $t_{n\text{-}1}$. To sustain a healthy cell growth for an extended period, a culture medium exchange rate was performed on a daily basis. To exchange media, the tubes were centrifuged at about 230 × *g* for 5 min following which the spent medium was removed and the tubes were fed with the corresponding percentage of the fresh medium. The experiments described in Sections 2.4, 3.2 and 3.3 were performed using the same protocol: starting from day one post-inoculation and until day 3, 50% of the spent medium was exchanged with fresh medium, while a refeed of 70% was performed on days 4 through 6 after inoculation. On day 7 and until the end of the culture, ~100% of the reactor volume was exchanged per day (RV/d). The removed spent media were used immediately to assay for metabolites by YSI Biochemistry Analyzer (Yellow Springs Instruments, Inc., Yellow Springs, OH), or stored at −80 °C until the enzyme activity and product quality assays were performed to determine product titer and its affinity, respectively. The volumetric and specific glucose and glutamine consumption rates were calculated using:

$$q_{x,n} = \frac{C_{x,n}}{(\mathrm{IVCD}_n - \mathrm{IVCD}_{n-1})} = \frac{\left(c_{x,n-1} \times (1 - D_n) + (m_x \times D_n)\right) - c_{x,n}}{\Delta \mathrm{IVCD}_n}, \tag{3}$$

where *x* is glucose (glc) or glutamine (Gln), $q_{x,n}$ and $C_{x,n}$ are, respectively, the specific rates and the volumetric glucose and glutamine consumption at time t_n, while the glucose and glutamine concentrations measured in the spent medium at time $t_{n\text{-}1}$ or the fresh medium are indicated as $c_{x,n}$ and m_x, respectively; D_n is the daily dilution at time t_n, glucose concentration (g/L) was converted to molar using M(glc) = 180.2 g/mol, and $\Delta\mathrm{IVCD}_n$ is the integral of viable cell densities calculated between time t_n and $t_{n\text{-}1}$. The specific lactate and recombinant product productivities were determined using:

$$q_x = \frac{\mathrm{p}_{x,n}}{(\mathrm{IVCD}_n - \mathrm{IVCD}_{n-1})} = \frac{\mathrm{p}_{x,n} - \left(\mathrm{p}_{x,n-1} \times (1 - D_n)\right)}{\Delta \mathrm{IVCD}_n}, \tag{4}$$

where *x* is product (p) or lactate (lac), q_x is the cell specific production rate of *x*, $\mathrm{p}_{x,n}$ and $\mathrm{p}_{x,n\text{-}1}$ are, respectively, the enzyme's activity or lactate concentration measured in the spent medium at time t_n or $t_{n\text{-}1}$; lactate concentration (g/L) was converted to molar using M(lac) = 90.1 g/mol. Lactate yield from glucose was calculated from Eqs. (3) and (4) using the corresponding specific metabolic rates: