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A perfusion-capable microfluidic bioreactor for assessing microbial heterologous protein production

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Abstract

We present an integrated microfluidic bioreactor for fully continuous perfusion cultivation of suspended microbial cell cultures. This system allowed continuous and stable heterologous protein expression by sustaining the cultivation of *Pichia pastoris* over 11 days. This technical capability also allowed testing the impact of perfusion conditions on protein expression. This advance should enable small-scale models for process optimization in continuous biomanufacturing.

Methods for the continuous cultivation of cells have the potential to improve the efficiency of processes for manufacturing biopharmaceuticals. One mode is cultivation by perfusion: fresh media flows continuously through a bioreactor while biomass remains in the bioreactor. Perfusion offers advantages over traditional batch-style manufacturing, enabling consistent production of proteins over time and reduced cultivation volumes. Development of robust processes using perfusion, however, requires a rigorous understanding of key experimental parameters influencing overall productivity.

Approaches that use quality-by-design (QbD) for process development often employ scaled-down systems for cultivation to gain experimental understanding and build rigorous models of the process. Scale-down models for batch and fed-batch modalities are widely employed in the biopharmaceutical industry. Many technologies exist that enable a range of operational modalities, volumes, and strategies for control. Shake flasks, nonetheless, remain a primary model for complex modes of cultivation: these experiments may use baffled glass

vessels with volumes greater than 1 L or microtiter plates adapted for automated high-throughput studies. The simplicity of shake flasks makes them accessible, but they are a poor model for the conditions present in a bioreactor. It is difficult to monitor critical parameters and no control is possible. In contrast, multiplexed bench-scale mimics of large stirred tank vessels incorporate many features and controls typically employed in commercial bioreactors, including dynamic gassing, biomass monitoring, and closed-loop control of temperature, pH, and dissolved oxygen. Both reusable glass-based (*e.g.* Sartorius Biostat Q6+) and disposable polymer-based (*e.g.* Eppendorf DASbox) vessels provide working volumes less than 1 L. Some platforms allow many (12 - 24) reactors to test multiple conditions in parallel (*e.g.* Sartorious ambr250). None of the currently available bench-top bioreactors, however, are capable of routine cultivation by perfusion without extensive retrofitting.

Single-use microfluidic devices hold substantial promise as screening tools to optimize conditions for cellular cultivation and manufacturing processes. To date, microfluidic systems have been developed to mimic the monitoring and control capabilities of full-scale bioreactors for batch culture. Recently, a polydimethylsiloxane-polycarbonate (PDMS-PC) device with a 1 mL working volume and an online analytics suite capable of monitoring and controlling temperature, pH, dissolved oxygen (DO), and cell density for chemostat and turbidostat continuous culture was demonstrated. No microfluidic bioreactors capable of supporting dense suspensions of cells by continuous perfusion have been reported to date. Here, we present a microbioreactor system that supports continuous perfusion and show that this system: (i) supports robust microbial growth; (ii) provides a well-regulated environment for cultivation and expression of therapeutically-relevant heterologous proteins; and (iii) serves as a platform technology for testing conditions for cultivation by perfusion that promote improved protein expression.

The perfusion-capable microscale bioreactor chip ("perfusostat") comprises a spin-coated 80 μm PDMS membrane chemically bonded to flanking layers of CNC machined PC with integrated valve architecture and analytics as described previously (Fig. 1). Briefly, three interconnected growth chambers create a combined effective working volume of 1.0 mL. Four automatically refillable 40 μL on-chip reservoirs sourced by pressurized media bottles supply fresh media to the growth chambers. Periodic pressure-driven deflections of the PDMS membrane facilitate homogeneous mixing of the culture. Dissolved oxygen control was achieved by regulating the concentration of oxygen in the gas mixture used to drive mixing, thereby promoting diffusion of oxygen through the PDMS membrane. Perfusion was achieved by collecting sterile culture fluid passed through a 1 cm diameter 0.2 μm polyethersulfone (PES) membrane integrated into one of the three growth chambers. A separate outlet channel permitted unfiltered sampling of the growth chamber contents for offline analysis.

Cultivations in the perfusion-capable device were monitored and controlled using a combination of on- and offline sensors as previously described using an integrated microbioreactor control module. "Fluorescence lifetime" measurements of DO and pH were acquired using sensor patches embedded in the growth chamber (Fig. 1). Optical density was assessed at 630 nm using an LED and a photodiode detector integrated into the

hardware module on opposing sides of the bioreactor chip. Online temperature was measured using an integrated circuit temperature sensor.

Both mammalian and microbial cells are used commercially to make biologic drugs. *Escherichia coli* and *Saccharomyces cerevisiae* are common microbial hosts for producing biopharmaceuticals, but the methylotrophic yeast *Pichia pastoris* is an alternative platform used to manufacture two FDA-approved therapeutic proteins. Moreover, *P. pastoris* represents a promising alternative to traditional mammalian hosts owing to its genetic stability, capacity for cultivation at high densities, and capability to produce fully-humanized post-translational modifications. Given its industrial relevance and our own experience with this host, we elected to use *Pichia pastoris* to demonstrate the capabilities of the perfusostat device.

Perfusostat fermentations were performed with strains of *Pichia pastoris* (NRRL 11430) modified to express either recombinant human growth hormone (rhGH) or recombinant interferon alfa-2b (rIFNα-2b) under the control of the alcohol oxidase-1 (AOX1) promoter. Working cell banks (WCBs) of these strains were prepared as described in the ESI. Except where otherwise noted, fermentations were performed using a three-phase fermentation protocol. Briefly, WCB aliquots were thawed, re-suspended in buffered glycerol-complex medium (BMGY; see ESI for protocol), and inoculated at a cell density of $\sim$0.1 OD as measured offline (600 nm, 1 cm pathlength). Cultures were then grown in BMGY (25 °C, 20% air. sat., and 1.0 mL/h perfusion rate). Upon reaching a cell density of 0.75 OD as measured on-line (630 nm, 508 um pathlength), closed-loop feedback control was enabled and periodic metered ejections allowed maintenance of this optical density. Cultures continued under biomass control for $\sim$9 h (3 doubling times) to acclimate the cells to the cultivation environment and setpoints. Exchange of BMGY media for buffered methanol-complex medium (BMMY; see ESI for protocol) was performed by filling and evacuating the growth chamber 30 times in 30 minutes. This exchange induced protein production, and cultures were then maintained at 25 °C, 20% air. sat., and a perfusion rate of 0.75 mL/h. pH was adjusted during cultivations by addition of fresh buffered media at pH 6.0. Mixing of growth chamber contents occurred at a membrane deflection frequency of 2 Hz. A schematic of this three-phase cultivation protocol is provided in the ESI (Fig. S1).

Cellular viability during cultivation is a common critical parameter in bioprocess development. We first confirmed that the bioreactor provided a suitable cultivation environment for *Pichia pastoris* when operated as a perfusostat. Duplicate cultivations of both drug-producing strains in the chips grew in an exponential fashion, as expected, and stabilized at a preset online OD_{630} of 0.75 under closed-loop biomass control (Figs. S2a,b). Minor variations in the cell densities at inoculation likely altered the time required to reach the stated optical density setpoint for each culture. The mean calculated specific growth rate (μ, h^{-1}) across all four cultivations (Fig. 2) was 0.306 h^{-1} ± 0.006 h^{-1}. These data agree well with reported values for μ in *P. pastoris* grown in shake flasks (0.17 h^{-1}). They also suggest that our perfusostat bioreactor chip provides a growth environment comparable to a typical shake flask.

We then confirmed that cultures of *Pichia pastoris* in the perfusostat chips displayed normal kinetics of protein expression. Working cell bank aliquots of the hGH-secreting strain were reconstituted directly in BMMY induction media at an offline cell density of ∼12 OD_{600}. Replicate perfusostats were seeded with these suspensions and operated at a perfusion rate of 1.0 mL/h. Immediately following inoculation, both cultures experienced a brief (∼3 h) exponential growth phase (data not shown), likely due to the mixed carbon source (glycerol/methanol) initially available in the system. Measurable levels of secreted hGH were detected from both cultures within 4-6 hours of inoculation (Fig. 3a). Furthermore, after initial detection of protein, both perfusostats continued to yield comparable and consistent rates of hGH accumulation for an additional 16 h (Fig. 3b). These results suggest that our perfusion-capable bioreactors provide a suitable environment for protein expression and secretion by *P. pastoris*.

We then evaluated the ability of the perfusion-capable microfluidic bioreactors to screen conditions that promote enhanced protein expression. Given that induction of heterologous protein expression by methanol feeding is critical to developing conditions for fermentation with *P. pastoris*, we first screened a set of non-standard induction media. Specifically, we tested whether or not induction of protein production with methanol concentrations greater than 1% (v/v) could improve secreted titers of hGH. Four devices were inoculated with separate aliquots of the hGH working cell bank and outgrown in pre-mixed BMGY. In each device, a separate BMMY blend (i.e. 1%, 2%, 5%, or 10% methanol (v/v)) was mixed in real-time from concentrated stock solutions of individual media components. Cultivation under 2% methanol (v/v) conditions yielded an improvement in hGH expression rates over standard BMMY (1% methanol) within ∼48 h post-induction (Fig. 4a). Concentrations of methanol above 5% (v/v) negatively impacted protein expression, likely resulting from decreased culture viability due to prolonged exposure to methanol. Nonetheless, the rate of hGH accumulation under each separate methanol concentration tested remained essentially constant with time (Fig. 4b). These observed differences in stable secreted protein accumulation rates may indicate achievement of different conditionally dependent metabolic states by each culture tested.

In addition to media development, optimizing conditions for perfusion requires matching the cell density in the bioreactor with the perfusion rate through the bioreactor to ensure proper nutrient addition and metabolite removal. Maximum titers of secreted protein are typically achieved using a "push-to-low" strategy where the highest sustainable cell density at a given perfusion rate is determined. We applied the push-to-low methodology to hGH fermentations. Two perfusostat devices were inoculated and outgrown to a stable online OD_{630} of either 0.5 or 0.75 at a perfusion rate and mixing frequency of 1.0 mL/h and 2 Hz, respectively. Media was exchanged to induce the cultures and the perfusion rates were lowered stepwise from 1.0 to 0.25 mL/h at a mixing frequency of 4 Hz. Each perfusion rate was maintained for a period of approximately one day (∼8 doubling times) to enable acclimation of the cultures to the new cultivation conditions. Samples of perfused cell culture fluid were collected during the last 8 hours (∼3 doubling times) of each period and analyzed for protein titer by ELISA (see ESI). As expected, the titers increased as the perfusion rate decreased to 0.5 mL/h for both cell densities tested (Fig. 5). Furthermore, the magnitudes of the stepwise increases in titer were similar across both cell densities,

potentially indicating that the nutrient demands or metabolite production do not differ significantly with these densities. Titers of hGH measured at a perfusion rate of 0.25 mL/h did not trend as expected based on the measurements at other perfusion rates. We believe that this outcome likely resulted from inadequate mixing. Culture viscosities and cell viabilities were also non-optimal at this lowest perfusion rate.

Many of the benefits typically associated with perfusion—smaller equipment volumes, reduced operating expenses, and streamlined integration of unit operations—are predicated on the ability of these processes to sustain cultivations under stable conditions. As such, any system designed for perfusion cultivation must maintain key cultivation parameters and cellular productivity at constant levels for extended periods of time. To this end, we sought to demonstrate that the perfusostat devices could maintain a *P. pastoris* culture at stable levels of OD, DO, and protein secretion for an amount of time comparable to our previously reported non-perfusion bacterial growth studies (~500 h). To test the long-term stability, we used duplicate fermentations of both hGH- and IFNα-2b-secreting strains (1% methanol (v/v) and 0.75 mL/h perfusion rate). Online OD_{630} and DO setpoints were stably maintained for ~280 h post-induction (Fig. S3) with oscillatory deviations in DO of comparable amplitude to those commonly reported for small-scale *Pichia* fermentations. All four cultures achieved similar levels of protein secretion within 24 h of induction (Fig. 6a,b). Moreover, all four cultures continued secreting either hGH or IFNα-2b at stable levels through an additional 260 h of fermentation time.

Conclusions

Here, we present a perfusion-capable microfluidic bioreactor that provides a suitable environment for sustained recombinant protein expression from a microbial host organism. To our knowledge, this is the first demonstration of heterologous protein production from a microbe cultivated under fully continuous perfusion conditions. Based on our testing of various media formulations and perfusion rates, we believe that our system can serve as a platform to screen for conditions that improve secreted protein titers. By implementing rigorous feedback control over key process parameters (e.g. OD, DO, perfusion rate), our devices maintained cultures of hGH- and IFNα-2b-expressing strains of *P. pastoris* in regimes of stable protein production for ~11 days. This capability should enable scale-down studies to be run for the same periods of time as typical industrial processes (7 - 30 days). Traditional scale-down models such as shake flasks and microtiter plates do not offer consistent control for these lengths of time. Moreover, the ability of our system to provide a controlled environment over long time-scales, coupled with its small working volume (1 mL) and simplicity of multiplexing, could enable high-throughput, parallelized studies of cell biology and genetics (*e.g.* genetic drift) under actual process conditions. Consequently, moving forward, we aim to demonstrate the comparability of our system to larger reactor systems, using both microbial and mammalian hosts, to fully enable its use in design-of-experiment studies for industrial process development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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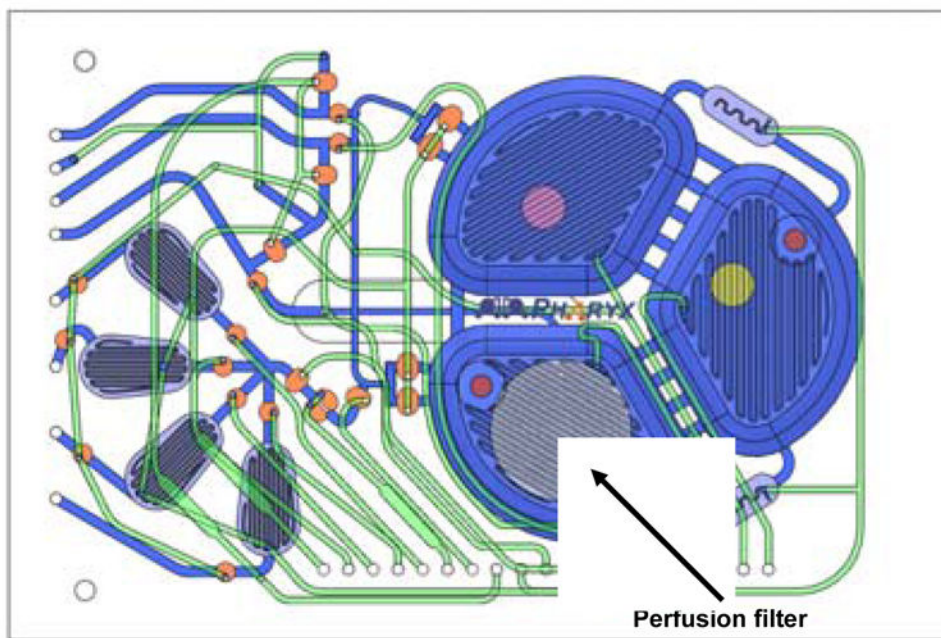
Perfusion filter

Fig. 1. Schematic representation of the perfusostat device
Fluid enters the growth chambers (blue) through four 40 uL reservoirs (purple) by the selective actuation of various valves (orange). Product-containing perfused media exits the device through a 0.2 um PES membrane (grey; labeled). Dissolved oxygen and pH measurements are acquired by patch-based sensors (pink and yellow) embedded within the top- and right-most growth chambers. Each chip incorporates two 508 μm path lengths (red) for OD_{630} sensing.
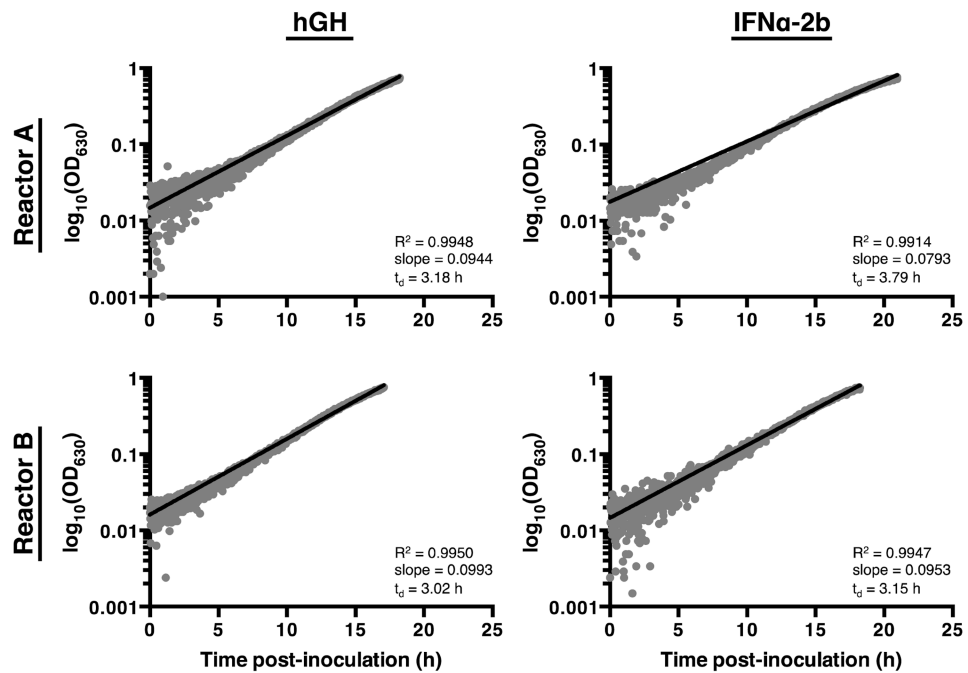
Fig. 2. Determination of *P. pastoris* growth characteristics under perfusion conditions
hGH- and IFNα-2b-secreting strains of *P. pastoris* were inoculated in duplicate at an offline OD_{600} of ~0.1 and grown under perfusostat conditions on non-inductive BMGY media. The "exponential regions" of all four growth profiles were log_{10} transformed and linear regressions were fit to the transformed data. For all growth curves, the "exponential region" was defined as the portion of the profile between t = 1 h and the initiation of closed-loop biomass control at online $OD_{630} = 0.75$.
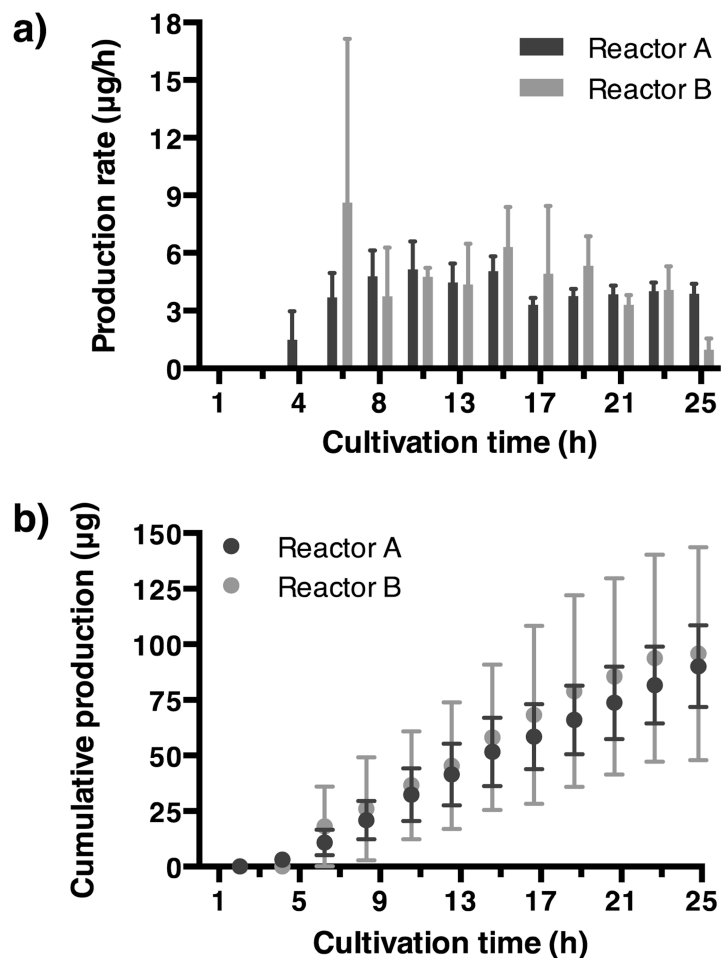
Fig. 3. Determination of the kinetics of heterologous protein expression by *P. pastoris* under perfusion

a) hGH production rate (µg/h) as a function of total cultivation time (h) for two parallel perfusostat fermentations of *P. pastoris*, as determined by capillary electrophoresis. Both cultures were inoculated directly in induction media (BMMY) at an offline OD_{600} of ~12. Cultures were grown to a steady-state online OD_{630} of 0.75 and maintained at this cell density for the duration of the experiment. b) Offline OD_{600} normalized values of total hGH produced (µg) as calculated from the data presented in a) and plotted as a function of time post-induction (h). Error is represented as the range in technical duplicate capillary electrophoresis measurements.
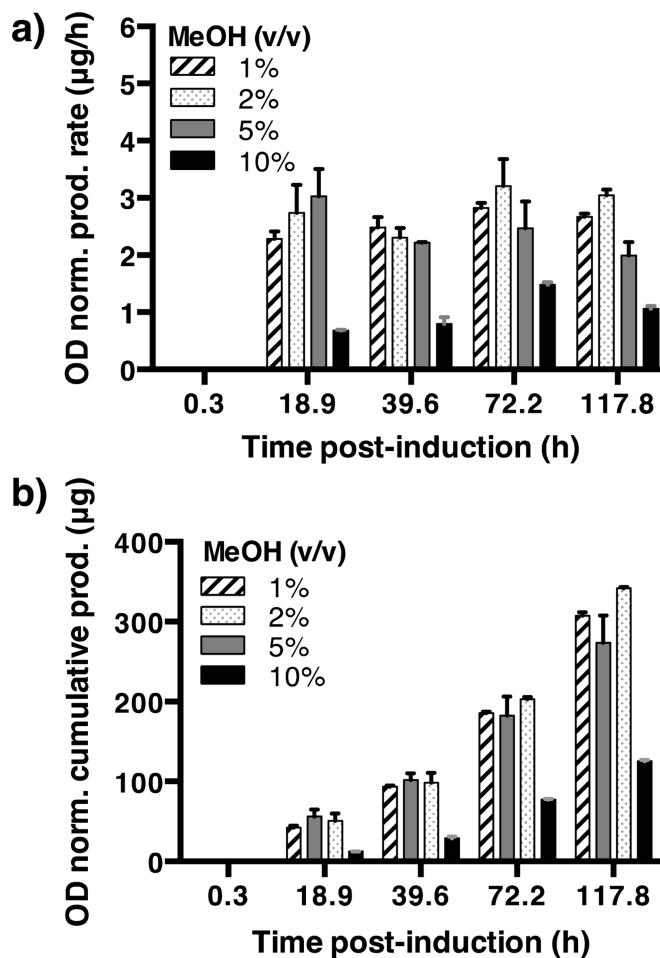
Figure 4. hGH expression by *P. pastoris* as a function of inducer (methanol) concentration
a) Offline OD_{600} normalized hGH production rate (µg/h) versus time post-induction (h) for each of four separate cultivations using induction media (BMMY) with distinct methanol concentrations, as determined by capillary electrophoresis. b) Offline OD_{600} normalized values of total hGH produced (µg) as calculated from the data presented in a) and plotted as a function of time post-induction (h). Error is represented as the range in technical duplicate capillary electrophoresis measurements.
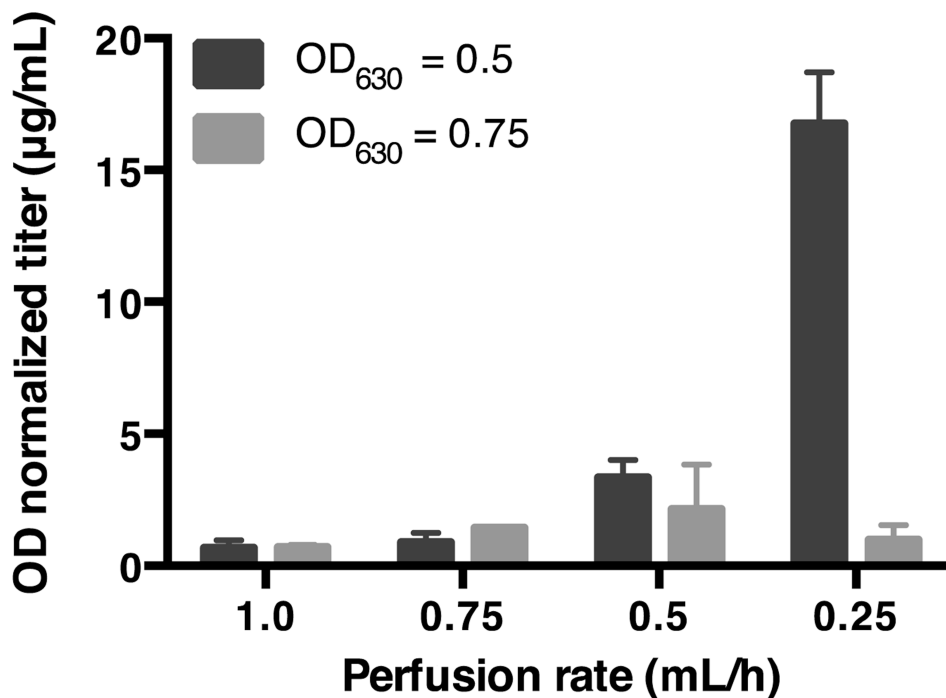
Fig. 5. hGH expression by *P. pastoris* as a function of cell density and perfusion rate
Offline OD_{600} normalized hGH titer (µg/mL), as determined by sandwich ELISA, and plotted as a multivariate function of perfusion rate (mL/h) and online OD_{630}. Each of two perfusostats was set to maintain an online OD_{630} setpoint of either 0.5 or 0.75 and was stepped down through perfusion rates from 1.0 mL/h to 0.25 mL/h in 0.25 mL/h increments. Error bars represent 1σ.
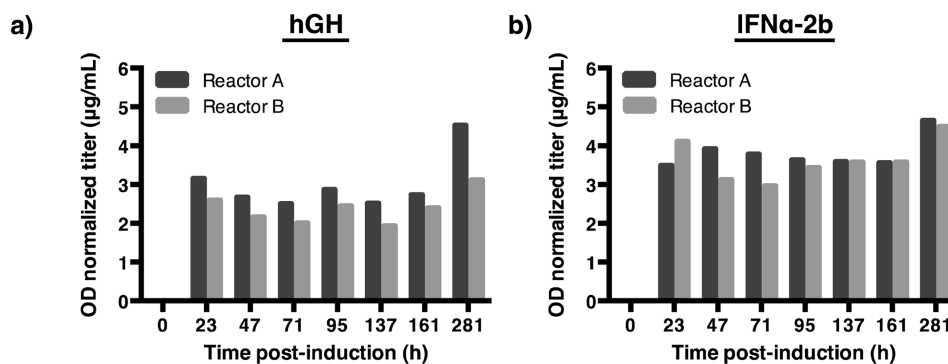
Fig. 6. Perfusostat cultivation sustained long-term culture productivity

a) Offline OD_{600} normalized hGH titer (µg/mL), determined by capillary electrophoresis, plotted as a function of time post-induction (h), and presented in biological duplicate. b) Offline OD_{600} normalized IFNα-2b titer (µg/mL), determined by capillary electrophoresis, plotted as a function of time post-induction (h), and presented in biological duplicate.