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TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Productivity Studies Utilizing Recombinant CHO Cells in Stirred-Tank Bioreactors:

A Comparative Study Between Pitched-Blade and Packed-Bed Bioreactor Systems

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The pitched-blade system is traditionally the choice for stirred tank bioreactors in large-scale production of animal cells in suspension culture. The packed-bed basket technology developed by New Brunswick Scientific provides a shear-free environment for large-scale (up to 100 L) production of animal cells. At present, little information is available on the utility of this system for the production of secreted proteins, especially in perfusion mode of operation.

The perfusion process provides a homeostatic environment for optimal cell growth similar to that experienced by cells *in vivo*. In contrast, the batch culture approach does not appropriately model this homeostatic environment due to the depletion of nutrients and accumulation of waste products in the culture system. Thus, the objective of this study was to compare the growth and productivity of alkaline phosphatase (ALKP)-secreting Chinese hamster ovary (CHO) cells cultured in these two bioreactor types: pitched-blade bioreactors operated in batch mode versus packed-bed bioreactors operated in perfusion mode. CHO cells cultured in the packed-bed bioreactor, operated in perfusion mode, produced greater amounts of ALKP compared to cells cultured in the pitched-blade system run in batch mode.

These observations suggest that continuous exposure of cells to fresh culture media and the shear-free culture environment provided by the Fibra-Cel growth matrix disks offered more favorable growth conditions for CHO cells, allowing for either greater cell proliferation (higher density) or greater protein production on a per-cell basis. Overall, the results of this comparison study suggest that packed-bed bioreactors provide significant advantages for moderate-scale production of cells. The benefits of this bioreactor system may translate to large-scale cell culture for generating secreted protein products useful in medical applications.

Introduction

The biomedical industry employs bioreactors for the medium- to large-scale production of cells and cell products such as pharmacological drugs and antibodies. The most common type of bioreactor is the pitch-blade (or stirred-tank) system, operated in either batch or perfusion mode, in which cells are cultured in suspension with constant media agitation. However, there are significant limitations for application of this bioreactor type to the generation of cells in culture, including the inability to grow adherent cells, the adverse effects of shear stress on cells constantly in motion, and the limitation on ultimate cell density. The packed-bed bioreactor system provides an alternative to the pitched-blade system that addresses some of these key deficiencies. The packed-bed bioreactor creates a homeostatic environment similar to the environment found *in vivo*, where waste products are constantly removed and fresh nutrients are replenished. Batch cultures do not provide a homeostatic environment due to the depletion of nutrients and the accumulation of waste products.[1]

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Cells cultured in packed-bed bioreactors are not exposed to hydrodynamic forces like pitched-blade bioreactors, thus allowing for maximum cell growth and protein expression.[2] Long run times allow the packed-bed bioreactor to decrease the constant need for reseeding cells and reestablishing seed cultures thus reducing setup time and labor dramatically. Secreted products are automatically separated from cells in perfusion, eliminating filtration and membrane fouling. Perfusion techniques in pitched-blade bioreactors are cumbersome and complex, usually requiring one of three techniques: centrifugation, filtration, or sedimentation. The packed-bed bioreactor eliminates the need for all of these techniques as the cells are trapped in-between the Fibra-Cel growth matrix disks (New Brunswick Scientific) allowing for media changes without the loss or damage of cells.[3]

Packed-bed bioreactors have been compared with microcarrier systems[2] and perfusion systems have been compared to fed-batch systems.[4, 5] Perfusion and batch cultures are two common modes of operation for scale-up of mammalian cells in culture. However, to date, no studies have compared the productivity of protein-secreting cells in packed-bed bioreactors in perfusion mode to pitched-blade bioreactors in batch mode. Therefore, the objective of this study was to determine whether cells cultured in a packed-bed bioreactor in perfusion mode would generate more protein product than cells cultured in a pitched-blade bioreactor in batch mode. We expect that the results of this research project will aid in the introduction of packed-bed bioreactors in perfusion mode as a desirable and productive approach to large-scale animal cell culture.

Materials and Methods

Culture Procedures

In order to evaluate the impact of these bioreactor systems on protein secretion by cultured cells, we employed a recombinant ALKP-secreting CHO cell line (rCHO), generously provided by CDI Bioscience, Inc. The rCHO cells were engineered with the isopropyl β-D-1-thiogalactopyranoside (IPTG)-regulated RP shift vector so that the rCHO cells stop replicating and shift to protein production when induced with IPTG. CD-CHO medium (Gibco, Life Technologies) was used throughout these experiments. The media contains 6.3 g/L glucose and was supplemented with 8 mM L-glutamine and 100 µg/mL of an antibiotic/antimycotic solution (Invitrogen, Life Technologies). Frozen rCHO cells were thawed and transferred to T-75 flasks with chemically-defined CHO serum-free medium and allowed to expand. Once a sufficient number of cells was achieved, sterile disposable spinner flasks were utilized to further expand the cells. Subculture of the cells continued until a sufficient number of viable cells was achieved for use as a seed culture at the density of approximately 5×10^5 cells/mL. Two New Brunswick Scientific BioFlo® 310 advanced benchtop stirred-tank bioreactors were utilized to grow the rCHO cells.

Pitched-Blade Impeller Bioreactor Operated in Batch Mode

The first bioreactor system utilized the pitched-blade impeller. The blades on the pitched-blade impeller are flat and set at a 45° angle. This blade orientation provides good axial and radial mixture of the media while also increasing the oxygen mass transfer rate and disruption of bubbles released from the sparger. The pitched-blade impeller is designed to minimize stress of mixing on shear-sensitive cells.[6]

Three experimental trials were performed using the pitched-blade bioreactor in batch mode in a 2.2 L total volume vessel (1.7 L working volume). For each trial, the bioreactor was allowed to operate until the cell concentration reached approximately 2×10^6 cells/mL at which time the cells were induced with IPTG. All of the experimental trials had the following parameters: propeller speed set to 120 rpm (± 5 rpm), dissolved oxygen at 35% (±1%), temperature at 37.0°C (±0.1°C), and pH at 7.10 (±0.01).

Packed-Bed Basket Impeller Bioreactor Operated in Perfusion Mode

The second bioreactor system utilized the packed-bed basket impeller. This bioreactor system is suitable for both anchorage-dependent and suspension cells, and this system does not require the adaptation of anchorage-dependent cells to suspension culture. The packed-bed basket impeller is commonly used in the production and collection of extracellular proteins.[7] The packed-bed basket impeller incorporates a basket with two horizontally positioned perforated metal screens. Fibra-Cel disks are placed in between the metal screens creating a bed to entrap suspension cells or provide a surface for attachment of anchorage-dependent cells. The Fibra-Cel disk bed provides a culture environment that allows freshly oxygenated media to slowly pass over the cells while also providing protection from external shear forces.[6] The rotation of the impeller creates a negative pressure that draws media up through the hollow center shaft where the sparger introduces oxygen to the media. The packed-bed bioreactor is the ideal system to use when a product is secreted out of the cell. Because cells are immobilized in the Fibra-Cel bed, samples of media can easily be removed without cell loss or culture disruption.

Three experimental trials were performed using the packed-bed bioreactor operated in perfusion mode in a 2.2 L total volume vessel (1.75 L working volume). The vessel was equipped with a basket, as described earlier, containing 85 g of Fibra-Cel disks. The perfusion process was initiated once the cells reached the exponential growth phase. On day 1 of perfusion, 0.5 L of the media was removed from the vessel and replaced with fresh medium using a peristaltic pump. On day 2, 1 L of media was similarly replaced; subsequently, 2 L of culture media was replaced every other day (up to day 15). Otherwise, all experimental trials had the same growth conditions (temperature, oxygen, pH) as the batch process.

Biomarkers of Cell Growth and Productivity

Cell productivity was assessed by measuring activity of the secreted ALKP protein using an enzyme assay (AnaSpec, Inc.) according to the manufacturer's protocol. A unit (U) of ALKP activity was defined as the amount of enzyme that hydrolyzes 1 µmol of p-nitrophenylphosphate to p-nitrophenol in a total reaction volume of 1 mL in 1 minute at 37 °C. The YSI 2700 Select Biochemistry Analyzer (YSI, Inc.) was utilized to monitor the glucose and lactate levels in the culture media every 24 hours for the duration of each trial.

Results

Pitched Blade Impeller Bioreactor Operated in Batch Mode

Cell Density and Viability

Figure 1 shows cell growth and viability in three independent experiments in the pitched-blade bioreactor. Seeding densities were $0.29 - 0.57 \times 10^6$ cells/mL as calculated by trypan blue staining utilizing the Countess® Automated Cell Counter (Invitrogen, Life Technologies). A slight decrease in cell density occurred in experiments 1 and 2 between days 3 and 4, suggesting that the optimal time for induction occurred on day 3. At the time of induction, cell densities were 1.5×10^6 cells/mL in both experiments 1 and 2 (Figure 1A-B). The maximum cell density observed in experiment 1 (2.7×10^6 cells/mL) was relatively lower than in experiment 2 (3.5×10^6 cells/mL). Due to the rapid exhaustion of glucose in experiment 3, it was necessary to induce the cells on day 3. Cell density in experiment 3 was 2.0×10^6 cells/mL at the time of induction, and the maximum cell density observed was 2.2×10^6 cells/mL (Figure 1C). Cell viability was greater than 90% in all experiment trials with the pitched-blade bioreactor.

Glucose Utilization and Lactate Production

Glucose is the main energy source for cell proliferation and ALKP production. Thus, glucose levels and cell densities were expected to directly correlate with ALKP production in each experiment. Because lactate is a

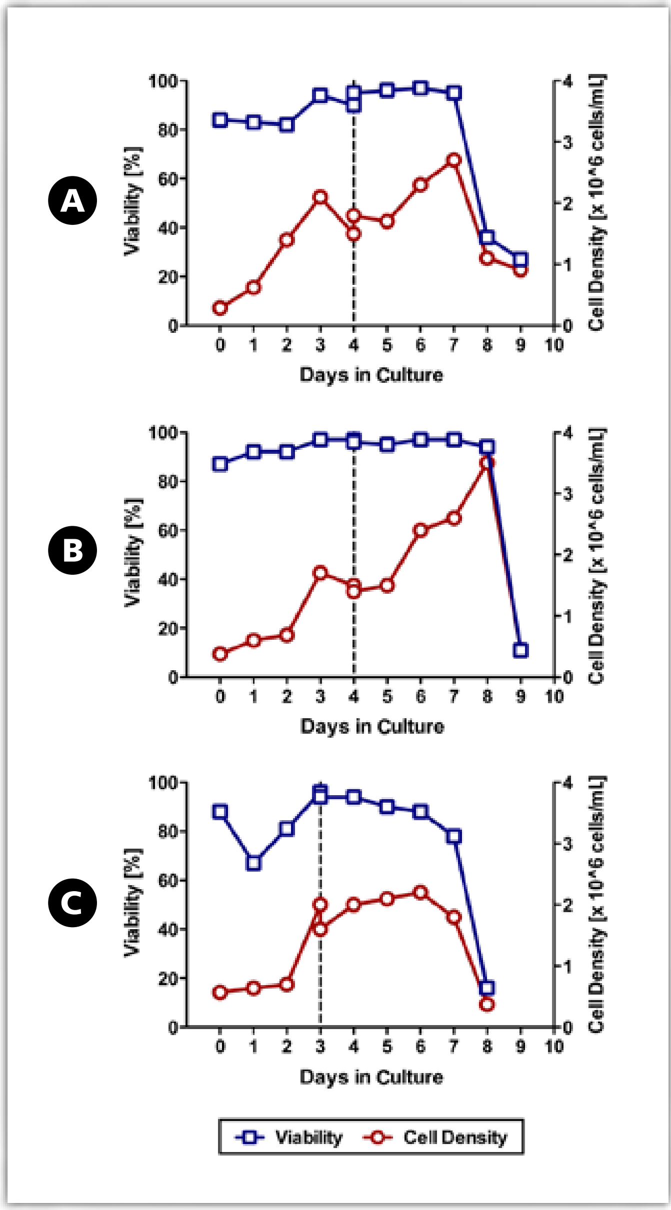

FIGURE 1. **Growth of rCHO cells in pitched-blade bioreactor system.** Values shown are the cell density and viability on each day of culture, and each panel represents an independent experiment trial [(A) trial 1; (B) trial 2; (C) trial 3]. The dashed line indicates the time of induction of ALKP production by IPTG.

secondary energy source, lactate levels were expected to decline following this initial increase and the utilization of glucose in the media. Lactate metabolism is beneficial to the system by reducing a major metabolic by-product, glucose, from the system.[8, 9] Glucose levels measured at the time of induction were 2.6 g/L in experiments 1 and 2, while glucose levels measured at the time of induction in experiment 3 were nearly depleted at 0.8 g/L (Figure 2). Glucose levels in the first two experiments were sufficient to sustain ALKP production. However, glucose consumption by the cells in experiment 3 depleted amounts necessary to support the production of ALKP. Lactate levels in experiment 3 were nearly 1.5 g/L higher than the previous experiments, suggesting that cell replication may have been inhibited (Figure 2C). Similar results were reported by others using CHO and NS0 cells.[8]

ALKP Production

Figure 3 shows the concentrations of ALKP measured daily or at the time of cell harvest in each of the three independent replicated experiments utilizing the pitched-blade bioreactor. ALKP concentrations steadily increased

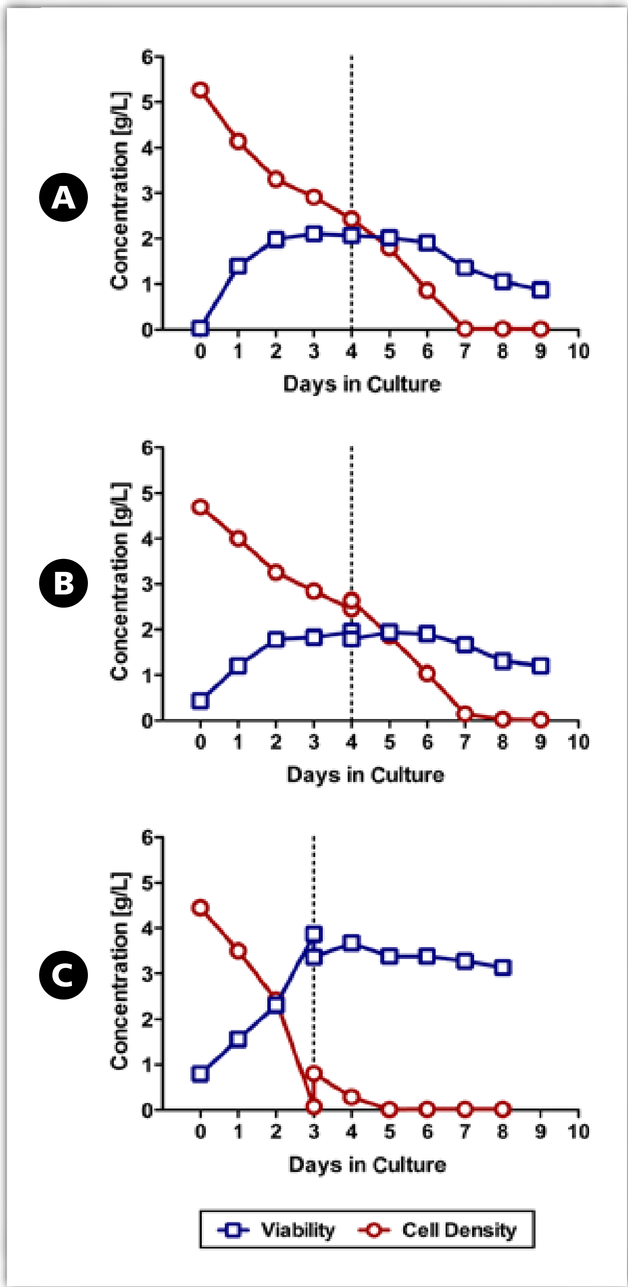

FIGURE 2 (right). **Glucose consumption and lactate production by rCHO cells cultured in the pitched-blade bioreactor system.** Values shown are the concentrations of glucose and lactate in the culture media measured daily, and each panel represents an independent experiment trial [(A) trial 1; (B) trial 2; (C) trial 3]. The dashed line indicates the time of induction of ALKP production by IPTG.

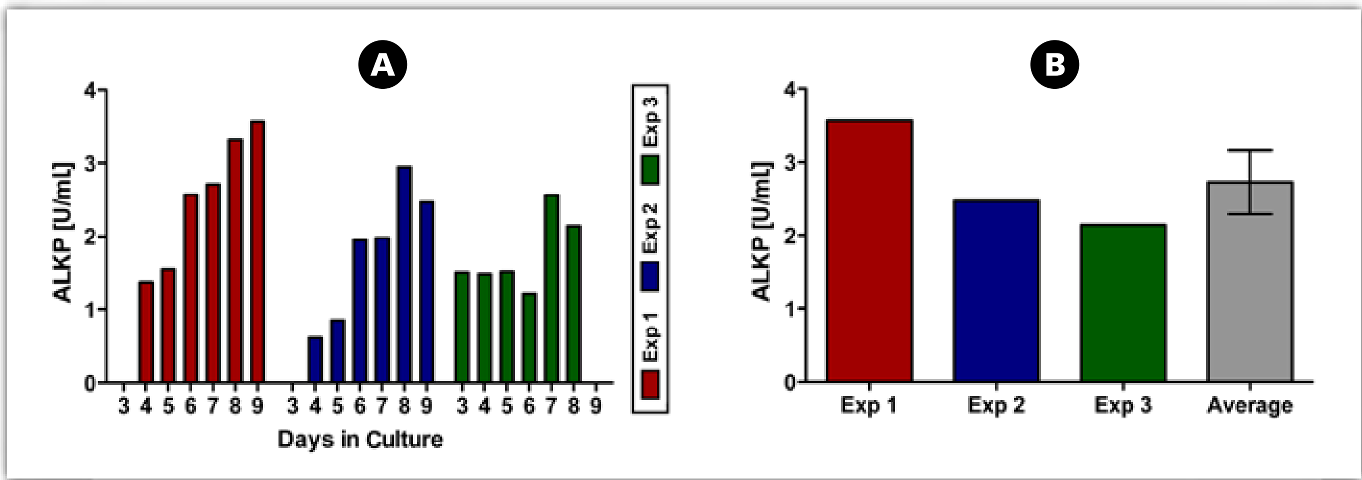

FIGURE 3 (below). **ALKP production by rCHO cells cultured in the pitched-blade bioreactor system.** (A) ALKP concentration in culture media measured each day of each experimental trial. IPTG induction of ALKP occurred on culture day 4 for experimental trials 1 and 2 and on day 3 for experiment 3. (B) Values shown are the media ALKP concentrations at time of cell harvest for all three experimental trials; also shown is the mean total production of ALKP ± standard error of measurement (SEM) for all three experimental trials.

over the six culture days post induction. However, a decrease in ALKP activity was observed at the time of harvest in trials 2 and 3, possibly due to degradation of the ALKP protein at the end of the experiment.[10] Because serum-free media was utilized for growth of rCHO cells in this study, ALKP was susceptible to the action of proteases made by the rCHO cells.[11] As compared to the first trial, the increased cell density observed in experiment 2 indicates that cell proliferation was more predominant than the production of ALKP (Figure 3). As reported above, glucose levels and cell density in trial 3 were the lowest of all the replicate experiments, and these observations likely account for the low amount of ALKP detected.

Packed-Bed Basket Impeller Bioreactor Operated in Perfusion Mode

Cell Density and Viability

The packed-bed bioreactors in all three replicate experiments were seeded with 5.0×10^5 cells/mL. However, because of the presence of the Fibra-Cel discs, it was not possible to sample the cells directly during culture to determine yield and viability. Therefore, cell count and viability were not monitored on a daily basis; rather, the rate of glucose consumption was used as a surrogate to approximate changes in cell density.[12] The growth of cells in the packed-bed reactor was estimated using the average glucose consumption rate and growth rate of cells in the pitched-blade bioreactor. Glucose consumption rates were markedly similar in both the pitched-blade and packed-bed bioreactor systems up to day 4 (Figure 4). However, after day 4 of culture, glucose utilization in the packed-bed bioreactor continued to increase exponentially while the trend for glucose consumption in the pitched-blade bioreactor increased linearly. Increased glucose consumption observed on culture day 5 suggests that cell density in the packed-bed bioreactor had likely increased.

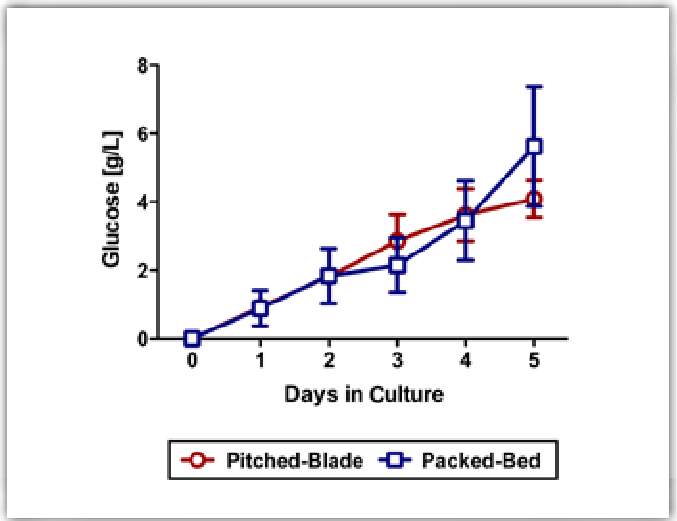

FIGURE 4. **Comparison of glucose utilization by rCHO cells in pitched-blade and packed-bed bioreactor systems.** Values shown are the mean daily glucose media concentrations ± SEM of three replicate experiments.

Glucose Utilization and Lactate Production

Most of the glucose consumption by the cells in experiment 1 was observed prior to media exchanges during the perfusion process (Figure 5A). Cells in experiment 2 utilized the least amount of glucose in the media prior to repeat media exchanges (Figure 5B) whereas the majority of glucose used by the cells in experiment 3 occurred within the first 24 hours after each media exchange (Figure 5C). As previously observed with the pitch-blade bioreactor system, media lactate concentrations increased in response to decreasing glucose availability in all three trials (Figure 5).

ALKP Production

Concentrations of ALKP in the three independent experiments utilizing the packed-bed bioreactor are shown

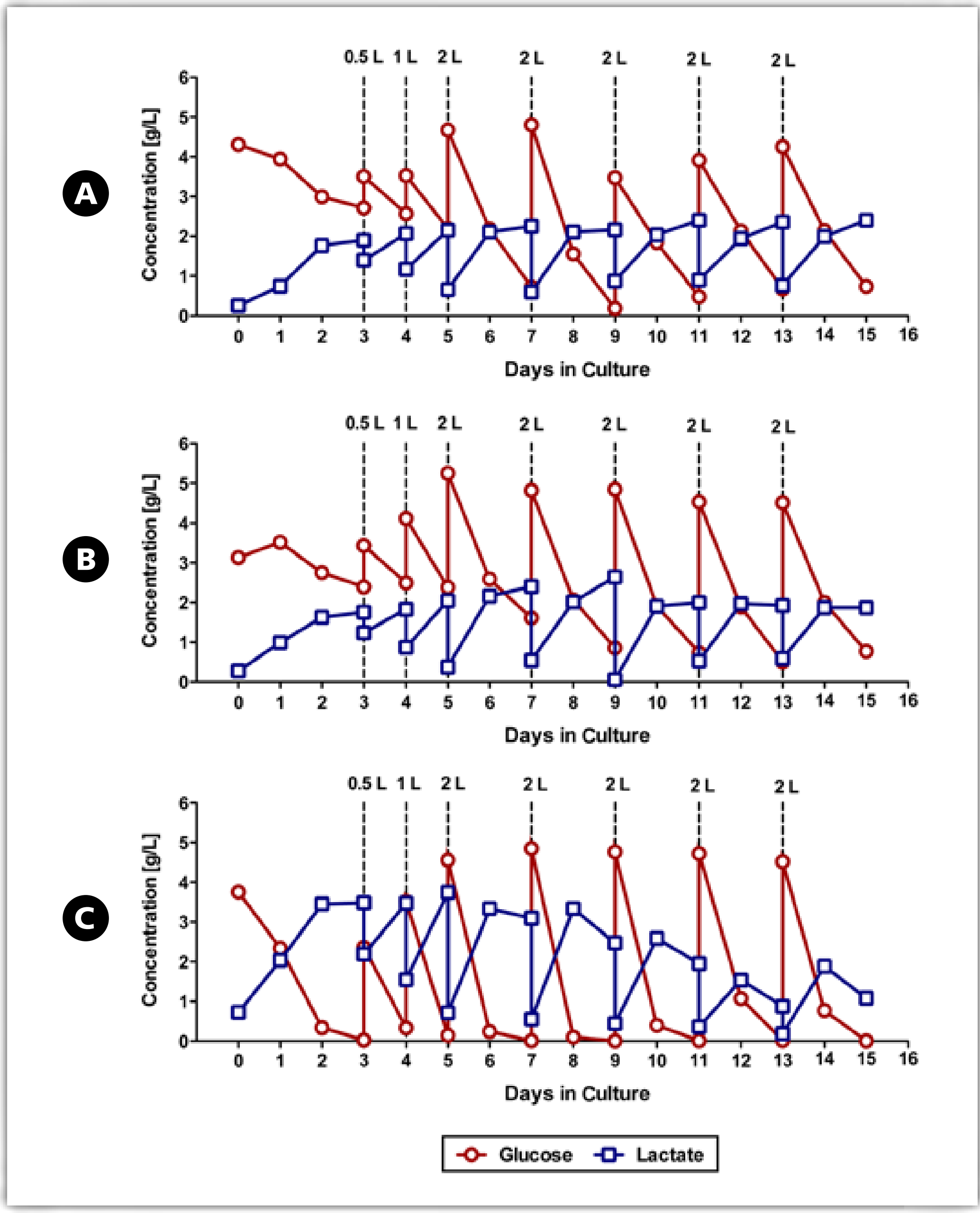

FIGURE 5. **Glucose consumption and lactate production by rCHO cells cultured in the packed-bed bioreactor system.** Values shown are the amounts of glucose and lactate measured in the culture media at each media exchange. The time and volume of the media exchange is indicated at each dashed line. Induction of ALKP activity by IPTG began on culture day 5 and continued every two days throughout the remainder of the experiment. Results of three experimental trials are shown [(A) trial 1; (B) trial 2; (C) trial 3].

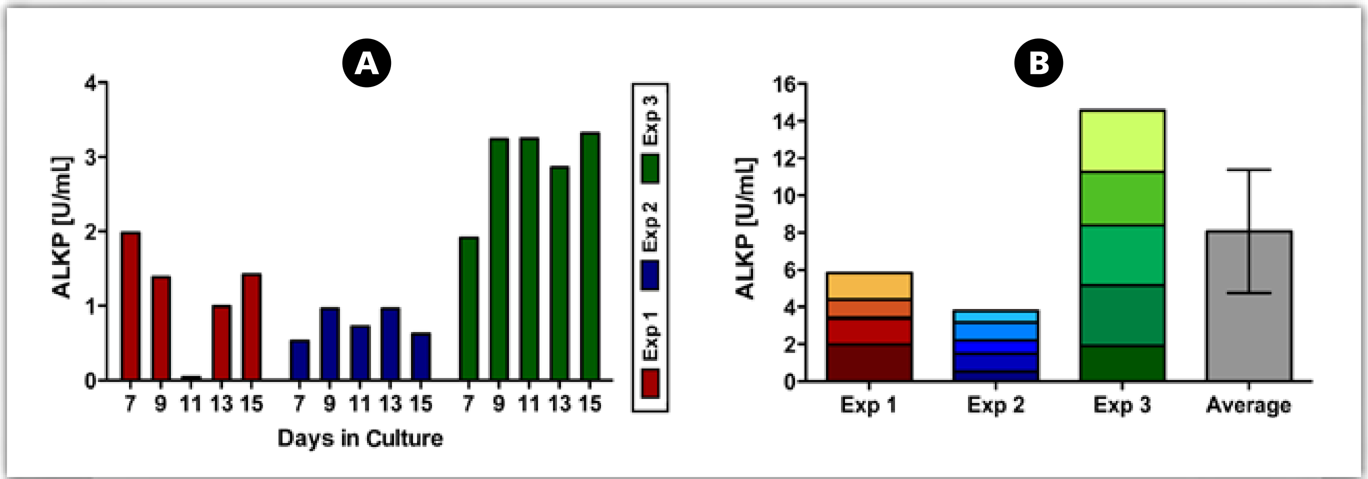

FIGURE 6. **ALKP production by rCHO cells cultured in the packed-bed bioreactor system.** (A) ALKP concentration in culture media measured each day of each experimental trial. IPTG induction of ALKP began on culture day 5 and continued every two days for the remainder of the experiment. (B) Stacked bar charts show the cumulative production of ALKP throughout the experiment, with each bar representing a perfusion. Also shown is the mean total production of ALKP ± SEM of all three experimental trials.

in Figure 6. Following initial expansion culture of rCHO cells for five days, ALKP production was induced every two days with a media exchange containing IPTG. We determined previously that continuous culture with IPTG in the media yielded greater production of ALKP compared to a transient exposure to the inducing agent (data not shown). A modest increase in ALKP production was observed at each media exchange although the level of ALKP varied by induction day and by experiment trial (Figure 6A). The correlation between glucose utilization and ALKP production observed previously supports the inference that the rate of glucose consumption in experiments 1 and 2 was conducive to the production of moderate amounts of ALKP. However, the pattern of glucose utilization and ALKP was notably different in trial 3 (Figure 6B). The rapid exhaustion of glucose and a presumed high cell density were likely contributing factors to the noticeably greater amounts of ALKP observed in experiment 3 compared to the other trials.

Comparison of Bioreactor Systems for ALKP Production

A major objective of this study was to compare the pitched-blade and the packed-bed bioreactors run in their routine operating modes (batch and perfusion, respectively). The average total ALKP production per experiment trial is shown in Figure 7. Overall, the packed-bed bioreactor system produced ALKP to a much greater extent (nearly 3-fold greater) compared to the pitched-blade bioreactor. This pattern is retained even when accounting for different volumes of culture media used in the two bioreactor systems (1.7 L for the pitched-blade system compared to 1.3 L for the packed-bed system). The total amount of ALKP measured after five media exchanges in the packed-bed bioreactor was 10,500 U compared to 4,640 U in the pitched-blade bioreactor, or approximately a 2.25-fold greater production of ALKP in the pitched-blade bioreactor.

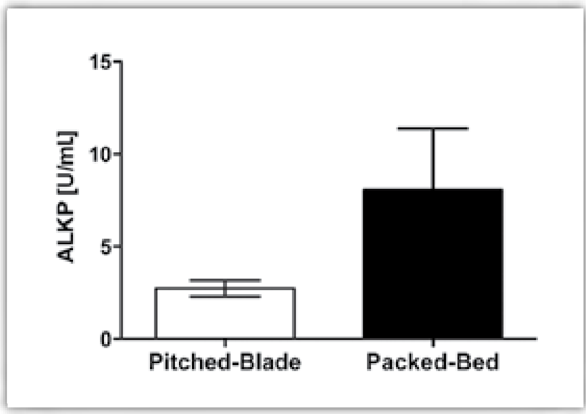

FIGURE 7. **Comparison of ALKP production by rCHO cells cultured in the pitched-blade and packed-bed bioreactor systems.** Values shown are the mean ALKP concentration ± SEM for three replicate, independent experimental trials using either the pitched-blade or packed-bed bioreactor systems.

Discussion

Although the bioprocessing industry is currently dominated by the well-proven batch culture process, results of this study suggest that a perfusion mode of culture with a packed-bed culture environment may prove superior in some applications. The perfusion mode of cell culture offers some significant advantages to large-scale cell producers, such as lower set-up and maintenance costs, and greater performance.[13, 14] By virtue of its design, the packed-bed bioreactor may be used continuously for months at a time when operated in perfusion mode. The three-dimensional environment of the Fibra-Cel disks provides optimal culture conditions for cell growth and allows for new infusions of media into the system without cell loss. Also, the shear-free culture environment of the packed-bed system offers more flexibility for researchers utilizing anchorage-dependent cell lines for the production of proteins or vaccines. Cell line maintenance is reduced since preparation of new seed cultures is not required. Moreover, initial setup efforts associated with the packed-bed bioreactor are substantially less than would be required using the pitched-blade system which requires multiple, shorter cultures to equal the production of a single culture with the packed-bed system. The perfusion process also offers several advantages such as the removal of toxic metabolites or excreted proteases and the infusion of fresh nutrients to maintain optimal culture conditions. Protein product quality is likely to be higher with the perfusion system as freshly secreted cellular proteins may be removed periodically by media exchanges.

However, one minor disadvantage of the packed-bed bioreactor is limited scalability. The packed-bed system is currently limited to a maximal operating volume of about 100 L, although larger-scale, packed-bed reactors may be available in the future. Also, when considering the cost-benefit of the packed-bed system, the additional cost of Fibra-Cel disks should be taken into account.

Although the pitched-blade bioreactor requires less monitoring over the duration of an experiment, as cells are cultured until nutrient depletion has occurred, this approach does require significant initial input in terms of labor and resources. Multiple culture runs, and thus multiple seed cultures and system preparations, are required to match the output of the packed-bed system. In addition, application of a perfusion operation mode to pitched-blade systems requires pumping the cells through a membrane filtration device (*e.g.*, hollow fiber columns) to prevent cell loss.[15] This process can impair bioreactor sterility and cause potential damage to the cells. Finally, cell product concentration may be comparatively low, as exemplified in this study, compared to the initial resources (volume of culture media) invested in the culture system. Given these drawbacks of the pitch-blade system, the added cost of the Fibra-Cel disks ultimately may be irrelevant in a cost-benefit analysis.

In summary, the results of this study show that the packed-bed bioreactor operated in perfusion mode is superior to the pitched-blade bioreactor operated in batch mode for growth of rCHO cells secreting ALKP. Given the greater productivity of cells cultured in the packed-bed bioreactor and the multitude of advantages of this system operated in perfusion mode, researchers desiring to scale up mammalian cell culture for protein production should strongly consider this strategy.

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