Performance Characteristics of Mammalian Cell Culture Process Operating Continuously with Protein-Free Medium

R. BLIEM,* R. OAKLEY, K. MATSUOKA, AND V. TAIARIOL

Bio-Response, Inc., 1978 West Winton Avenue, Hayward, CA 94545; Present affiliation: Bristol-Myers Squibb Co., PO Box 4755, Syracuse, NY

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ABSTRACT

In this communication we briefly describe a continuous industrial process for the production of Tissue Plasminogen Activator (tPA), operating with a standard, basal medium (DME/F12). Process data of two culture runs has been chosen to present a performance profile over a production period of more than 3 mo. Developmental aspects of glucose utilization (as a process parameter) are briefly discussed.

INTRODUCTION

Continuous cell culture processes may offer a variety of advantages over batch processes, such as increases in the efficiency of labor and equipment utilization (e.g., smaller peripheral vessels), improved process control, and facilitated process optimization (*see further 1–3*). Continuous culture is therefore gaining interest as a means of improving the cost efficiency of industrial cell culture processes. However, its utility places stringent requirements on a variety of process components, such as aseptic operations or cell line stability.

*Author to whom all correspondence and reprint requests should be addressed.

Another area that the process improvements may achieve is the application of protein-free culture media. Most culture media used in industrial processes still comprise protein supplements, that constitute 3–20 times the cost of the protein-free basal medium.

The industrial development of many products is hampered, and the development of some products (e.g., human urokinase from diploid cells or chorionic gonadotropin from transformed cells) has been discontinued as a result of the high cost of such medium supplements.

Other benefits of a protein-free medium accrue both upstream (e.g., quality control and assurance), as well as downstream (product purification) of the cell culture process.

Although the application of protein-free media is a development goal in animal cell technology, there are potential constraints that must be considered. For example, the addition of supplementary proteins may be necessary to quench the activity of cellular proteases released into the supernatant, or to reduce cell adhesiveness when small aggregates or single cells are desired.

The conventional approach to developing serum-free and protein-free medium formulations is to screen and select the medium components to suit the requirements of the cell line (*5,6*). For practical reasons, this is often carried out under culture conditions that bear little resemblance to conditions prevalent in the production reactor.

Integration of Cell Line, Medium, and Reactor Development

An alternative approach is to integrate the selection and development of the cell line, the medium, and the bioreactor conditions. This approach formed the starting point for the process described in this paper.

Integration was approached by selecting the cell line to suit a standard medium under process conditions. The successful application of this protein-free medium was initially attributed to the properties of the cell line alone that reportedly had been selected for growth in protein-free medium (*7,8*). Subsequent investigations, however, have shown that the particular reactor design and process conditions described in this paper, play at least an equally important role in enabling the cell to express its ability to be propagated in protein-free medium. This is based on the following observations:

1. protein-free F12/DME is only able to support the propagation of this line in T-flasks for a few generations, and
2. other cell lines (hybridomas)j that have not been selected for growth in protein-free medium, can still be sustained in this medium in packed bed reactors (PBRs) for periods extending well beyond those that are possible in stirred reactors, roller bottles, or T-flasks (manuscript in preparation).

Despite successful developments at the laboratory scale (*9; see further 5,6*), we are not aware of any industrial applications of a standard, protein-free basal medium for the continuous propagation of human cells.

MATERIALS AND METHODS

Cell Line

A strain of Bowes melanoma (a human cell line), that was originally selected for growth in protein-free medium (*7*). The line propagates well in basal medium under the process conditions described below, but exhibits a comparatively lower propagation capacity in protein-free F12/DME in T-flasks or in free suspension (spinner flasks). The seed stock was therefore amplified in 5% FCS in F12/DME (1:1) medium in roller bottles. At the time of reactor seeding, cell viability that was determined by Trypan Blue dye exclusion, was greater than 90%.

Growth and Production Perfusion Medium

Standard Coon's F12/Dulbecco's Modified Eagle's Medium (Gibco) at a 1:1 mixture (1.33 g/L glucose, 2.09 g/L sodium bicarbonate) with no additional small or large mol wt supplements (i.e., protein-free).

Bioreactor System

The bioreactor system comprises a packed bed with an external recycle loop that incorporates a silastic membrane oxygenator. The schematic layout of the system is presented in Fig. 1. The bed packing consisted of 3 mm solid glass beads. The two reactors had a bed vol of 12 (reactor used in run A) and 48 L (reactor used in run B), and a total liquid vol of the packed reactor of 6 and 20 L, respectively; the bead packing alone possesses an average void vol of about 34%.

The reactors were operated in a continuous mode, whereby the medium feed rate was increased over the culture growth phase and subsequently maintained at a constant rate (Figs. 2,3). The reactors were designed to minimize gradients across the reactor bed and to facilitate scaleup. This (basket-type) reactor design (Fig. 2) therefore differs from that of the single-bed type, previously reported by Brown et al. (*8*), Whiteside and Spier (*10*), and Griffiths et al. (*11*), in that the bed height is held constant, or within a narrow range, whereas the bed number and bed diameter are increased for the purpose of scaleup. This minimizes the scaleup difficulties posed by substrate and biomass gradients (e.g., *8*) in some long-term processes.

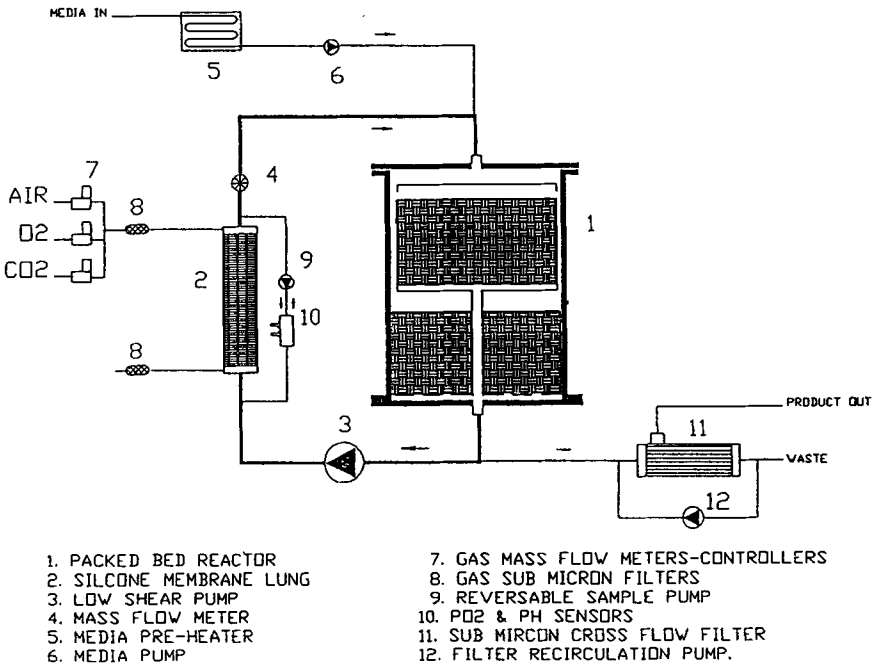

Fig. 1. Schematic layout of bioreactor system.

Operating Conditions

Process parameters that were routinely monitored and regulated include: pH, pO_2, pCO_2 (Instrument Laboratory System 1301); glucose and lactate concentration (using YSI membranes; instrument Model 2000); cell density in the recirculating loop (haemocytometer); the medium recycle rate, medium feed rate, gas flowrate, inlet O_2 and CO_2 gas composition (on-line pH and pO_2 probes (Ingold) were only used on run B). The medium recirculation rate is operated between 0.5–2 bed void vol changes per min, yielding a reactor dispersion number ranging from 0.018 to 0.04 over the course of the growth phase (for the bead packing alone) (D/uL; D, axial dispersion coefficient; u, bulk liquid velocity; L, bed height) (*12*).

Product Assay

Double chain tissue plasminogen activator (tPA) was determined by the amidolytic release of a fluorogen, and is expressed in relative units; single chain tPA is determined by plasminolytic conversion of single chain to double chain tPA 1(*13*).

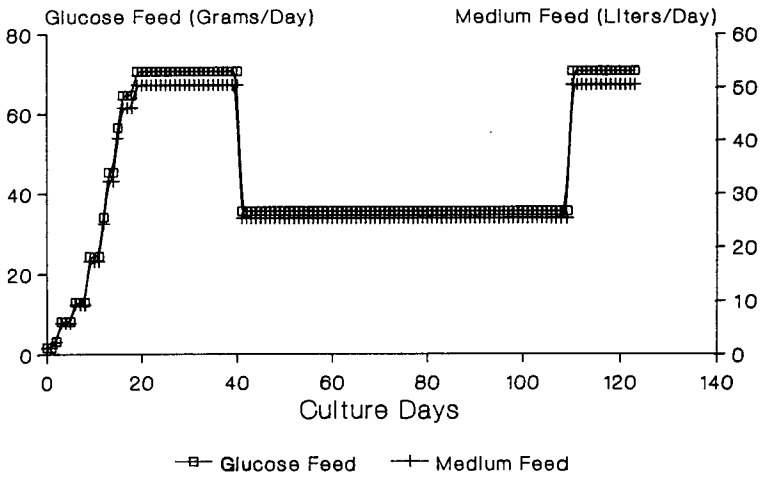

Fig. 2. Run A: medium and glucose feed rates.

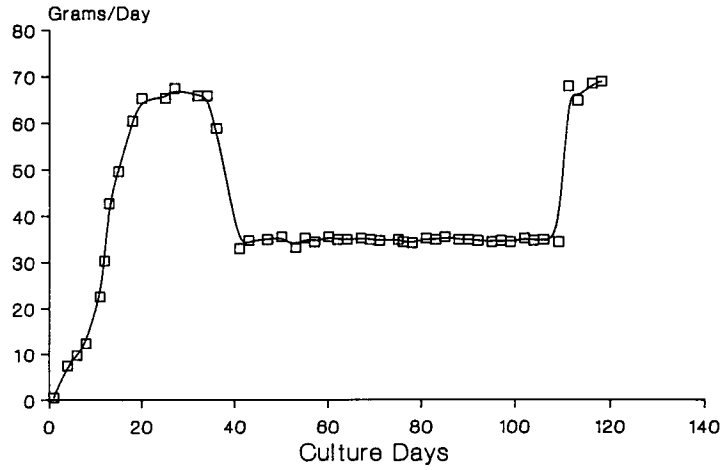

Fig. 3. Run A: glucose utilization.

RESULTS

Growth Phase

Approximately 24 h after the reactor had been inoculated with 3.3 E9 cells per L (reactor bed with greater than 90% viability), perfusion with protein-free medium was initiated. In the two present runs the cells were inoculated into 5% FCS in F12/DME. Although this Bowes strain can be inoculated into protein-free medium, the initial presence of serum does

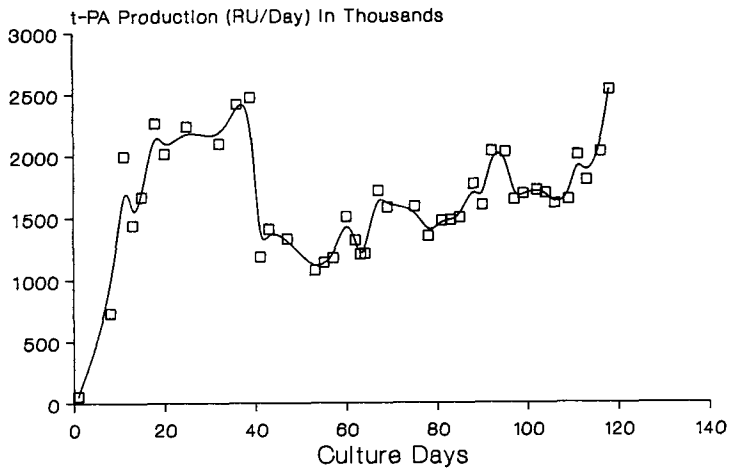

Fig. 4. Run A: t-PA production.

reduce the lag phase. Although the inoculum is seeded into 5% serum, prior determinations on other reactors had shown that the culture supernatant is practically serum-free as a result of washout within the first week of operation.

The medium feed rate was increased from an initial 0.17 L (d×L reactor bed) to 50 L/d for the reactor used in run A and 220 L/d 48 L for the reactor used in run B, respectively, within 3 wk (Figs. 2,8).

Production Phase

In run A the maximum perfusion rate was held for approx 3 wk and subsequently reduced by 50% in order to test its effect on reactor productivity and stability (i.e., the output of single chain tPA). the reduction in feed rate resulted in a proportionate decrease in reactor productivity, although the tPA concentrations and percent single chain tPA (the desired product) were not significantly affected by the step change. This is important as single-chain tP is susceptible to proteolytic cleavage (yielding double-chain tPA), that could possibly have resulted from a release of cell-associated proteases following such a dramatic reduction in the medium feed rate. Figures 4, 5, and 6 depict the profiles of reactor productivity, tPA concentration, and percent single chain tPA (run A). After approx 2.5 mo, the perfusion rate was increased again for a short period in order to assess the responsiveness to a renewed increase before terminating the run. The profiles depicted in Figs. 1 and 2 illustrate the relatively "robust" performance characteristics of this production system. In view of the high cell densities, the limiting medium conditions, and the protein-free medium, it is surprising how well the culture tolerated the magnitude of this reduction in medium supply.

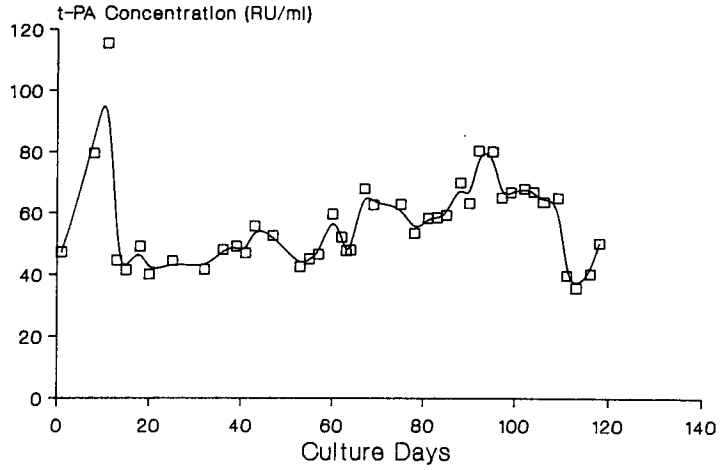

Fig. 5. Run A: t-PA concentration.

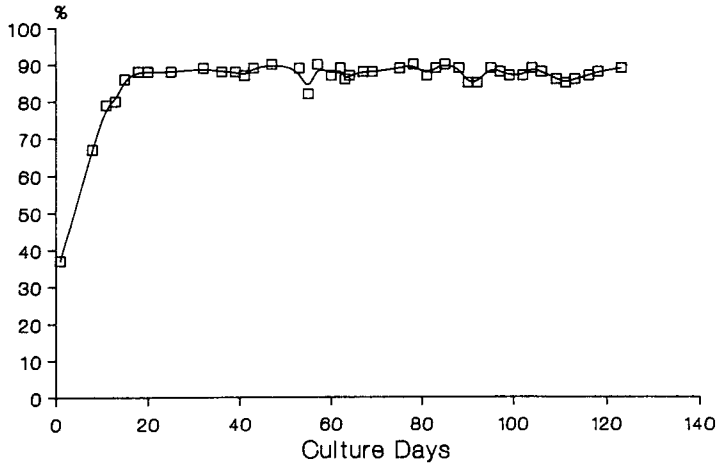

Fig. 6. Run A: Percent single chain t-PA.

Based on the cell mass, which was continuously washed out with the effluent, the population doubling time of the cells during the production phase was estimated at approx 10 d, amounting to some 10–15 population doublings over the three month period (including the growth phase).

Under routine production, glucose and oxygen consumption serve as initial indicators of culture performance. The most sensitive performance indicator for this process, however, remains tPA itself. The gradual increase in tPA production between days 40–100 (run A, Fig. 5) is attributed to a corresponding deliberate increase in the topical oxygen concentration of the medium. This effect, that has also been observed to occur in suspen-

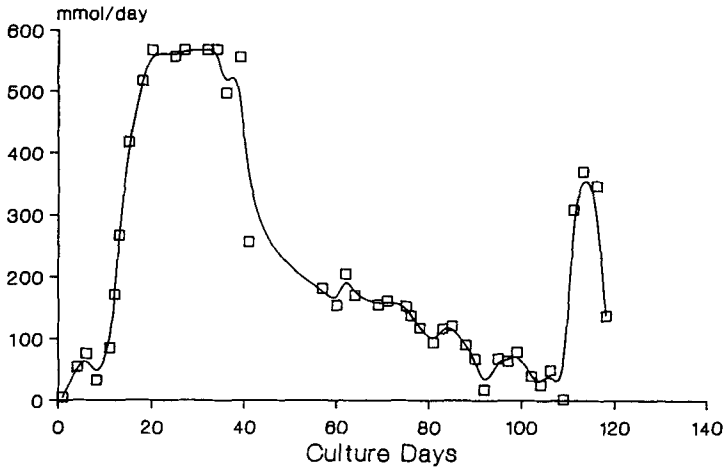

Fig. 7. Run A: lactate production.

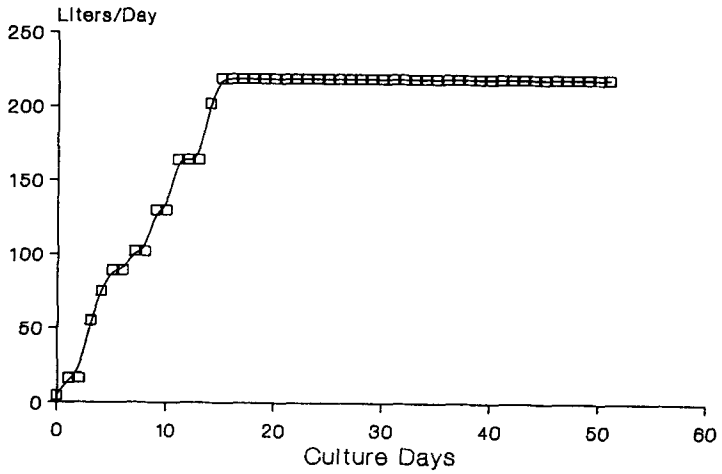

Fig. 8. Run B: medium feed rate.

sion culture (*14*), is demonstrated more clearly in run B (Figs. 8–11). After the tPA production had been maintained at a constant level for a period of 10 d (days 19–29, Fig. 9), the oxygen tension in the culture supernatant was stepped up from (about) 60–80% air saturation (Fig. 10), resulting in a twofold increase in the reactor productivity (tPA output per reactor per day) (Fig. 9). It was calculated that, owing to the high oxygen consumption rates of the cell line (approx 0.9–2 E-16 mol O_2 per cell per s), oxygen transfer through the full depth of the cell aggregates (~0.5 mm) was diffusion limited. However, we have not observed such a strong dependence of product formation on the oxygen tension, at above 50% saturation and

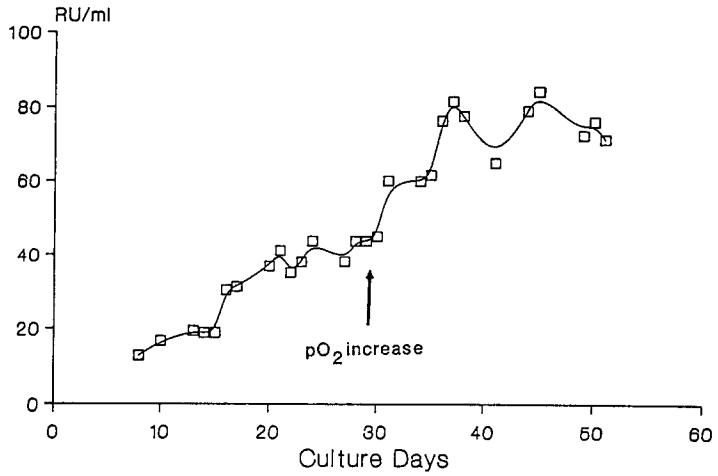

Fig. 9. Run B: t-PA concentration.

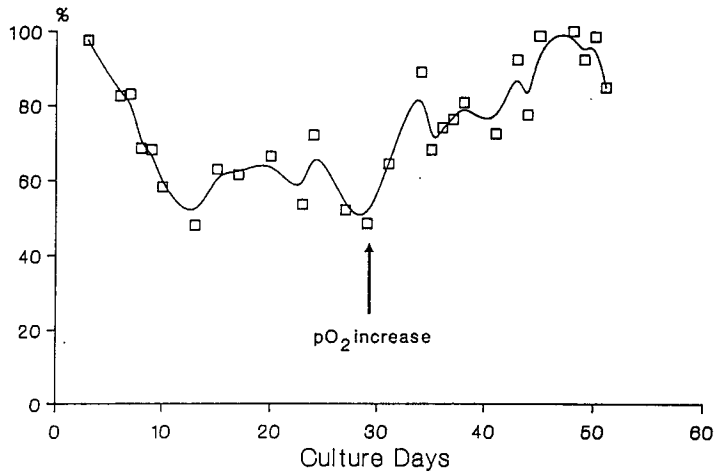

Fig. 10. Run B: Percent air saturation.

under comparable conditions, with other cell lines (such as C127 or hybridomas).

Glucose utilization was also affected by the changes in oxygen tension. Glucose utilization is widely used as a process parameter for three main reasons:

1. As the main carbohydrate component of the culture medium glucose may serve as a broad indicator of medium exhaustion with respect to other components, particularly in substrate-limited continuous cultivation;

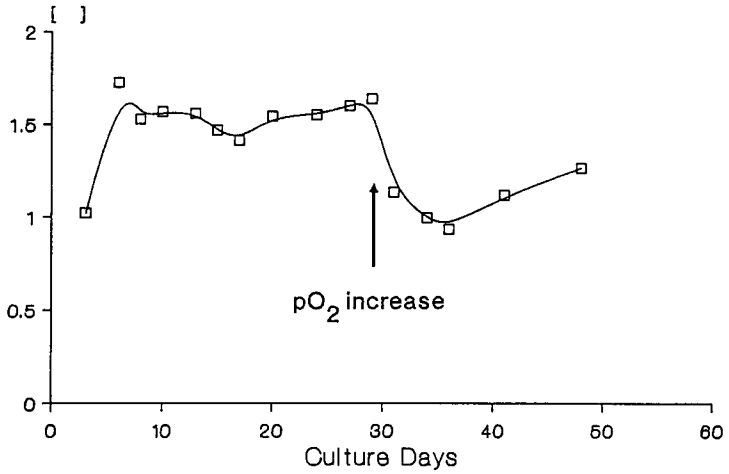

Fig. 11. Run B: lactate production/glucose utilization.

2. Glucose plays a central role in primary metabolism; and
3. It is easy to measure under routine conditions.

In addition, supernatant parameters play a more important role in heterogeneous culture systems since it is impractical to rely on representative cell density measurements under routine conditions. Unfortunately, in our experience with various cell lines (such as hybridomas, insect cells, recombinant BHK, CHO, or C_{127}; various culture systems), glucose utilization is not sufficiently sensitive to serve as an indicator of product formation, and is therefore commonly interpreted as a broad measure of cell growth and metabolic status, and in continuous culture, it also serves as a first measure of performance stability.

In contrast to our experience with other cell lines, we have observed that both tPA production as well as glucose utilization are oxygen limited at pO_2 levels (of the medium exiting the oxygenator), corresponding to less than 60% air saturation (this is still an appoximate value). The following brief discussion outlines the initial observations that form the basis for further development and optimization work.

During the production period, culture growth and reactor productivity were limited by the medium feed rate. Figures 3 and 7 (run A) show a time-course profile of the glucose consumption and lactate production. Although glucose consumption was relatively stable during the production phase, lactate production decreased, indicating a slow, but continuing metabolic shift. During this period, the oxygen tension in the medium was increased. Whereas the glucose consumption remained stable (Fig. 3) (limited by the glucose feed rate), the apparent efficiency of glucose utilization, as indicated by the lactate yield (lactate production/glucose con-

sumption), changed considerably over the 3 mo time course (days 45–105, Run A: compare Figs. 3 and 7). This effect was even more pronounced in Run B (Figs. 10 and 11). It is known that nonreplicating cells produce less lactate, but they also consume less glucose (*16*). Also, the fact that apparently only the lactate production rate responded to an increase in the oxygen tension (days 40–110, Fig. 7) suggests that this phenomenon does not simply constitute a Pasteur Effect, that involves changes in both glucose and lactate metabolism (*15*).

Although both glucose utilization and tPA production were oxygen limited below 60% air saturation, the effect on glucose utilization has sofar only been observed at topical glucose concentrations of less than 0.2 g/L. On the other hand, increasing the glucose feed rate, and thereby increasing the topical glucose concentration above 0.2 g/L does not affect tPA production.

DISCUSSION

The present data provides a performance profile of a continuous production process for single-chain tPA, using protein-free medium over a period of more than 3 mo. Although the Bowes cell line may be propagated in the form of cell aggregates in free suspension, this form of cell propagation requires the addition of serum or other protein supplements to the basal medium. In low-protein medium the Bowes cell line does not perform well as an aggregate culture; under these conditions, the cell line is therefore preferably treated as an anchorage-dependent line, and propagated on a solid matrix, such as in a packed bed system.

The bed packing functions as a cell retention system, thereby increasing local cell densities to 5E7 cells/mL. Despite these high cell densities and the heterogeneous character of the reactor, the medium feed rate may be used to regulate reactor output. Culture A was downregulated primarily to demonstrate the "robustness" of this culture system to large variations in medium feed rate; however, under routine conditions, this means of performance control is rarely applied. Neither the downregulation of the medium feed rate, nor other performance variances significantly affected the proportion of single-chain tPA, which remained between 80 to 90% of the total tPA throughout the 3 mo course.

Glucose utilization is of interest both in terms of its central metabolic role, and as a major nutrient that is easily measured. We are currently examining various aspects of glucose utilization, both to improve the overall efficiency of medium utilization, as well as to improve on the application of glucose as a process parameter. It seems that in the presence of elevated oxygen tension (> 60% air saturation), the Bowes line responds to a limitation of glucose (and possibly other components) by altering its carbohydrate consumption pattern in the direction of higher efficiency.

Reuveny et al. (*17*) made some similar observations with a hybridoma line in single cell suspension (as opposed to a cell aggregates in a PBR). They noted that both the productivity and the efficiency of glucose utilization increased as the cell density increased to above 1×E7 cells/mL (perfusion culture). Both the medium feed rates (~120 mL/[E9 cells×d]) and the oxygen tension were relatively low (~10% air saturation), although the topical oxygen tensions of PBRs and homogeneous suspension reactors are not strictly comparable in terms of oxygen availability to the cells. The observations of Reuveny et al. also suggest that below a certain glucose concentraiton, and probably other medium components, the efficiency of carbohydrate utilization improves. These threshold values for glucose are likely to vary between reactor systems (and, of course, cells).

CONCLUSION

The present results illustrate an industrial application of a PBR system for a continuous process. To our knowledge, this is also the first demonstration of an industrial cell culture process, using human cells and operating continuously with a standard basal medium alone. This tPA process currently provides clinical grade material required for an Investigational New Drug.

During the early part of our investigations, it appeared that it was the properties of the cell strain that alone enabled its continuous propagation in protein-free medium. Subsequent investigations, however, have indicated that it is the reactor design and operating conditions, that promote the cell line potential for protein-free propagation.

This packed bed system is now routinely operated continuously for up to 6 mo (at Bio-Response). It has been scaled up to operate at approx 250 L per reactor per day (approx 5–10 E11 cells per reactor), and has been found to be very "robust", both with respect to operational variations, as well as scalability.

In principle, the process represents a substrate-limited continuous culture with cell recycle. In this mode of operation, an understanding of the effects of substrate limitation and variations in the medium feed rate are key elements in the development and optimization of the medium conditions process parameters. Glucose utilization is being investigated both for its value as a general process indicator, as well as its specific features with respect to this process, i.e., the close association of oxygen tension and glucose utilization. This has now formed the basis for a broader program into the utilization of carbohydrates, amino acids, and oxygen under substrate-limited conditions, both in heterogeneous (PBRs) and homogeneous (STRs) systems.

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