Continuous Production of Prourokinase in Feed Harvest and Perfusion Cultures

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The production of prourokinase (PUK) by a human kidney tumor cell line is studied in long term cultures. Cells are grown on microcarriers which are retained inside the reactor by sedimentation or with a spin filter. Two modes of operation are compared: feed harvest at an average medium exchange rate of 0.3 d^{-1} and continuous perfusion at a higher dilution rate of 1.5 d^{-1}. In the two systems a stable production of PUK has been maintained for more than 400 h. Kinetics of cellular growth, nutrient consumption, and metabolite and PUK excretion are similar. After an initial rapid growth period, one observes a 10-fold reduction in all the cellular metabolic activities during the stationary phase. Continuous perfusion yields a higher cell density (7×10^6 cells·mL^{-1}) than feed harvest (3×10^6 cells·mL^{-1}), which results in a twofold increase in the reactor productivity. But higher final enzyme activities, about 250 ru·mL^{-1}, are obtained in the feed harvest recovered medium than in the perfusion medium, 100–150 ru·mL^{-1}. The cumulative medium consumption per mass of product is the same in the repeated batch and in the continuous operation mode.

INTRODUCTION

Perfusion reactors with immobilized or confined cells are more and more widely used in mammalian cell technology for the production of proteins. Compared to the classical batch reactors, they are usually operated at higher cell densities and often yield several advantages, such as higher productivities, increased longevity, or reduced serum utilization.

Reactors with immobilized or retained cells can be operated with continuous or intermittent substrate feeding. The first mode is the continuous perfusion of the reactor with nutrients, the spent medium being removed at the same rate. Continuous reactors are often run near steady state, at a relatively constant medium composition for the cells.[1,2]

A second mode of operation is feed harvest, where the reactor is operated as a sequence of batches with partial replacement of the medium when the main nutrients are exhausted. Medium exchange is usually performed at a lower equivalent dilution rate than in perfusion reactors. As a main difference with continuous perfusion, in feed harvest cells are subjected to a constantly varying environment: the nutrient concentrations steadily decrease during the batch operation and then suddenly increase when medium is exchanged.

Whereas the respective merits of batch and continuous operation of mammalian cell reactors have been extensively investigated,[3,4] little attention has been given to the comparative performances of continuous perfusion and feed harvest with retained cell reactors.

In this article the two modes of operation are compared for the production of prourokinase (PUK) by an anchorage-dependent human kidney tumor cell.[5] Like other plasminogen activators, such as t-PA or streptokinase, urokinase, the activated form of prourokinase, leads to the dissolution of blood clots and has therapeutical potential for the treatment of deep venous thrombosis. The PUK productivity of the used TCL 598 cell line has previously been studied in different systems such as roller bottles, microcarrier-stirred tanks, and perfused ceramic matrix.[6,7]

With cells grown on microcarriers long term cultures can be achieved either by feed harvest operation, the carriers being recovered at medium exchange by sedimentation, or by continuous perfusion of the stirred reactor using a spin filter for cell retainment. A main variable to be optimized for a long term culture is the exchange rate of medium. If high dilution rates have a favorable effect on the reactor productivity, they also result in more diluted products and larger quantities of used medium.

In this comparative study of the reactor kinetics and performances under the two modes of operation, a main em-

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phasis was given to medium utilization. The feed harvest and continuous perfusion reactors were operated at two very different medium exchange rates in order to gain a better understanding of the influence of this key variable on the kinetics of cell growth and PUK synthesis.

MATERIALS AND METHODS

Cell Line

The human kidney tumor cell line TCL 598 was obtained from Sandoz Ltd., Basel. Cells are treated by trypsin (0.05 $g \cdot L^{-1}$ trypsin + 0.02 $g \cdot L^{-1}$ EDTA; Gibco Europe) every 3 days at a ratio of 1:3. They are propagated at 37°C in 750-cm^2 tightly capped roller bottles initially sparged with a gas mixture of 5% CO_2, 20% O_2, and 75% N_2. The seeding density is about 4×10^4 $cell \cdot cm^{-2}$. For the reactor inoculation, the roller bottles are trypsinized just before confluence. The trypsin action is stopped by medium addition and the cell suspension immediately transferred into the bioreactor. The cell line only secretes prourokinase (PUK). The appearance of urokinase (UK) in the medium results from an extracellular proteolytic cleavage.[8]

Medium

The growth medium for propagation cultures consists of DMEM (Gibco), containing 25 m*M* glucose and 2 m*M* glutamine, and supplemented with 5% (v/v) heat-inactivated fetal calf serum (FCS, Gibco). For the reactor cultures different media are used: DMEM or a 75% DMEM/25% Ham's F12 mixture, both supplemented with 2 m*M* glutamine and variable levels of glucose (11–25 m*M*) and FCS (0–5%). Penicillin (100 $U \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$) are also added.

Reactor

A 4-L reactor (SGI-France) equipped with silicone tubing (3 $m \cdot L^{-1}$) for bubble-free aeration is used. The rotating speed is maintained at 40 rpm, the temperature at 37°C, the pH at 7.1, and the dissolved oxygen between 40 and 60% of air saturation.

Cytodex 3 microcarriers (Pharmacia, Sweden) are prepared as described by the manufacturer. The carriers and the culture medium are incubated in the culture vessel a few hours before the start of the culture. Cell inoculation density is between 3×10^5 and 5×10^5 $cells \cdot mL^{-1}$ for a 5-$g \cdot L^{-1}$ microcarrier concentration.[9]

Feed Harvest Operation

Medium exchange is performed by following the depletion of glucose. Thus each day 25–50% of the culture supernatant is exchanged, after microcarrier settling, with preheated new medium. Over the whole culture this represents an equivalent dilution rate of 0.3 d^{-1}. At medium exchange the time required by the system to return to the initial set point is about 15 min.

Continuous Perfusion Operation

Fresh medium is fed at a fixed flow rate with a peristaltic pump, and the culture volume is kept constant by means of level sensors acting on a harvest pump. The microcarriers are retained in the culture vessel by a 60-μm mesh inox filter.

Analytical Methods

Culture samples are taken twice a day. Total cell density is evaluated from the enumeration of crystal violet stained nuclei on a haemacytometer.[10] Cell viability is checked by the trypan blue exclusion method after trypsination of the cells from the carriers. After removal of the microcarriers and the cells by centrifugation, the supernatant is frozen at −20°C for subsequent analysis of glucose, lactate, glutamine, and $NH4^+$. For PUK + UK analysis, the period of storage of the samples (at 4°C) does not exceed 1 week.

Glucose, L-lactic acid, and glutamine concentrations are enzymatically assayed (Boehringer) as described in the product insert. Ammonia is measured with a specific electrode (Orion) after adjustment of the sample pH to 12 by the addition of NaOH 0.5*N*.

The total PUK + UK activity in the medium is measured by first activating the PUK into the UK form by plasmin cleavage. Twenty-five microliters of the culture medium are diluted with 75 μL of buffer *A* (50 m*M* Tris–50 m*M* EDTA–38 m*M* NaCl, pH 8.8, 2% Tween 80). Then, 30 μL of a plasmin solution (1.25 $IU \cdot mL^{-1}$, Kabi vitrum) are added and the sample incubated at 37°C for 10 min. Second, the urokinase activity is measured on the chromogenic synthetic substrate pyro-Glu-Gly-Arg-pNitroanilin (S2444, Sigma). The substrate is diluted into a 5000 $KIE \cdot mL^{-1}$ Trasylol solution at a concentration of 0.75 $mg \cdot mL^{-1}$. Fifty microliters of the substrate solution is added to the UK sample, and the mixture is incubated at 37°C for 25 min. The pNitroanilin released is measured with a spectrometer (Multiscan-Flow) at 405 nm. The A_{405} is corrected by phenol red adsorption at 540 nm. The standard used for the calibration of the assay is UK from Japon Chemical Research Corporation. The values reported in this study are all expressed in relative units.

RESULTS AND DISCUSSION

Feed Harvest Culture

A long term feed harvest culture of human kidney tumor cells is performed by exchanging about one-third of the culture medium every day. Microcarriers are retained inside the reactors by sedimentation. The composition of the feed and the resulting variations of the medium concentration in the reactor during 425 h of operation are shown in Figure 1. The culture is started with DMEM supplemented with

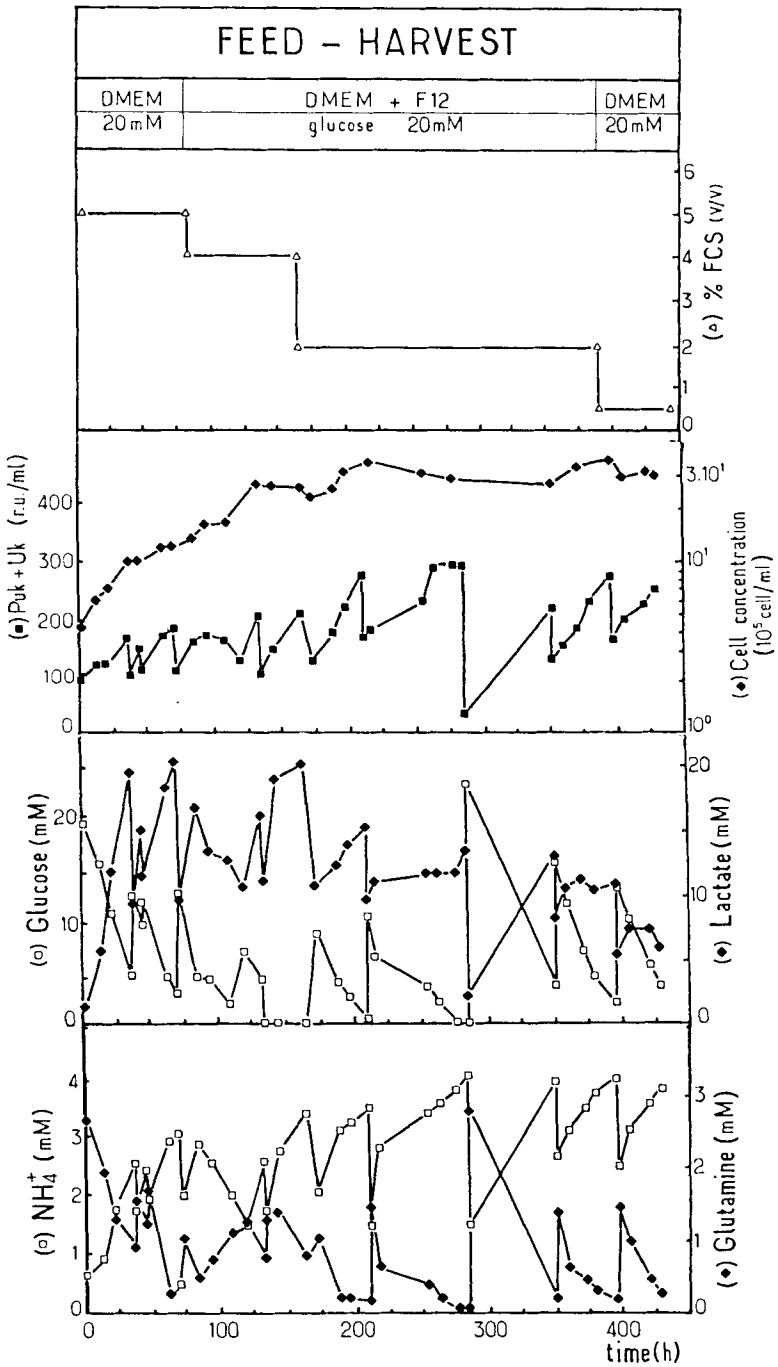

Figure 1. Time evolution of the cell density, PUK + UK, glucose, lactate, glutamine, and ammonia concentrations during a feed harvest culture at an equivalent dilution rate of 0.3 d^{-1}. The feed medium is DMEM or DMEM/Ham's F12 supplemented with 20 m*M* glucose and 2 m*M* glutamine. The FCS level is progressively reduced from 5 to 0.5%.

5% FCS for the first 75 h. Then the reactor is replenished with a mixture of DMEM and F12, and the serum level is reduced to 4 and 2%. After 400 h a DMEM medium with only 0.5% serum is used.

The cells grow rapidly on microcarriers during the first 220 h until a concentration of 3×10^6 cells·mL^{-1} is achieved. Afterward there is no further increase in cell density. From microscopic observations one sees that microcarriers are completely covered at about 2×10^6 cells·mL^{-1}. At higher densities cells grow on multilayers and form aggregates. The viability of the attached cells was found to be relatively constant during the whole culture and always larger than 70%. During the successive batches glucose and glutamine concentrations decrease from their initial 10 m*M* and 1.5 m*M* levels. At the same time lactate and ammonia levels increase up to 10–20 m*M* and 2–4 m*M* respectively.

Prourokinase is secreted both during the growth and stationary phases. The PUK plus UK concentration fluctuates between 100 and 250 ru·mL^{-1}. The secretion of PUK continues even when the serum level is finally reduced to 0.5%.

To have a better understanding of the culture kinetics, the evolution with time of the specific rates of growth (μ), glucose consumption (ν_{glc}), and enzyme production (π_{PUK+UK}) have been calculated with respect to the total cell concentration and are reported in Figure 2. As seen, the three rates are the highest during the initial growth phase. They subsequently decrease, reaching a relatively constant value after 220 h of culture. Interestingly, during the stationary phase the specific production rate of PUK plus UK is 5–10 times lower than for the initial growth period.

Continuous Perfusion Culture

An alternative long term operation mode of the cell reactor is continuous perfusion: medium is continuously fed and removed from the reactor, the microcarriers being retained with a filter. Figure 3 shows the operating conditions and the resulting performance of such a perfusion culture. Cells are first grown batchwise with 5% FCS in DMEM medium.

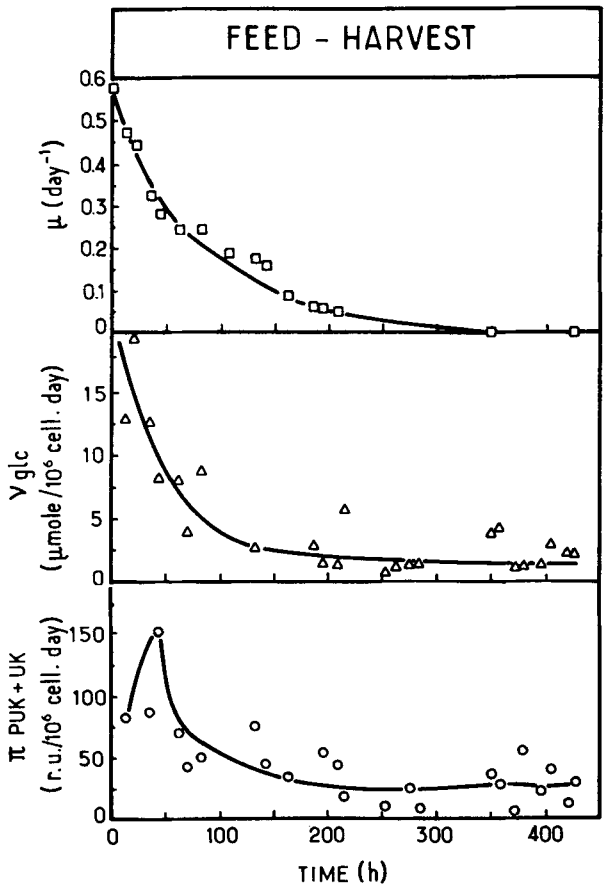

Figure 2. Evolution with time of the specific growth rate μ, the specific glucose consumption rate ν_{glc}, and the specific PUK + UK production production rate π_{PUK+UK} during the feed harvest culture. The rates are calculated per 10^6 total attached cells counted by the crystal violet method.

After 70 h, the cell density reaches 2×10^6 cells·mL^{-1}, and the perfusion is started with a medium containing 75% DMEM and 25% Ham's F12. The dilution rate is maintained at about 1.5 d^{-1} until 550 h, when it is lowered to 1 d^{-1} before being increased again to 2.3 d^{-1}. The serum level is stepwise decreased to 0.5% at 375 h and to 0% after 500 h.

Under these conditions the cell density increases during 350 h and reaches a stationary level of 7×10^6 cells·mL^{-1}. Aggregates of diameters up to 500 μm are observed during the stationary phase. The viability of the cells remains larger than 70% during the whole culture. Glucose and lactate concentrations are relatively stable during the continuous mode of operation. The glucose level in the feed was stepwise increased from 11 m*M* to 25 m*M* in order to check the possibility of glucose limitation on cellular growth. Thus two steady states are observed: the first at 3 m*M* glucose and 12 m*M* lactate and the second, after 400 h, at about 8 m*M* glucose and 22 m*M* lactate. As seen, a higher level of glucose has no significant influence on the cellular density. Meanwhile the glutamine concentration fluctuates between 0.5 and 2 m*M* and the ammonia level between 1.2 and 3 m*M*. The PUK plus UK concentration reaches 300 ru·mL^{-1} at the end of the initial batch culture. Under perfusion it then decreases and remains at a stable level around 100 ru·mL^{-1} even when serum is completely removed from the feed.

Figure 4 shows the corresponding time variation of the specific rates of growth (μ), glucose consumption (ν_{glc}), and enzyme production (π_{PUK+UK}) with respect to the total cell concentration. The obtained curves are very similar to those previously obtained in feed harvest. All specific rates are highest during the initial growth phase and then gradually decrease with time. During the stationary phase one finds an approximate 10-fold reduction in the specific rates of nutrient consumptions and PUK + UK production as compared to the initial growth period.

Comparison of Culture Kinetics

The comparison of the culture kinetics under the two different operational modes shows a similar overall behavior, with an initial rapid growth phase followed by stationary phase characterized by a reduced metabolic activity. The major difference between feed harvest and continuous perfusion is the final cell concentration. The perfused culture reaches 7×10^6 cells·mL^{-1} while the growth is limited at 3×10^6 cells·mL^{-1} with feed harvest. Since feed harvest was operated at a lower average medium dilution rate, the reduced final cell density probably results from limitations in essential nutrients such as glucose and amino acids or to the accumulation of some toxic metabolic products.[10–12] This has been verified by operating the feed harvest reactor at higher equivalent dilution rates of 0.7 and 1 d^{-1}. As shown in Figure 5, increasing the dilution rate indeed results in a final cell density increase of from 3×10^6 to 5×10^6 cells·mL^{-1}.

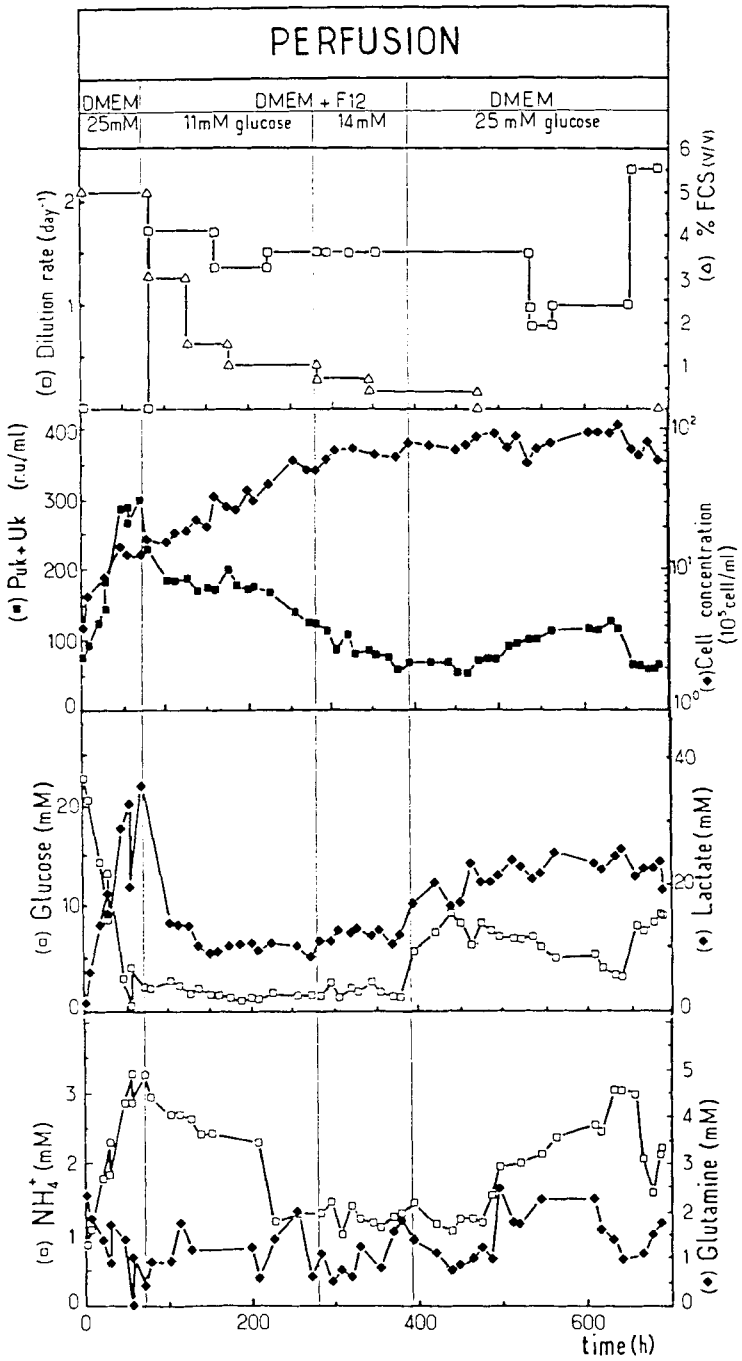

Figure 3. Time evolution of the cell density, PUK + UK, glucose, lactate, glutamine, and ammonia concentrations during a perfusion culture at a dilution rate of 1.5 d^{-1}. Perfusion is started after an initial batch phase of 70 h. Feed medium is DMEM with 25 m*M* glucose or DMEM/Ham's F12 (3:1) with 11, 14, or 25 m*M* glucose. Both media are supplemented with 2 m*M* glutamine. The FCS level is progressively reduced from 5 to 0% in the feed medium.

When comparing the reactor performance with respect to PUK + UK production, the main criteria to be considered are the enzyme concentration in the recovered medium, the reactor productivity, and the quantity of spent medium per unit amount of produced enzyme. In feed harvest the PUK + UK concentration increases from 100 to 250 ru·mL^{-1} between two medium exchanges, whereas with continuous perfusion it stays at about 100–150 ru·mL^{-1}. This results from the much lower average dilution rate in the feed harvest experiment.

Figure 6 compares the cumulative enzyme production for the two cultures. During the initial growth phase, enzyme productivities are very similar. During the stationary phase, on the contrary, the perfused culture gives better results,

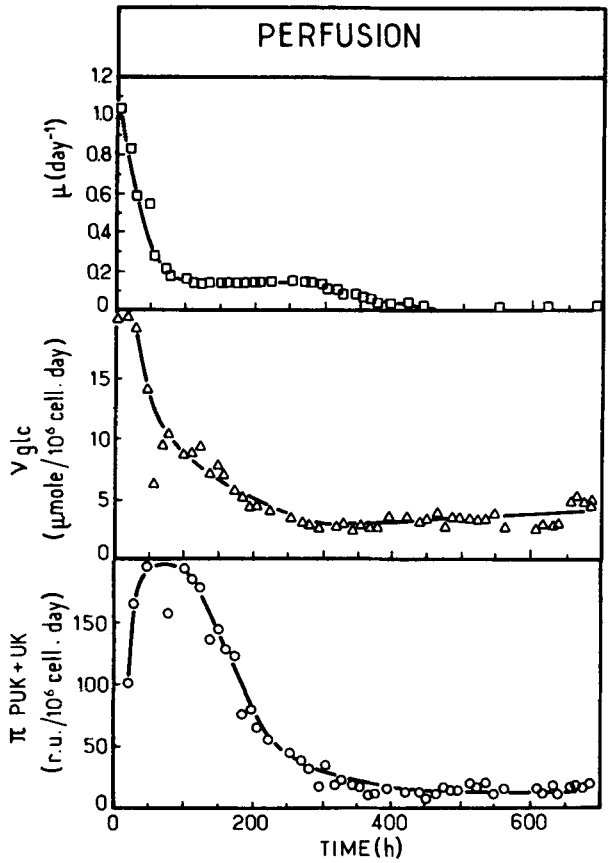

Figure 4. Evolution with time of the specific growth rate μ, the specific glucose consumption rate ν_{glc}, and the specific PUK + UK production production rate π_{PUK+UK} during the feed harvest culture. The rates are calculated per 10^6 total attached cells counted by the crystal violet method.

allowing a productivity of 320×10^3 ru d^{-1} compared to the feed harvest productivity of 150×10^3 ru d^{-1}. As the specific PUK production rates during the resting phase are similar for the two modes of operation, the superiority of the continuous perfusion can be attributed to its higher cell density.

An additional criteria of comparison is the efficiency of medium utilization. In Figure 7, the total PUK + UK

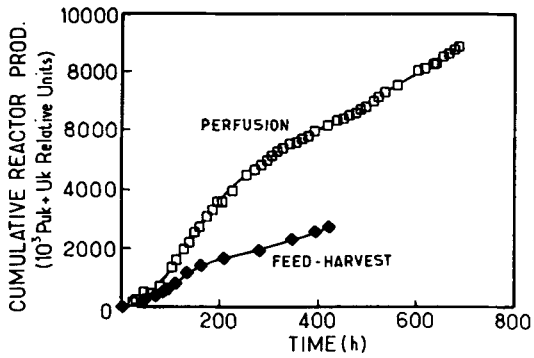

Figure 6. Comparison of the cumulative PUK + UK reactor production with time for the two culture modes: feed harvest (◆) and perfusion (□).

formed as a function of the spent medium is compared for the two reactors during 400 h culture time. One sees that the perfusion reactor which produces larger quantities of enzymes also consumes larger volumes of medium. As a result, the cumulative medium consumption per mass of produced enzyme is very similar in each operating mode: both reactors consume about 15 L of medium to produce 3×10^6 units of PUK + UK.

CONCLUSION

The long term continuous production of PUK by a human kidney tumor cell line can be achieved by retaining inside the reactor cells grown on microcarriers and feeding the reactor either intermittently or continuously. The feed harvest reactor with a relatively low dilution rate yields the highest enzyme concentrations. The continuously perfused reactor at a higher dilution, on the contrary, gives the highest cell concentration and enzymes productivities. With respect to the efficiencies of medium utilization for protein synthesis, the two reactors are essentially equivalent. A more detailed economical analysis would be needed to assess which of the two operation modes shows a superiority in overall performance.

Improvements in both reactors may still be expected by further optimization of the operating conditions. Provided

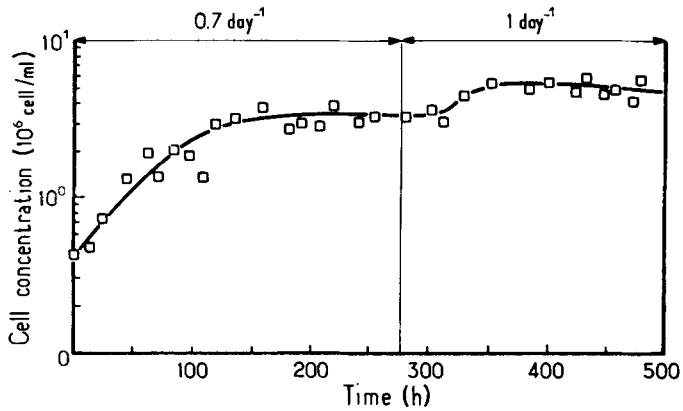

Figure 5. Time evolution of the cell density during a feed harvest culture at two different equivalent dilution rates: 0.7 and 1 d^{-1}.

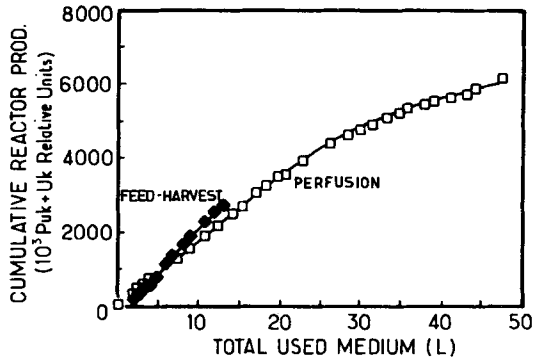

Figure 7. Comparison of the cumulative PUK + UK reactor production relative to the whole spent medium for the two culture modes: feed harvest (◆) and perfusion (□).

the biochemical or physical factors which presently limit cellular growth are well identified, higher cell densities and reactor productivities should be reached.[13-15] A critical aspect is the optimization of the feed medium with respect to serum and other essential growth factors. As demonstrated in this study, after an initial growth phase the serum level can be significantly reduced without a detrimental effect on the PUK synthesizing capacity of cells in the stationary phase. To optimize the serum or growth factor feeding strategy, additional studies have to be performed on the effect of reduced serum on cell viability and enzyme synthesis in prolonged cultures.

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