A tubular segmented-flow bioreactor for the infection of insect cells with recombinant baculovirus

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Abstract

A continuous process of insect cell (*Sf9*) growth and baculovirus infection is tested with the sequential combination of a CSTR and a tubular reactor. A tubular infection reactor enables continuous introduction of baculovirus and therefore avoids the 'passage effect' observed in two-stage CSTR systems. Moreover, a tubular reactor can be used to test cell infection kinetics and the subsequent metabolism of infected insect cells. Unlike batch and CSTR culture, cells in a horizontally positioned tubular reactor settle due to poor mixing. We have overcome this problem by alternately introducing air bubbles and media and by maintaining a linear velocity sufficient to keep cells suspended. This article addresses the development of the tubular reactor and demonstrates its use as an infection system that complements the two-stage CSTR.

Introduction

The baculovirus expression system is now established as a powerful and versatile tool for the expression of a variety of eucaryotic proteins. Not only does it provide for high-level expression, but it performs post-translational processing such as glycosylation and phosphorylation. There have been many bioreactors proposed to grow insect cells and produce proteins of interest, such as packed-bed bioreactors (Shuler *et al.*, 1990), helical ribbon impeller bioreactors (Maiorella *et al.*, 1988), continuous stirred tank reactors (CSTR) with direct sparging (Wang and Bentley, 1994), semi-continuous reactors (Hink and Strauss, 1980) and two-stage bioreactors (de Gooijer *et al.*, 1992; Kompier *et al.*, 1988; van Lier *et al.*, 1990; van Lier *et al.*, 1992; van Lier *et al.*, 1994) where the first CSTR is used for cell growth and the second for viral infection and protein production.

Tramper and co-workers demonstrated the two-stage CSTR for β -galactosidase production, but also noted a rapid decrease in production after four weeks of operation due to both the passage effect and selective pressure common in CSTRs (de Gooijer *et al.*,

1992; van Lier *et al.*, 1990; van Lier *et al.*, 1992; van Lier *et al.*, 1994). The passage effect occurs in the infection reactor and is characterized as defective interference caused by the occurrence of deletion mutants of the virus (van Lier *et al.*, 1992; Wickham *et al.*, 1991). These mutants may lack up to 40% of the viral genome and are therefore replicated faster than the intact nonoccluded viruses (NOVs). It is hypothesized that upon coinfection of a cell with defective and intact virus, the faster growing mutants compete favorably for replication factors and thus interfere with intact virus replication (de Gooijer *et al.*, 1992; van Lier *et al.*, 1992). Due to the differential in replication rate, the intact NOVs are gradually eliminated from the second reactor. To overcome this second-reactor instability, Taticek and Shuler (1992) proposed a three-stage system with a middle CSTR that serves as an infection reactor. Virus stock solution is continually introduced into the middle reactor operating with a residence time equivalent to that necessary for virus infection. In this way, all cells are infected by intact NOVs prior to the production reactor. Note, however, that a continuously operated stirred vessel will have a residence time distribution with the *average* residence time being the

inverse of the dilution rate. Even after two residence times, roughly 15% of the fluid introduced remains in the vessel. Thus, there will be a finite probability that some cells will be retained in the infection reactor for a sufficiently long time that they begin to produce progeny virus. This will again result in virus instability due to the passage effect; a successful 3-stage system was not reported. By incorporating a continuous flow of virus in a linear reactor, specifically placed as an infection reactor between two CSTR's one may be able to circumvent the defective virus instabilities noted by Tramper and co-workers (de Gooijer *et al.*, 1992; van Lier *et al.*, 1990; van Lier *et al.*, 1992; van Lier *et al.*, 1994) and Taticek and Shuler (1992) because there will be no retention of progeny virus in the reactor zones where infection occurs.

In this article, we report on a two-stage bioreactor system consists of an upstream CSTR that provides cells continuously to a downstream tubular reactor with segmented plug flow for viral infection. Virus stock solution is introduced at the beginning of the tubular reactor which was run with a mean residence time of up to 10.3 hours. The infection process can be monitored by taking samples at different distances down the tubular reactor. Although evaluated for viral infection, by operating at longer residence times the reactor could potentially be used to monitor changes in cell physiology during the infection and protein production phases. In addition to describing the infection process, we report on practical constraints that we encountered.

Materials and methods

Cell lines, virus, media and analytical

Spodoptera frugiperda, Sf-9, cells (ATCC, No. CRL 1711) were maintained in Ex-Cell 401 serum-free media (JRH Biosciences) and 100 mL spinner flasks (Bellco) supplemented with 0.1% Pluronic F-68 (JRH Biosciences), 100 mg/l ampicillin (Sigma), 2.5 µg/mL fungazone (Advanced Biotechnologies, Inc.) and 0.02% antifoam (Dow Corning 10% FG-10 antifoam emulsion, food grade). Ampicillin was used in lieu of gentamycin described in the literature (Summers and Smith, 1987) because gentamycin was found to interfere with antifoam, forming gel-like substances. The recombinant virus, vIBD-7, expressing β -galactosidase and infectious bursal disease virus (IBDV) structural proteins was described previously (Vakharia *et al.*, 1993) and was graciously provid-

ed by Dr. Vakharia (College of Veterinary Medicine, Center for Agricultural Biotechnology, University of Maryland). Total cell counts were performed with a hemacytometer (VWR), and viability was determined by trypan blue dye exclusion using a 0.04% solution (Sigma). β -galactosidase activity was measured using ONPG as described previously (Bentley *et al.*, 1994). Glucose and lactate concentrations were determined using an automated analyzer (Yellow Springs Instruments, model 2700).

Bioreactor setup

The overall reactor consists of two parts, a CSTR and a tubular reactor. The CSTR is composed of a glass-blown jacketted spinner flask shown in Figure 1 as described previously (Wang and Bentley, 1994). The CSTR temperature was maintained at 28 °C using a circulating water bath (VWR). The headplate consisted of a rubber stopper (No. 10) drilled with seven holes for stirring, media inlet, level control, vent, sampling, gas flow, and cell outlet. Experiments were performed in two sets: the first utilized a vertical exit port in the rubber stopper headplate. For the second, the reactor was modified so that the exit port protruded from the side of the reactor at 45 °C angle downward from the vertical.

The tubular reactor was comprised of a microprocessor-controlled peristaltic pump with six rollers (Watson Marlow, model 505Du) which could be operated as slowly as 0.2 rpm while accommodating a 4-channel pumphead (model 205BA) and MasterFlex no. 14 silicone tubing (length = 7.6 m, ID = 1.6 mm). The long tube was coiled as a series of concentric circles in a horizontal plane and placed in a laminar flow hood. Unless otherwise noted, the entrance and exit of the tubular reactor were used as the sampling points shown in Figure 1. Watson-Marlow silicone tubing (ID=1.14mm) was used for precise pumping of air into the tubular reactor through a Y-junction in the inlet line. The pH was not controlled because under the operating conditions the insect cell medium has a strong buffer capacity and the pH does not change significantly. As the normal insect cell growth temperature is room temperature, no additional temperature control was needed other than placing the tubular reactor in the hood. Note, the CSTR was maintained at 28 °C by a water jacket. In all experiments, the CSTR (working volume = 200 ml) was operated without air sparging at a dilution rate of 0.0315 hr⁻¹ as previously described (Wang and Bentley, 1994).

Figure 1. Schematic diagram of the CSTR-tubular two-stage bioreactor system. The expanded panel of the tubular reactor illustrates the bubble/liquid flow pattern and the expected velocity profile in the liquid segment.

Validity of segmented flow

Our work is predicated on having intimate contact between the virus and cell. Consequently, the reactor hydrodynamics desired include a well-mixed liquid element, totally segregated from the next, that moves linearly down the reactor's length. To assure that the actual flow pattern was a well-mixed liquid but well-segmented, a series of preliminary experiments was conducted. Initially water was introduced until the establishment of the proper air bubble/liquid mix. Then, a step increase in dye solution was introduced into the tube, separated by air bubbles analogously to the formal experiments with insect cells (see Figure 2). The tube exit was connected to a spectrophotometer equipped with a flow cell for instantaneous measurement of absorbance ($\lambda = 420$ nm). The spectrophotometer was also connected to a data acquisition board (Data Translation DT2801) and the signal (volt-

age) was transmitted to an IBM computer, on which the software, LabTech Notebook (Laboratory Technologies Corp.), was used to record the voltage over time. The measured voltage was proportional to the absorbance or optical density (OD). Our on-line measurement of OD (see Figure 2(A)) showed sharp step changes when the dye solution front and air bubbles passed the detector, indicating that the liquid concentration in the small segments of dye solution was uniform. Moreover, the OD for each liquid segment was virtually identical to the others, also each air bubble had a characteristic OD, demonstrating uniformity in the established flow pattern. Further, our findings demonstrate that there was negligible liquid absorbing to the tubing walls so that each liquid segment was similar, which was consistent with our visual observation.

We performed an analysis of the ideality of plug flow by both the axial dispersion model and the stirred tank in series model (Hill, 1977). The estimated Peclet

Figure 2. (A) Dynamic measurement of spectrophotometer voltage change during flow of air/liquid mixture in tubular reactor. (B) Left panel: axial dispersion model for step change in dye concentration as a function of Peclet number. Data for the first liquid element with dye are indicated as dots. Right panel: CSTR-in-series model indicating equivalent numbers of CSTR's and our data. F is the normalized concentration (ratio of OD to the OD prior to the dye). t/t^* is the ratio of actual time divided by mean residence time.

number (ratio of convective to dispersive flows) determined by our analysis was generally greater than 8,000, and the equivalent number of CSTR's was greater than 4,000 indicating nearly ideal plug flow (see Figure 2(B)). Thus, the desired flow was established.

Baculovirus infection

To test the infection efficiency in the tubular reactor, virus stock solution was introduced at the reactor entrance at an MOI of ≈ 60 (controlled by fixing the flow rate ratio of cells and virus stock solution). Infection was evaluated at three dilution rates (residence times $\tau = 1.6, 3.2$ and 5.1 hrs). Infected cells from the tubular reactor were collected and washed with PBS, resuspended in fresh medium, and incubated in a 25 cm^2 T-flask at 27°C for later measurement. A control was performed by taking the same number of cells directly from the CSTR and infecting at the same MOI in a T-flask where they were shaken for a time corresponding to the residence time in the tubular reactor, and then washed in the same manner as the previous cells and incubated until later measurement.

This procedure was repeated six times for measurements of β -galactosidase at 72, 96, 120, 144, 168, and 192 hrs post-infection. As noted above, the residence time was then changed to 3.2 and 5.1 hrs and the above experiments were repeated.

Results and discussion

Reactor Dynamics – no infection

Figure 3(A) and (B) show the time-course of cell density at the entrance and the exit of the tubular reactor run without virus stock addition. As stated previously, air bubbles were introduced for aeration and to assist in driving the cells down the tubular reactor. In this way, we taken advantage of bubble surface tension in addition to the liquid convective flow to propel the cells down the tube. In addition, since the Reynolds number is of order 1 (see Table 1), we expect a viscous flow profile that will maintain or continually resuspend cells in the liquid element (see Figure 2).

Figure 3. Cell density versus time in (A) set I and (B) set II. Vertical lines denote different residence times. Symbols include: ($\circ$), density at the exit of the tubular reactor; ($\bullet$), density at the entrance of the tubular reactor; ($\diamond$), viability at the exit of the tubular reactor. Panel (C) indicates glucose and lactate concentrations at the entrance ($\bullet$) and exit ($\diamond$) of the reactor for the experiment shown in Panel (A). RT is the residence time.

Table 1. Velocity, re and mean cell density for experimental sets I and II

Exp. no.	RT (cm/min)	Re	Cell density (set I) (10^5 cells/ml)	Cell density (set II) (10^5 cells/ml)
1	2.25	1.6	6.4	6.8
2	5.49	0.65	6.2	6.4
3	6.33	0.57	5.8	5.9
4	10.3	0.35	5.2	5.4

RT = mean residence time

Re = Reynolds number, $\rho \cdot D \cdot u / \mu$

The measured cell density difference between the entrance and the exit was negligible, indicating that the bubbles created a segmented flow effective in car-

rying the cells. From our visual observations, at the flow rates indicated, no significant settling occurred. Note that several tube diameters, liquid flow rates and air flow rates were attempted before we were able to demonstrate effective segmented flow without cell settling along the length of the tube. Additionally, the success of this reactor hinges on the controlled introduction of equally spaced air bubbles; without bubbles, we were unable to keep cells suspended for the reactor's length (7.6 m).

Besides ensuring the segmented flow profile, air bubbles also provide oxygen, an essential nutrient for the cells. Indeed, since there is such close contact between cells and air, we expect no oxygen limitation; the consistency of lactate level shown in Figure 3(C) confirms our assumption. Also, the glucose level in the

medium was measured at the entrance and exit of the tubular reactor. As shown in Figure 3(C), the glucose concentration was consistently higher than 15 mM, and was not limiting.

Ideally, the cell density in the tubular reactor should equal that of the CSTR ($\approx 7\text{--}7.5 \times 10^5$ cells/mL). In our first experiment (Figure 3(A)), cells settled in the tubing between the CSTR exit and the peristaltic pump most likely due to the lack of both suitable mixing and air bubbles (air bubbles were added at the pump). In addition, with a vertical withdrawl tube one might expect retention of cells in the CSTR due to settling and internal recycle (Batt *et al.*, 1990). As shown in Figure 3(A), the density in the tubular reactor for the first 100 hours was lower than in the CSTR because of this cell accumulation in the transition space. This cell accumulation also caused the low viability at the exit of the tube. The accumulated cell layer became thicker as more cells deposited, until a pseudo-steady state was reached. Then, all cells leaving the CSTR enter the tubular reactor (at high viability). Accordingly, in the first portion in Figure 3(A) (residence time = 10.3 hr), the cell density climbed gradually to around 5×10^5 cells/mL, then remained constant for approximately two days at which time it dropped sharply (≈ 175 hrs). A clump of cells was released from the CSTR exit/transition space, and was transported down the tubular reactor. This is shown in Figure 3(A) by a drop off in cell number density and a near zero viability. As the clump unleashed the accumulated cell layer, the exit sample was principally dead cells. Subsequently, we changed the speed of pump 3 (Figure 1) and lowered the mean residence time in the tubular reactor in several steps. Again, on several occasions at different residence times, a clump of cells was released and upset the tubular reactor pseudo-steady state.

To improve this conditions, the CSTR was modified so that the cells were no longer withdrawn from the vertical tube where a thin layer of cells had often agglomerated and died (based on visual observation). Instead, a thin glass tube (≈ 2 mm ID) was annealed to the CSTR at a downward angle of 45° from the vertical. This prevented cells from flowing directly upward, partially settling, and sticking to the tubing walls; a second set of experiments was performed. The total length of tubing between the CSTR and pump 3 for these two sets of experiments was the same (25 cm). The results shown in Figure 3(B) were slightly improved in terms of time necessary for the reactor to reach pseudo-steady state, but cell layers prior to the tubular reactor inlet pump still collapsed occasionally.

Since the pseudo-steady state was formed more quickly with the inclined withdrawl tube, we suspect that cell layers on the withdrawl tubing were not as thick. In addition, the cell densities measured in the second experiment set were higher than those in the first set, implying that there was less retention in the downward sloped tube and the cells flowed through the reactor system more smoothly.

The mean cell density for each pseudo-steady state is depicted as a function of residence time in Figure 4(A). For comparison, we have also depicted the cell densities in the CSTR for both experiment sets as a function of time after reaching the first pseudo-steady state. The cell densities in the CSTR for both sets were roughly similar, varying from 7 to 7.5×10^5 cells/mL. A lower residence time in the tubular reactor resulted in a higher viable cell density at the reactor exit. At lower residence times (higher liquid shear), there was less accumulation of cells prior to the pump inlet as expected. Note also that the average cell density in set II (angled withdrawl port) was slightly higher than in set I (vertical withdrawl port); again illustrating the advantage of a downward sloping withdrawl port in ensuring similar cell densities as the CSTR.

We repeated these experiments for four mean residence times, all the data obtained were compiled into Figure 4(B) where cell density is depicted as a function of linear velocity. Our results suggest that cells settled more at low linear velocity and settled significantly once the linear velocity dropped below 2 cm/min. Below this level, one of two conditions took place: either (1) the pseudo steady state cell density dropped sharply; or (2) a pseudo-steady state was never achieved. Thus, a linear velocity of 2 cm/min appears to be a minimum threshold necessary to keep cells suspended within the liquid element and moving down the reactor's length. Interestingly, Stokes settling velocity for a cell ($\approx 15 \mu\text{m}$, $\approx 1.4 \text{ g/cm}^3$) in this medium ($\mu \approx 0.011 \text{ g/cm}^2\text{sec}$) is roughly 0.2 cm/min, which is roughly one tenth the linear velocity in the trailing vertical region of a liquid element.

In summery, a tubular reactor was established that effectively transported Sf-9 insect cells in a plug-segmented flow pattern while maintaining high cell viability. This was accomplished due to the alternate introduction of air and liquid elements. Occasionally, a clump of agglomerated cells prior to the tubular reactor inlet pump was released into the tubular reactor. Efforts to minimize this problem by altering the withdrawl tube design were partially successful. Further efforts such as minimizing the tubing length and

Figure 4. Panel (A): Mean cell density at the exit during pseudo-steady state versus residence time. Panel (B): the relationship between cell density and linear velocity in the tubular region.

adding surfactants to prevent agglomeration may minimize the clumping further. Nonetheless, we were able to establish the appropriate hydrodynamic flow patterns after the peristaltic pump and in the tubular reactor so that well-mixed fluid elements were carried down the length by sequential addition of air bubbles, and the steady state period was sufficient, compared to the residence time, to evaluate the virus infection.

Infection and expression

Infection was initiated by the introduction of virus and cells at the entrance of the tubular reactor. As described in the Methods section, the experimental control was provided by cells taken directly from the CSTR and infected in a T-flask. During these experiments, we changed the ratio of air and liquid so that the profile looked more like sections of liquid film traversing along between air bubbles. As discussed previously,

Figure 5. Panel (A): the cell density in the tubular reactor when conducting infection experiments. Panel (B): time course of β -gal activity in cells infected for different residence times: ($\circ$), β -gal yield for cells infected in T-flasks; ($\bullet$), β -gal yield for cells infected in the tubular reactor. For both infection protocols, cells were washed in PBS and resuspended in fresh media during the expression stage. RT is the residence time.

we have taken precautions to avoid oxygen and/or glucose limitation. The MOI was controlled by fixing the flow rate ratio of virus solution and cells. Also, we ran the reactor at high MOI (≈ 60) to ensure that all cells were infected by primary infection. Consequently, infection efficiency is determined by the increase in activity of β -galactosidase relative to the control. Figure 5(A) shows the cell density curve in the tubular reactor exit while conducting the infection experiments. Note that the cell densities were stable for at least seven residence times prior to sampling so that a well established flow pattern for infection was evaluated. Panel (B) shows the time course of β -galactosidase activity for cells infected in both the tubular reactor and T-flask at three different residence or infection times. The yield of β -galactosidase was consistently higher from the cells infected in the tubular reactor, thus the infection of cells in the tubular reactor was comparable to or better than cells infected in T-flasks.

Our results also indicate that at lower residence times, primary infection was incomplete in both the tubular and T-flask reactors. This can be seen in Figure 5(B) by lower β -gal yield in the 1.6 hr experiment than in the others. At higher residence times for the tubular reactor ($\tau = 3.2$ and 5.1 hr), there were no significant differences in either the rate of increase or the level of β -gal activity, indicating that the infection process was completed prior to 3.2 hours post-infection. On the other hand, the T-flask measurements rose slightly from the 3.2 to 5.1 hr infection experiments, suggesting infection was still incomplete at 3.2 hrs. Normally, there is no shaking upon infection in T-flasks, our experiments were done with slow shaking which would provide better mixing and distribution of virus particles upon infection than in normal T-flasks infection. Thus, these results indicate that the infection in the tubular reactor was superior to the T-flask, likely due to the intimate contact of the cells and virus.

The segmented flow pattern developed eliminates the hydraulic residence time distribution common in CSTR's and hence minimizes the infection time distribution noted by other researchers (Bentley *et al.*, 1994; Licari and Bailey, 1992; Power *et al.*, 1994). In this way, we might be able to track cell physiology during the post-infection period without interference from population distributions by taking samples at various distances down the reactor's length. Further, our results suggest that incorporating a linear infection stage between the two reactors of a two-stage CSTR should be feasible, even with the occasional clump of cells leaving the first reactor and traversing down the tube to the second. To do this, the waste jar in Figure 1 can be replaced with another CSTR which is used as the protein producing reactor, while the first CSTR serves as the cell growth reactor. By far, the majority of cells entering the second reactor would be infected by intact NOV's establishing a steady stream of protein producing cells in the second reactor.

In summary, the infection of insect cells was demonstrated in a linear segmented flow bioreactor. Less than 3 hours was needed for complete infection at MOI 60. Although not tested, we expect this reactor could be operated for longer residence times at the same linear velocity (≥ 2 cm/min), so that cell metabolism could be effectively elucidated during the post-infection period. Some possible solutions to the clumping problem include the introduction of air bubbles in the CSTR exit, increasing mixing by an inline static mixer between the CSTR exit and the tubular reactor inlet pump, enclosing a small diameter permeable tube in the bottom of the tubular reactor for generating air bubbles continuously (Moser, 1991; Hammarberg *et al.*, 1991), and adding methylcellulose or dextran sulfate to alleviate cell agglomeration. Several of these ideas are currently being evaluated.

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