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Continuous production of baculovirus in a cascade of insect-cell reactors

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Summary. Insect cells *(Spodoptera frugiperda)* were cultured in a continuous stirred-tank reactor. The effluent was led to a cascade of another two reactors, each containing half the volume of the cell-growth reactor, where the cells were infected with *Autographa californica* nuclear polyhedrosis virus. For about 10 days production of 10^7 polyhedra (virus particles embedded in a protein capsule) per cm^3 was achieved. This short production time compared to previous experiments involving an analogous system with a single infection vessel of equal volume to the cell-growth vessel is ascribed to the accelerated occurrence of the so-called passage effect (a decrease of infectious virus with time). From the results of a computer model it was concluded that this passage effect was accelerated by the change in residence time distribution as compared to the earlier experiments.

Introduction

Baculoviruses occur almost exclusively among specific families of insects, in which they are lethal. They offer an interesting possibility as biological insecticides (Martignoni 1984). Baculoviruses can also be used as expression vectors for foreign genes, coding for proteins of medical, pharmaceutical or veterinary importance (Luckow and Summers 1988).

Two infectious forms of the virus occur: polyhedra, which are protein capsules in which the rod-shaped virus particles are embedded, and the non-occluded virus (NOV) form. Because of their stability polyhedra provide protection of the occluded virus particles in the environment. However, in the midgut of larvae they dissolve, due to the alkaline conditions, thereby releasing the virus particles.

NOVs are important for the spread of the infection in the insect host and in cell cultures. Cells start to produce NOVs about 12–14 h after infection, which are secreted (ca. 200 NOVs per cell, de Gooijer et al. 1989), until about 24 h after infection. In a second phase virus particles, which continue to be produced, are occluded into polyhedra. This process ends about 48–72 h after infection when the cell starts to disintegrate.

Baculoviruses are routinely produced on a small scale using insect larvae (Shapiro 1986). A process based on this production method is difficult to control and hard to scale up. It is, however, also possible to produce baculoviruses in insect-cell cultures. This is a more promising approach to large-scale production.

The production of *Autographa californica* nuclear polyhedrosis virus has been achieved in various cell lines (see for a review: Weiss and Vaughn 1986). This production was either batch-wise or semi-continuous. The replication cycle of baculoviruses offers the possibility of producing these viruses in a continuous system (Tramper and Vlak 1986). Such a system may consist of an insect-cell-producing bioreactor and one or more bioreactors in series (Fig. 1) in which baculoviruses or recombinant proteins are produced (Tramper et al. 1990).

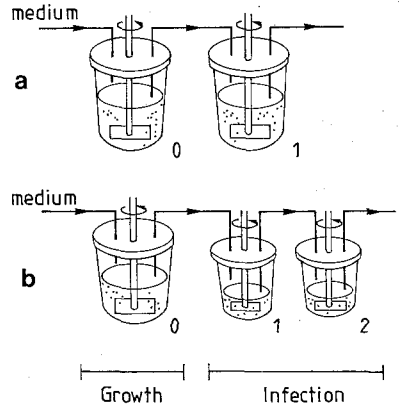

Fig. 1a, b. Reactor configurations for continuous baculovirus production. **a** Configuration described by Kompier et al. (1988). **b** Configuration described in the present study

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The run-time of the system will be limited by the so-called passage effect (Tramper and Vlak 1986). After prolonged passage through multiple infection cycles the infectivity of the virus diminishes, resulting in fewer polyhedra per cell and an increasing number of polyhedra with abnormal morphology (MacKinnon et al. 1974). These effects become significant after ten passages and severe when the number of passages is beyond 25 (Faulkner 1981).

Continuous production of baculovirus has been shown to be possible in a two-reactor system (Fig. 1a) for about 1 month (Kompier et al. 1988). Then the number of polyhedra and NOVs produced per cell and the fraction of cells containing polyhedra decreased, which was ascribed to the passage effect. During the pseudo-steady states of the two experiments described, values of 26% and 55% were observed for the fraction of cells containing polyhedra (Kompier et al. 1988).

The most efficient infection of cells occurs in a plug-flow reactor. Here the residence time does not show a distribution and can be chosen such that every cell stays in the reactor during one complete infection cycle and leaves the reactor before it lyses. This will facilitate downstream processing. If the infection reactor in the system as described above is replaced by two reactors each containing half the volume of the cell-growth reactor (Fig. 1b), a relatively large step in the direction of a plug-flow reactor is made.

In this study we report on the continuous production of *A. californica* nuclear polyhedrosis virus in *Spodoptera frugiperda* cells in a system consisting of three reactors (one for growth of cells and two for virus production). We observed higher fractions of cells containing polyhedra, but the running time of the system was considerably shorter compared to the two-reactor system (Kompier et al. 1988). About 2 weeks after the infection started, the production of baculoviruses decreased rapidly. By means of a model (De Gooijer et al. unpublished data) we concluded that the passage effect was enhanced by the change in residence time distribution in the virus production part.

Materials and methods

Spodoptera frugiperda cells (Sf-AE-21) (Vaughn et al. 1977) were maintained in TNM-FH medium (Hink 1970) without egg ultrafiltrate but supplemented with 10% foetal calf serum and 0.1% (w/v) methylcellulose. The NOV form of the E2-strain of *A. californica* nuclear polyhedrosis virus (Smith and Summers 1978) was used.

The continuous system consisted of one 2-dm^3 (working volume: 1.2 dm^3) flat-bottomed fermentor (Applikon, Schiedam, The Netherlands) to culture the insect cells (reactor 0 in Fig. 1) and two 1-dm^3 (working volume: 0.6 dm^3) round-bottomed fermentors (Applikon) for production of virus (reactors 1 and 2 in Fig. 1). The reactors were equipped with marine impellers and air was passed through 0.2 μm filters into the headspace of the reactors at about 10 $dm^3 \cdot h^{-1}$. The temperature in the reactors was kept at 28° C. The reactors were separated from each other by drop formers. To connect the various parts of the system, silicone tubing and peristaltic pumps were used. The mean residence time in the insect cell reactor was set to 60 h by adding medium at 20 $cm^3 \cdot h^{-1}$. Consequently, the mean residence time in each virus producing reactor was 30 h.

Three experiments were performed with the set-up mentioned above. In the first experiment the cell-growth reactor (reactor 0) was inoculated with cells which had been subcultured 50 times in tissue-culture flasks. The infection process was started 30 days after inoculation of the cell-growth reactor. For the second experiment the same uninterrupted cell-growth reactor was used, 92 days after it had been inoculated with cells. The cell-growth reactor for the third experiment was inoculated with cells which had been subcultured 80 times. Infection was started 40 days after inoculation.

To start the infection process, the outlet of the cell-growth reactor was connected to the infection reactors (1 and 2). Cell suspension (300 cm^3) of the continuously operated cell-growth reactor was pumped into the first infection reactor to allow a good initial mixing of this reactor. An amount of virus resulting in a multiplicity of infection (number of infectious NOVs per cell) of about 2.5×10^{-2} median tissue culture infective dose ($TCID_{50}$) units per cell was added to the first infection vessel. About 30 h after inoculation the cell-growth reactor and then the first infection vessel became filled. At this point 300 cm^3 of the cell suspension of the first infection vessel was pumped into the second infection reactor.

The start-up procedure of the third experiment was slightly different. The infection reactors were filled as described above, but virus was added to the first infection reactor when all three reactors were completely filled and running continuously. The initial multiplicity of infection here was 1 $TCID_{50}$ unit/cell.

The production process was monitored almost daily by taking samples of about 5 cm^3 from each infection vessel and of about 1 cm^3 from the cell-growth vessel. The number of cells was determined microscopically, using an Neubauer haemocytometer. Cell viability was measured, using the exclusion of trypan blue (0.4% w/v in phosphate-buffered saline) as indicator.

In the samples of both virus-production reactors the fraction of cells containing polyhedra was determined. The presence of polyhedra in the nucleus was assessed using a microscope (magnification 400 ×). To determine the infectivity of NOVs, 4-cm^3 aliquots of the samples were centrifuged (1500 *g*, for 15 min) and

Table 1. Average values for cell concentrations and non-viable cell fractions, during pseudo-steady-state conditions

Experiment no.	Reactor 0		Reactor 1		Reactor 2	
	Cell conc ($10^5/cm^3$)	% Non-viable cells	Cell conc ($10^5/cm^3$)	% Non-viable cells	Cell conc ($10^5/cm^3$)	% Non-viable cells
1	9.5	3	8.6	18	8.2	46
2	9.4	5	8.2	45	8.1	52
3	10.5	2	11.5–11.2[a]	14	11.5–9.0[a]	38

[a] No stable value was reached: cell concentration declined slowly

the supernatant was filtered through a 0.45-μm filter. Infectivity was measured using an end-point dilution method (Vlak 1979). The pellet was resuspended and lysed (0.1% sodium dodecyl sulphate, for 45 min at 43° C) to release the polyhedra. The polyhedra were counted using a Neubauer haemocytometer.

Results and discussion

In Table 1 the average total cell concentration and cell viability reached in each of the three reactors are given for each experiment. In Fig. 2 cell concentration and viability with time are shown for experiment 1. The two apparent peaks in the graphs of the non-viable cell fraction in both infection reactors were due to brief malfunctioning of a pump. The total cell concentration in both infection reactors during the first two experiments was slightly lower than in the respective cell-growth reactors (Table 1). Considering the residence time distribution of the cells, this is probably due to lysis of cells with a long residence time. At the start of the third experiment cell concentrations in both infection reactors were higher than in the cell-growth reactor. Here growth of cells occurred due to the different start-up procedure. During this run cell concentrations in both infection reactors decreased slowly. The fraction of non-viable cells in both infection reactors increased rapidly about 4 days after infection in all three experiments until it reached a constant value (Table 1). After about 18 days the non-viable cell fraction slowly decreased in both reactors.

To assess how efficiently the cells were infected, we determined the fraction of cells containing polyhedra with time. In Fig. 3a this fraction is shown for the first infection reactor and in Fig. 3b for the second infection reactor, for all three experiments. If it is assumed that cells coming into the reactor are immediately infected and that polyhedra become microscopically visible 24 h after infection, the fraction of cells containing polyhedra can reach 45% in the first infection reactor and 81% in the second infection reactor, based on calculations of the residence time distribution (Levenspiel 1972). Considering that these are maximum values based on an ideal situation, the values of 30%–35% and 50%–70%

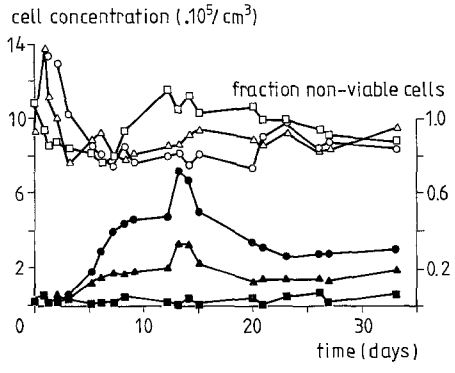

Fig. 2. Cell growth in all three reactors of the cascade for experiment 1: *open symbols,* total cell concentration; *solid symbols,* fraction of non-viable cells; □, ■, cell-growth reactor; △ ▲, first infection reactor; ○ ●, second infection reactor

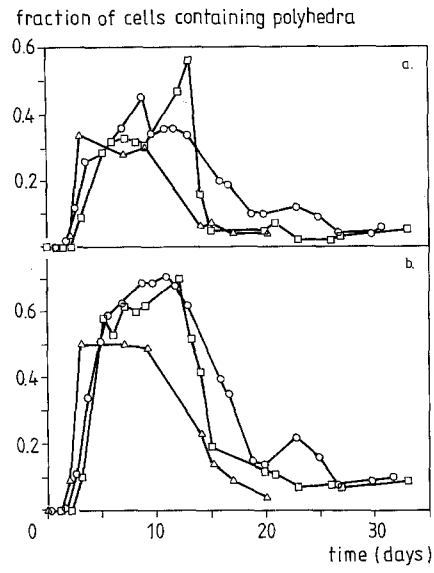

Fig. 3a, b. Fraction of cells which contained polyhedra. **a** First infection reactor. **b** Second infection reactor. □, experiment 1; △, experiment 2; ○, experiment 3

which we found in the first and second infection reactors, respectively, are in good agreement.

When a single infection reactor is used the fraction of cells which contain polyhedra decreases after about 25 days (Kompier et al. 1988). In the reactor system described here this fraction decreased after about 15 days. Apparently, the decrease was accelerated when mixing became more like a plug-flow system. To assess if the decrease in cells containing polyhedra was due to a passage effect a computer model (de Gooijer et al. un-

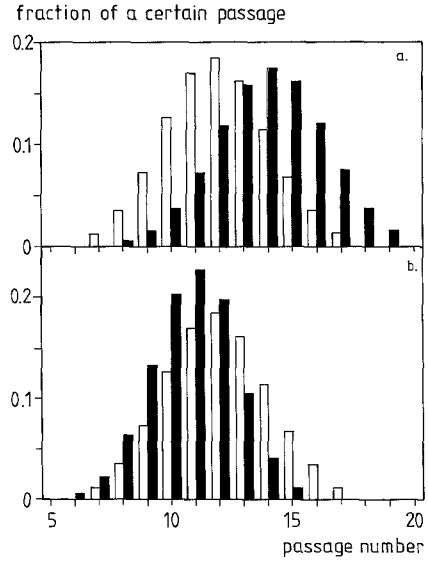

Fig. 4a, b. Comparison of the virus passage distributions between a reactor configuration with one infection reactor *(open bars)* and a configuration with two infection reactors *(solid bars).* **a** Both configurations 25 days after infection. **b** Configuration with one infection reactor 25 days after infection and configuration with two infection reactors 15 days after infection (approximate times of decrease of infection)

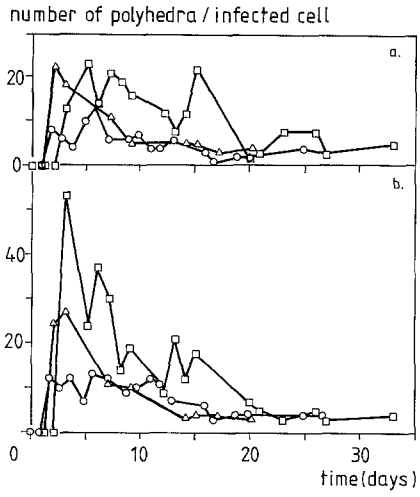

Fig. 5a, b. Number of polyhedra per cell containing polyhedra. **a** First infection reactor. **b** Second infection reactor. □, experiment 1; △, experiment 2; ○, experiment 3

published data) was used to calculate the fraction of virus from each passage in an infection reactor.

For each virus passage present in the second infection reactor 25 days after infection, the fraction was calculated and compared to the calculated passage distribution in a system with a single infection reactor (Fig. 4a). In the system with two infection reactors relatively more virus with a higher passage number was present. The cascade of two mixed reactors thus accelerated the occurrence of virus with higher passage numbers. A comparison between the predicted virus passage distributions in both reactor configurations when the decrease of infection occurred was also made and is shown in Fig. 4b. Both distributions showed a good similarity, considering the simplicity of the model.

In Fig. 5 the number of polyhedra per polyhedra-containing cell is shown for the first (Fig. 5a) and the second (Fig. 5b) infection reactor. The values for the second infection reactor were in the same range as found by Kompier et al. (1988). The decreasing number of polyhedra was concomitant with the decrease in the number of polyhedra-containing cells, with a 100-fold decrease of infectious NOVs (data not shown) and with the occurrence of high-passaged virus (Fig. 4). This strongly indicates the occurrence of a passage effect.

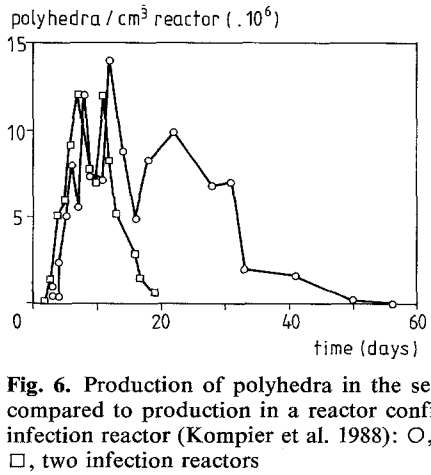

Fig. 6. Production of polyhedra in the second infection reactor compared to production in a reactor configuration with a single infection reactor (Kompier et al. 1988): ○, one infection reactor; □, two infection reactors

Figure 6 shows the number of polyhedra per cm^3 reactor volume for the second infection reactor. These data are from experiment 3. For comparison, data from Kompier et al. (1988) for a reactor configuration with one infection reactor are shown on the same graph. The maximum production level in both systems is similar (about 10^7 polyhedra/cm^3 reactor volume), despite the higher fraction of polyhedra-containing cells in a reactor configuration with two infection reactors. This is due to the lower cell concentrations in the cell-growth reactors in the present experiments.

In conclusion, replacing the infection reactor in a continuous culture, consisting of a growth reactor and one infection vessel, by a more plug-flow-like configuration of two continuous stirred-tank reactors increased the fraction of cells that contained polyhedra. After 15 days the number of polyhedra-containing cells, the number of polyhedra per cell and the number of infective NOVs decreased. This strongly suggests the occurrence of a passage effect. This decrease occurred 10 days earlier than observed in a reactor configuration with one infection reactor. This is in accordance with our calculations which predict that a plug-flow-like reactor configuration accelerates the occurrence of higher passage numbers.

Although a higher fraction of cells contained polyhedra, production, measured as polyhedra per cm^3 bioreactor, did not differ significantly when using two instead of one infection reactor. This was probably due to lower cell concentrations obtained in the cell-growth reactor. However, even when cell concentrations would have been the same, two infection reactors would not have been advantageous, because of the shorter running time as result of the faster passage effect.

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