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A CONTINUOUS PROCESS FOR THE PRODUCTION OF BACULOVIRUS USING INSECT-CELL CULTURES

R. Kompier, J. Tramper* and J.M. Vlak+

Agricultural University, Department of Food Science, Food and Bioengineering Group, Bomenweg 2, 6703 HD Wageningen,
+ Department of Virology, P.O.B. 8045, 6700 EM Wageningen, The Netherlands.

SUMMARY

A continuous culture of insect cells (*Spodoptera frugiperda*) was used for continuous production of baculovirus (nuclear polyhedrosis virus from *Autographa californica*). The system consisted of a cascade of two continuous stirred tank reactors (CSTRs). In CSTR I the insect cells were grown in suspension. This suspension was fed continuously to CSTR II where the virus infection occurred. For a period of about 25 days the average volumetric productivity was about 10^7 polyhedra (virus particles occluded in protein capsules) and 10^8 infectious NOVs (non-occluded virus particles) per cm^3 effluent. This is equivalent to 25 polyhedra and 250 NOVs per infected cell, respectively. In one case, the percentage of infected cells was 65%, which is close to the theoretical value of 68%. After a run-time of 32 days a decrease of process productivity was observed, probably due to the so-called passage effect, a degeneration of the virus DNA.

INTRODUCTION

Baculoviruses are insect pathogens that can be used as biocontrol agent for agriculturally important pest insects (Martignoni, 1984), as alternative to chemical insecticides. In addition, they can be used as expression vectors for eukaryotic and prokaryotic genes coding for proteins of medical, pharmaceutical and veterinary importance (Luckow and Summers, 1988).

Baculoviruses have a unique, bi-phasic replication cycle (Fig. 1) (Faulkner, 1981; Kelly, 1982). Initially, replicated virus particles are secreted into the medium in the form of non-occluded virus (NOV) particles. These NOVs are infectious for insect cells. Later in the replication cycle the rod-shaped virus particles are occluded in protein crystals, called polyhedra. Polyhedra are infectious for insects. NOVs and polyhedra are key parameters in determining the efficiency of the continuous baculovirus production process.

At present, commercial production of baculoviruses *in vivo* is achieved using insect larvae (Shapiro, 1986). Production on this basis is, however, expensive, time consuming and difficult to scale-up efficiently. More suitable for large-scale production are *in vitro* processes using insect-cell cultures. Batch-wise and semi-continuous production of *Autographa californica* nuclear polyhedrosis virus (AcNPV) in *Spodoptera frugiperda*, *Trichoplusia ni* and *Mamestra brassicae* cells have been reported (see for review: Weiss and Vaughn, 1986). However, a fully continuous production process has not yet been achieved.

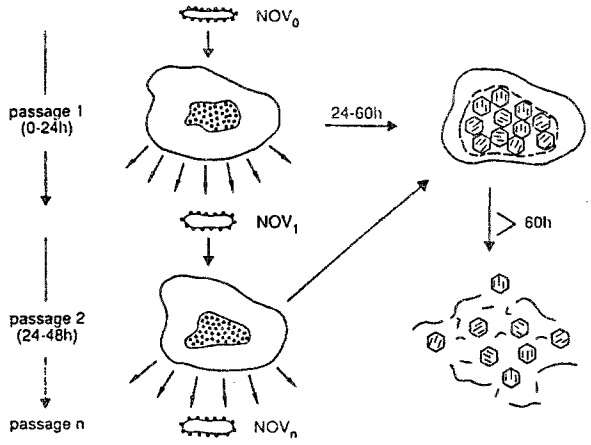

Figure 1. Infection of insect cells by baculovirus.

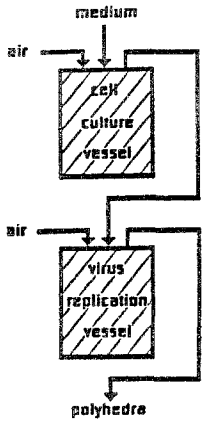

Figure 2. The continuous system.

The aim of this study is the establishment and analysis of a continuous baculovirus production process using two interconnected continuous stirred tank reactors (CSTRs) as proposed by Tramper and Vlak (1986) (Fig. 2). In the cell culture vessel (CSTR I) an established insect-cell line of *S. frugiperda* is propagated. This CSTR is connected to the virus replication vessel (CSTR II), in which the nuclear polyhedrosis virus of the alfalfa looper *A. californica* (AcNPV) is replicated. In order to describe the continuous production process, the cultures in these reactors were examined over time for cell concentration, cell viability, percentage of infected cells, production of polyhedra and production of NOVs.

MATERIALS AND METHODS

Cells and virus

A cell line (IPLB-SF-21) from pupal ovaries of the fall armyworm *S. frugiperda* (Vaughn *et al.*, 1977) was used. The nuclear polyhedrosis virus (NPV) from the alfalfa looper, *A. californica* (Ac) (Vail *et al.*, 1973), strain AcMNPV-E2 (Smith and Summers, 1978) was taken. The virus inoculum used was derived from the third passage in the IPLB-SF-21 cell line.

Fermentors

The continuous production system consisted of two 1 dm^3 round-bottom fermentors (Applikon) equipped with marine impellers, that were operated, after a start-up period, in a continuous fashion while flushing sterile air over the liquid surface. The air supply was passed through a Millipore sterile filter at 9 $dm^3.h^{-1}$. Stirrer speed was 400 rpm (Tramper *et al.*, 1986). Cells were maintained at 28^0 C in TMN-FH medium (Hink, 1970), supplemented with 10% fetal bovine serum and 0.1% (w/v) methylcellulose. By feeding this medium continuously at 13.3 $cm^3.h^{-1}$ to CSTR I, a main residence-time of 60 h was obtained in both CSTRs (working volume of each fermentor: 800 cm^3). All cell suspensions, virus and medium were transferred from vessel to vessel through silicone tubing and peristaltic pumps. CSTR I was separated from CSTR II by a drop former to prevent virus-infection in CSTR I.

Sample analysis

In the present study, duplicate runs have been performed. During each run the production process was daily monitored by the determination of several parameters. Using a Bürker-Türk counting chamber and the exclusion of Trypan Blue (0.4%) as indication of viability, cell concentration and viability were determined in samples from both reactors. Also, the percentage of infected cells in samples from CSTR II was

recorded. Cells were considered to be infected when upon microscopic inspection polyhedra were visible. In addition, samples of 4 cm^3 were taken from CSTR II and centrifuged at 1500 g for 15 min. The supernatant was filtrated through a 0.45 μm filter (Schleicher & Schuell) and then stored at 4^0 C until they were assayed for infectivity (see below). The cell pellet was analysed for the number of polyhedra using a Bürker-Türk counting chamber. Finally, the integrity of the polyhedra was investigated by electron microscopy. Samples, that were taken from CSTR II during the second run at 6 and 56 days post inoculation, were processed according to Van der Wilk *et al.* (1987).

Infectivity measurement

The infectivity of NOVs was measured using the end-point dilution method (Vlak, 1979), expressed as $TCID_{50}$ (Tissue Culture Infective Dose 50%) units. One $TCID_{50}$ unit causes infection in 50% of the cell cultures. The assay was performed in the presence of 0.1% methylcellulose in accordance with the actual situation in the bioreactor system; an assay without methylcellulose served as control.

RESULTS AND DISCUSSION

Start up

From experiments using batch cultures it was observed by light microscopy that about 50 h post infection polyhedra formation is completed. Taking the residence-time distribution into consideration, a mean residence-time of 60 h was chosen. Cell lysis is not yet significant then, which is an important aspect for the down-stream processing.

CSTR I was inoculated with cells, that had been grown in monolayer cultures (Falcon plastic flasks) with weekly subculturing. In CSTR I the cell density reached a steady state within two weeks of 7.5×10^5 and 9×10^5 cells.cm^{-3} for the first and second run, respectively. The cell suspension was then partly pumped from CSTR I into CSTR II; the virus inoculum was added to give an initial multiplicity of infection (MOI) of 8.7×10^{-8} $TCID_{50}$ units per cell in the first run. This is to ensure an optimum MOI at the time of continuous running. The initial MOI in the second run was increased to 4.4×10^{-6} $TCID_{50}$ units per cell because it appeared that 0.1% methylcellulose in the medium reduced the infectivity (unpublished results). The presence of methylcellulose is essential to reduce the shear stress in the bioreactors (Hink, 1982). After a fed-batch period for CSTR I and a batch and fed-batch period for CSTR II the system was operated in a continuous fashion for 25 and 56 days for the first and second run, respectively. The first run was terminated because of a system default.

First run

When steady state levels were reached the average cell concentration in CSTR I and CSTR II was about 7.5×10^5 and 9×10^5 cells.cm^{-3}, respectively (Fig. 3a). This difference in cell density can only be ascribed to growth of uninfected cells in CSTR II; contribution by infected cells is unlikely as virus infection inhibits cell division (Vail *et al.*, 1973).

The percentage of infected cells, as indicated by the presence of polyhedra in the cells, reached a maximum level of 60-65% within 10 days post inoculation (Fig. 3b). This percentage is in agreement with the theoretical maximum percentage of visibly infected cells, based on calculation of the residence-time distribution (Levenspiel, 1972). When we assume that the supplied cells were immediately infected and that polyhedra visibility started 24 h post infection, the theoretical maximum is 68%.

In accordance with the percentage of infected cells, the percentage of dead cells in the virus production vessel rose from 2% at the time of virus inoculation to 60% at day 8. The percentage of dead cells in CSTR I had a constant value of about 2% during the entire run (Fig. 3b).

The infected cells contained about 15 polyhedra per cell (Fig. 3b). This resulted in a volumetric productivity over time as shown in Figure 3c. After 7 days run-time the infectivity reached a steady state of about 10^7 $TCID_{50}$ units.cm^{-3} (MOI = 11) (Fig. 3c).

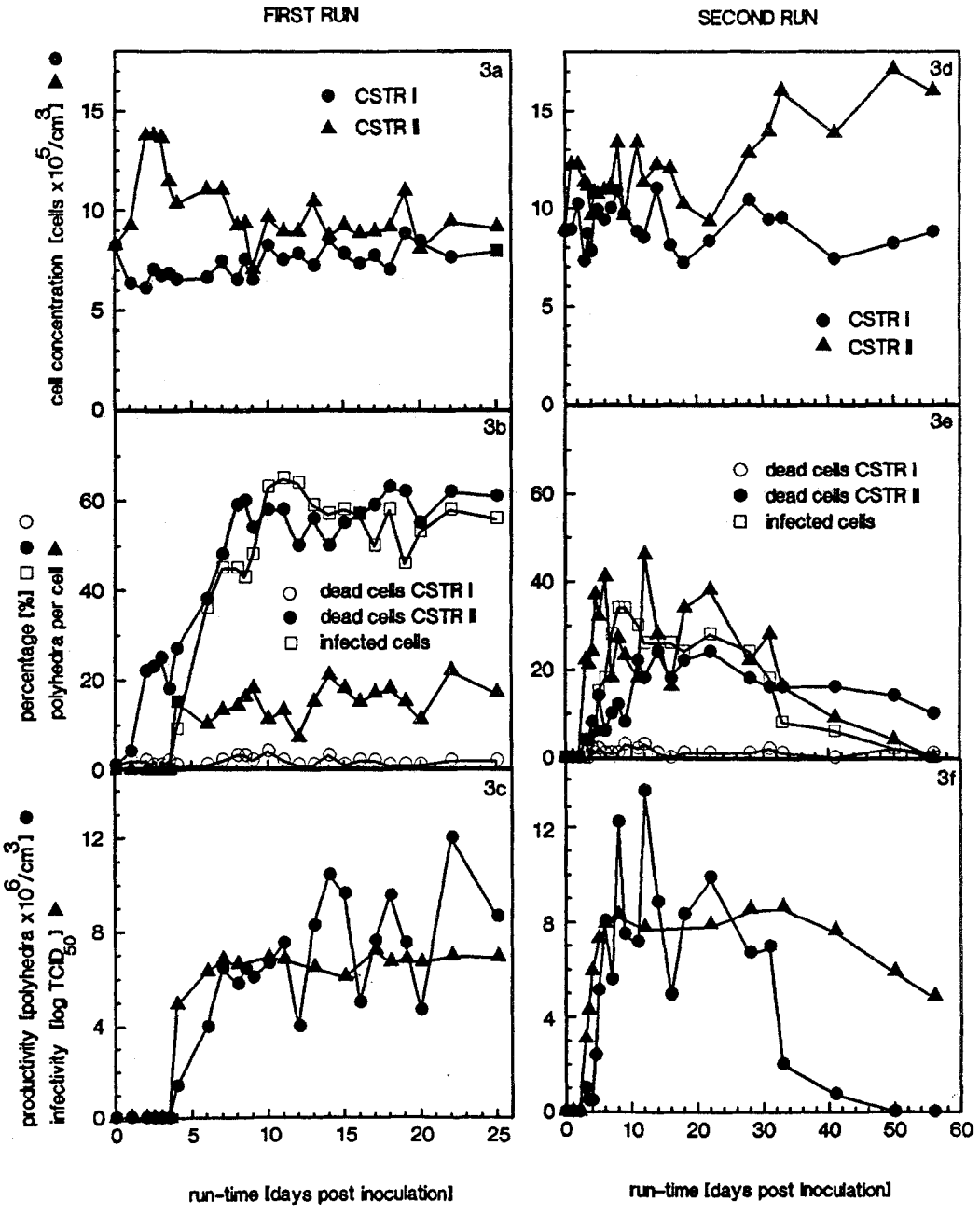

Figure 3. Experimental results of the continuous baculovirus production process.

Second run

The average cell concentration in CSTR I was 9 x 10^5 cells.cm^{-3} with a constant percentage of dead cells of about 2%. An increase of cell growth was observed in CSTR II: the number of cells increased from 1.1 x 10^6 at day 26 to 1.6 x 10^6 cells.cm^{-3} at day 56 (Fig. 3d).

The percentage of infected cells reached a maximum level of 30% within 7 days post inoculation (Fig. 3e). For unknown reasons this is lower than in the first run and about half of the theoretical value of 68% derived from our calculated residence-time distribution.

From day 7 until day 30 the process was in a rather stable condition. The infectivity reached a steady state of about 10^8 $TCID_{50}$ units.cm^{-3} (MOI = 90) (Fig. 3f). From this we can calculate that during this steady state an infected cell produces about 250 NOVs in its infected lifetime. This is in good agreement with the value of 200 NOVs, experimentally found for anchored insect-cells (De Gooijer *et al.*, 1988).

After 30 days post inoculation the percentage of infected cells and the average yield of 25 polyhedra per infected cell started to decrease (Fig 3e). After a run-time of 56 days polyhedra were rarely found and showed, when present, an atypical morphology with defective occlusions upon electron microscopic inspection. Almost no enveloped virus particles were occluded. Furthermore, a similar downward trend was observed for both the percentage of dead cells, falling from 25% to 10%, and the infectivity falling from 10^8 to 10^5 $TCID_{50}$ units.cm^{-3} from day 30 to day 56 (Fig. 3f). These data are a reflection of a decrease of the productivity in the system.
After day 56 no polyhedra were produced any longer in CSTR II, although 10^5 $TCID_{50}$ units.cm^{-3}, resulting in a MOI of 0.06, were present. Since the cells in CSTR II were fully susceptible to virus infection at day 56 (data not shown), it seems as if the virus had gradually lost its capacity to maintain high levels of productivity.

Passage effect

The reduction of productivity and the observed anomalies in polyhedron morphogenesis support the hypothesis that these phenomena are due to the passage effect. This effect is due to a degeneration of the virus when the number of passages increases (MacKinnon *et al.*, 1974). The result is a reduction in the number of polyhedra and the number of virus particles occluded in polyhedra. The passage effect becomes significant when the number of passages surpasses ten, and is very severe above passage twenty-five (Faulkner, 1981). By means of a computer model we have calculated the fraction of the various NOV generations as a function of the run-time (Tramper and Vlak, 1986). This model indicates that in the CSTR passage numbers higher than ten become significant after about a month, which affects the productivity. Detailed biological and molecular-genetical analysis of the virus present in CSTR II at various times after inoculation should further clarify this point.

CONCLUSIONS

To our knowledge this is the first report of a fully continuous process for the production of baculovirus in insect-cell bioreactors. Steady state production levels of polyhedra and NOVs were obtained for about 25 days. A volumetric productivity of about 10^7 polyhedra per cm^3 and a percentage of infected cells of 65% was achieved in the first run. The second run also reached a productivity of about 10^7 polyhedra per

cm^3, but at a lower percentage of infected cells. After a run-time of 30 days phenomena were observed similar to those described for the passage effect. A possible strategy for increasing the process productivity, is to alter the system configuration by replacing the infection vessel by a cascade of two CSTRs of half the volume (De Gooijer *et al.*, 1988). A better total residence-time distribution will thus be obtained (Levenspiel, 1972). The theoretical maximum percentage of visibly infected cells is then 81%. Experiments involving such systems, both for production of baculovirus and for recombinant proteins, are currently under way.

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