Two-stage baculovirus production in insect-cell bioreactors

Frank van Lier

Frank van Lier

Two-stage baculovirus production in insect-cell bioreactors

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen
in het openbaar te verdedigen
op vrijdag 3 november 1995
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

Stellingen

1 Door een lage MOI in een bioreactor te bewerkstellingen kan het "passage effect" uitgesteld worden.

Dit proefschrift.

2 Doordat bij insektecellen een tot expressie te brengen gen zich niet in de cel bevindt, maar er bij iedere produktie door middel van infectie met een recombinant baculovirus ingebracht dient te worden, is grootschalige toepassing van insektecel cultures onaantrekkelijk.

3 Het is kenmerkend voor de behoudendheid van de wetenschap dat de revolutionaire theorie van de "vormende oorzakelijkheid" nauwelijks enige serieuze aandacht krijgt.

Sheldrake, R. (1981) A new science of life. Blond and Briggs, London.

4 Met het begrip factor wordt, binnen de biologische wetenschappen, vaak de suggestie van een verklaring gewekt.

5 Het geeft te denken dat naarmate de leeftijd van een erkend gewetensbezwaarde militaire dienst hoger is, er meer getwijfeld wordt aan de aan de bezwaren ten grondslag liggende motieven.

6 Een idealist is iemand die iets doet ondanks dat anderen het laten, een realist is iemand die iets niet doet omdat anderen het laten.

7 De gangbare notie dat de mens een kuddedier is doet ten onrechte vermoeden dat een groep mensen vredelievend is.

8 Als de vijfdaagse werkweek al vanaf het begin was ingevoerd, had het er met de schepping heel wat beter voor gestaan.

9 Het is absurd dat veel leden van Scouting, de grootste en meest veelzijdige jeugdclub van Nederland, vaak niet voor hun lidmaatschap durven uitkomen.

10 De impopulariteit van dia's ten opzichte van foto's is het gevolg van de doorgaans slechte presentatie van dia's.

11 Een afstand van Groningen is psychologisch korter dan een afstand naar Groningen.

12 Ontkennende vragen kunnen beter niet naar waarheid beantwoord worden, de vrager verwacht doorgaans de ontkenning van het tegendeel dat gevraagd werd ter bevestiging.

Stellingen behorende bij het proefschrift "Two-stage baculovirus production in insect-cell bioreactors"

Frank van Lier

Wageningen, 3 november 1995

Promotor: dr. ir. J. Tramper
hoogleraar in de Bioprocestechnologie

Co-promotor: dr. J.M. Vlak
universitair hoofddocent in de Virologie

The research described in this thesis was carried out at the Department of Food Science, Food and Bioprocess Engineering group of the Wageningen Agricultural University, The Netherlands, in cooperation with the Department of Virology of the same university.
Financial support by the Dutch Programme committee for Agricultural Biotechnology is gratefully acknowledged.

ISBN 90-5485-397-2

Aan mijn ouders

Voorwoord

Naast de stellingen en de samenvatting is het voorwoord een van de delen van een proefschrift dat door het merendeel van de mensen die het proefschrift ter hand nemen gelezen wordt. De stellingen geven een indruk van de gevatheid van de schrijver, de samenvatting een vaag idee waar het allemaal over gaat en het voorwoord vertelt de lezer wie er nou eigenlijk verantwoordelijk waren voor het proefschrift.

Het in dit proefschrift beschreven onderzoek was onderdeel van een samenwerking tussen de vakgroep Virologie en de sectie Proceskunde. Mijn directe begeleider en promotor was Hans Tramper die naast al zijn inbreng in het onderzoek ook zorgde voor een goede werksfeer. Hans, je bent wat ze in de moleculaire biologie een sterke promotor noemen.

Een sterke promotor alleen is niet genoeg. Andere "Proceskunde componenten" van de groep waren Kees "model bouwer" de Gooijer en Fred "cel stresser" van den End. Heren het was een waar genot met jullie samen te werken, of zoals jullie zouden zeggen: ほめて!

Bij de vakgroep Virologie werd het onderzoek aangestuurd door copromotor Just Vlak. Just, bedankt voor je inbreng in mijn onderzoek en de hoeveelheden ~~inet~~inkt die je in mijn manuscripten verwerkt hebt.

Marcel Kool werd als AIO bij de vakgroep Virologie dikwijls omschreven als mijn "counterpart" of zelfs "alter ego". Marcel, ik heb met jou prima samengewerkt en zonder jouw onderzoek had dit proefschrift een heel andere inhoud gehad.

Magda Usmany was de steun en toeverlaat bij Virologie wanneer het ging om praktische problemen met de cellen. Magda, bedankt voor jouw bereidwillige hulp.

Naast de genoemde kern was er nog een fluctuerende groep mensen die aan het projekt meewerkten in het kader van hun afstudeervak of stage. In chronologische volgorde waren dat: Robert Olie, Willem van der Meijs, Nicole Grobben, Peter van der Laar, Jasper van Heemst, Miranda de Vaan, Hans van den Hombergh, Gerard van Duijnhoven, Mirjam den Boer en Arie

van Oorschot. Ongeacht wat er van jullie werk op papier is terecht gekomen: ik ben jullie dankbaar voor alles wat jullie voor het onderzoek gedaan hebben, wat jullie mij geleerd hebben en voor jullie bijdrage aan de sfeer.

Met die sfeer zit het wel goed bij de sectie Proceskunde; sla maar een willekeurig Proceskunde proefschrift open en lees het voorwoord (of in sommige gevallen het nawoord) er maar op na, wat kan ik daar nog aan toevoegen. Ga zo door Proceskunde.

Bij de Vakgroep Virologie, waar we toch met een voor Virologische begrippen flinke opstelling bivakkeerden in een gebouw dat al uitpuilde, werden we zonder meer geaccepteerd, bedankt hiervoor.

Zonder ondersteuning ben je nergens. Het behulpzame secretariaat van Proceskunde (Hedy, Joyce en Tonny) moet in dit verband dan ook zeker vermeld worden. Daarnaast heb ik dankbaar gebruik gemaakt van de ondersteuning van de tekenkamer en de fotolocatie van het "Biotechnion".

Hoewel ik na mijn vertrek uit Wageningen niet vergeten werd deed het mij toch goed in mijn nieuwe werkkring een aantal mensen te treffen die mij gestimuleerd hebben om door te gaan met mijn proefschrift; ik moet eerlijk bekennen dat dat soms moeilijk was: Gerben, Gerco, Kees, Koos, Leo, Menno, maar vooral Bianca, bedankt voor de aanmoedigingen.

En dan als laatsten, maar niet als minsten de twee mensen die er al vanaf het prille begin bij betrokken zijn. Ma en pa het feit dat dit proefschrift aan jullie is opgedragen zegt, hoop ik, genoeg.

Contents

Chapter 1 is an updated version of the publication: van Lier, F.L.J., Vlak J.M. and Tramper, J. (1991) Production of baculovirus-expressed proteins from suspension cultures of insect cells. In: Spier, R.E. and Griffiths, J.B. (eds.) Animal cell biotechnology - volume V. Academic Press, London, 169-188.

1

Production of baculoviruses and baculovirus-expressed proteins in insect-cell cultures

Summary

The baculovirus expression vector system is an established research tool for production of heterologous proteins. This expression system evolved from research aimed at application of baculoviruses in crop protection. To commercialise the production of proteins with a baculovirus expression vector or the production of baculovirus insecticides, large-scale insect-cell culture is necessary. This chapter deals with factors influencing cell growth and especially infection of insect cells and reviews the status of large-scale production with insect-cell cultures.

1. Introduction

Baculoviruses are a class of viruses which almost exclusively affect insects. Baculoviruses have a limited host-range; only a few related insect species are susceptible to a certain baculovirus (Gröner 1986). This makes baculoviruses very suitable for the selective control of pest insects. Several baculoviruses have been used as bio-insecticides. From those mentioned in a review by Huber (1986) only one virus, isolated from the insect *Heliothis zea*, has been commercialized. Others have been developed within the framework of governmental programmes. Especially in forestry, the use of baculoviruses is receiving much attention. Bottleneck in many applications is the production of large quantities of virus. Production usually occurred in insect larvae (Shieh and Bohmfalk 1980). On a large scale this way of production has several disadvantages: mass growth of insect larvae is labour intensive, it is difficult to keep the product free from contaminants, large facilities are necessary and the process is difficult to control automatically. On a large scale, production in insect-cell cultures may be a viable alternative (Tramper and Vlak 1986).

Recombinant-DNA techniques offer another application of baculoviruses. These viruses appear to be very suitable as vectors for the expression of foreign genes (Luckow and Summers 1988, Miller 1989, Luckow 1991). Expression of foreign genes via a baculovirus vector is usually high compared to recombinant protein expression in other eukaryotic systems (Vlak and Keus 1990). Post-translational modification of proteins is comparable to processing of proteins in mammalian cells. It is this application in particular which resulted in a renewed interest in insect cells. A major breakthrough in the field was the FDA approval to MicroGenSys to use a baculovirus-expressed gp160 envelope protein of human immunodeficiency virus (HIV) type 1 as a possible AIDS vaccine in clinical trials (Van Brunt 1987). This gp160 vaccine appeared to be safe and immunogenic in volunteer patients with early HIV infection (Redfield et al. 1991).

For scale up, suspension cultures offer the best possibility. In this chapter the current status of insect-cell culture for production of baculoviruses and baculovirus-expressed recombinant proteins is described with special emphasis on factors influencing cell growth and virus production. Some general (molecular) biological aspects concerning the interaction of baculovirus and insect cell and the application of the system will be presented in section 2. In section 3 factors influencing virus replication and cell growth will be discussed, whereas factors concerning scale up of production will be the subject of a fourth section. Section 5 will deal with modelling of the baculovirus/insect-cell system.

2. Baculoviruses and their application

2.1. Replication

Baculoviruses are characterized, as implied by their name (baculum is Latin for rod), by the presence of rod-shaped virus particles which are occluded in large cuboidal proteinaceous crystals. The genetic information is contained in a double-stranded circular DNA molecule of about 130 kilobase pairs in size. Baculoviruses have been isolated from over 500 species, mainly of the order of Lepidoptera (Federici 1986). The family Baculoviridae can be divided into two subfamilies: the Eubaculovirinae comprising two genera of occluded viruses: nuclear polyhedrosis viruses (NPVs) and granulosis viruses (GVs), and the subfamily Nudibaculovirinae comprising the non-occluded viruses (Francki et al. 1991). The cell- and molecular biology of *Autographa californica* (Ac) NPV has been studied in most detail and much of the information described in this chapter is taken from studies concerning this virus.

Virus particles of the Eubaculovirinae are embedded in a paracrystallin matrix consisting mainly of a single protein called polyhedrin. The occlusion body for NPV, called polyhedron, protects the enclosed virus

particles against rapid decay in the environment. To infect an insect a polyhedron has to be ingested by a larva. The polyhedron will dissolve in the midgut of the larva due to the high pH of 9.5 to 11.5 of gut juices (Granados 1980). The released virus particles infect cells of the midgut epithelium and are replicated there. Part of the replicated virus is budded into the haemolymph and causes a secondary systemic infection, thus spreading the infection through the insect. The viruses leaving the cell are called non-occluded virus (NOV) particles.

The replication cycle of an NPV (figure 1), which also occurs in cultured insect cells, starts with the entering of an NOV into the cell by endocytosis or fusion (Granados 1980). The viral DNA is replicated in the nucleus of the cell and about 12-14 hours post infection (p.i.) virus particles are released from the cell (Hink and Strauss 1976a, de Gooijer et al. 1989). NOVs are responsible for the spread of the infection to other cells. About 18 to 22 hours p.i. secretion of NOVs ceases and formation of polyhedra starts (Granados 1980). Two to four days p.i., the cell lyses and hence releases the polyhedra.

2.2. Baculoviruses in insect control

The host specificity of baculovirus makes the viruses save insecticides because other animal life is not affected (Gröner 1986, Liard et al. 1990). Apart from their host specificity there are several other characteristics which make baculoviruses attractive for use in pest control (Huber 1986). Baculoviruses, being biological agents, are able to multiply within the host population. Resistance to baculoviruses does not seem to be a problem, considering the experience gained until now (Gröner 1986, Huber 1986). In contrast to most other biological agents, baculoviruses can be distributed with conventional techniques.

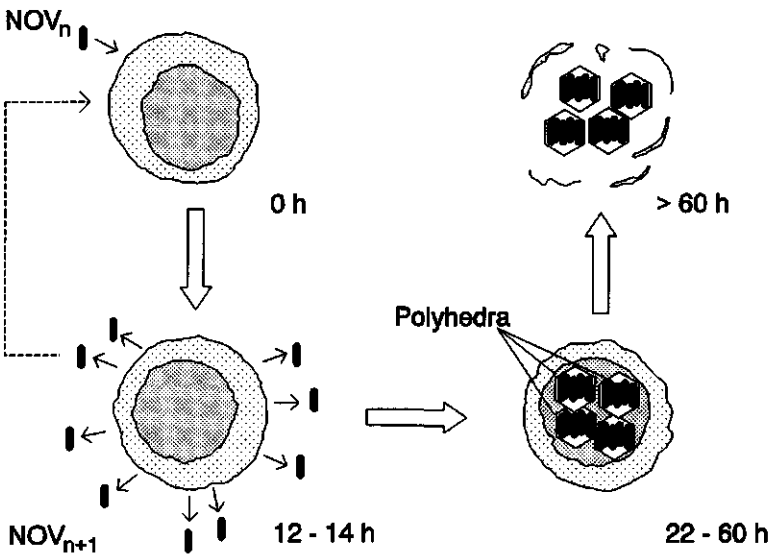

Figure 1.
Infection cycle of a nuclear polyhedrosis virus. NOV: non-occluded virus, the subscript denotes the passage number.

Several baculoviruses have been used as bio-insecticides in agriculture and forestry. In reviews by Huber (1986) and Wu et al. (1989) some examples of successful application of baculoviruses are given. There are however some limitations to the use of baculoviruses in insect control. The specificity of the viruses which was already mentioned as most attractive feature of baculoviruses is paradoxically also the major drawback in commercialization. For companies the specificity means small markets and for the farmer the specificity is a disadvantage if more than one pest has to be controlled.

Another problem is the relative slow speed of action: the insect will not stop feeding immediately after infection. Furthermore, often small amounts of virus are used to reduce costs and it will take some time before the viruses are distributed through the entire insect population. With the use

of recombinant DNA techniques the speed of action can be increased. When genes coding for insect-specific toxins, hormones or metabolic enzymes are introduced in the virus genome, the gene products interfere with the insect metabolism resulting in earlier death or cessation of feeding (Bishop 1989, Vlak 1990).

Biological agents differ from chemical compounds in the way they have to be used and stored. Being stored at ambient temperature baculoviruses will loose activity faster than chemical insecticides. The right moment of application is also more critical compared to chemical insecticides. Often the target insect is only susceptible for the virus during limited life-stages.

Finally, the present production of baculoviruses involves large-scale cultivation of host insects. This procedure is labour intensive, time consuming and difficult to keep free from contamination, resulting in high production costs. Production in insect-cell cultures instead of larva may reduce these costs.

2.3. Baculovirus-mediated expression

When recombinant DNA techniques were developed, expectations were high regarding large-scale protein production using genetically modified bacteria. The majority of the commercially attractive proteins, however, is from higher animals and it was soon realized that bacteria have limitations in their capacity to express eukaryotic DNA into (near) authentic, biologically active proteins. Low eukaryotic organisms like yeast and fungi also turned out to be less attractive systems for the expression of eukaryotic genes as they lack the ability to adequately perform the post-translational modifications which are usually necessary to produce biologically active proteins. Hence animal cells are now more frequently used to produce recombinant proteins.

Among animal cells, insect cells are of growing importance for the

production of recombinant proteins. A convenient and versatile viral expression vector system has been developed (Luckow and Summers 1988, Miller 1989). Insect cell lines are relatively easy to maintain: the cells grow equally well in suspension as on surfaces, they are relatively insensitive to environmental changes and they do not support growth of viruses or expression of oncogenes affecting humans (Agathos et al. 1990). However, information on the physiology of insect cells is relatively scarce.

Due to a number of unique properties, baculoviruses are very suitable as expression vectors of foreign genes. The polyhedrin gene is expressed at high level and is only necessary for production of polyhedra. It is not essential for virus replication in vitro (Smith et al. (1983). The polyhedrin gene therefore is ideal for the allelic replacement by the gene of choice (figure 2A). The additional DNA can be up to 25 kbp in length without disturbing the assembly into the rod-shaped virus particles. To insert a foreign sequence of DNA into the large baculovirus genome, transfer vectors are required (for reviews: Bishop et al. 1992, Davies 1994). These vectors contain a bacterial plasmid and the flanking regions of the polyhedrin gene. The foreign gene is usually inserted behind the polyhedrin promoter and is transferred from the vector to the virus genome by homologous recombination after transfection of insect cells. Recombinant viruses can be easily detected in an infected cell culture because of the absence of polyhedra (Smith et al. 1983) or via positive screening systems (Vialard et al. 1990, Zuidema et al. 1990).

Many proteins have been expressed in insect cells infected with a recombinant baculovirus (for reviews: Luckow and Summers 1988, Miller 1989, Vlak and Keus 1990, Luckow 1991). Post-translational processing in insect cells leads to proteins most of which are "genetically, immunogenically and functionally similar to their native counterparts" (Luckow and Summers 1988). This processing includes proteolytic cleavage, phosphorylation, amidation, myristoylation and both N- and O-glycosylation (Vlak and Keus 1990). The oligosaccharides are of the high mannose type. Kuroda et al. (1989) did not observe elongation of the

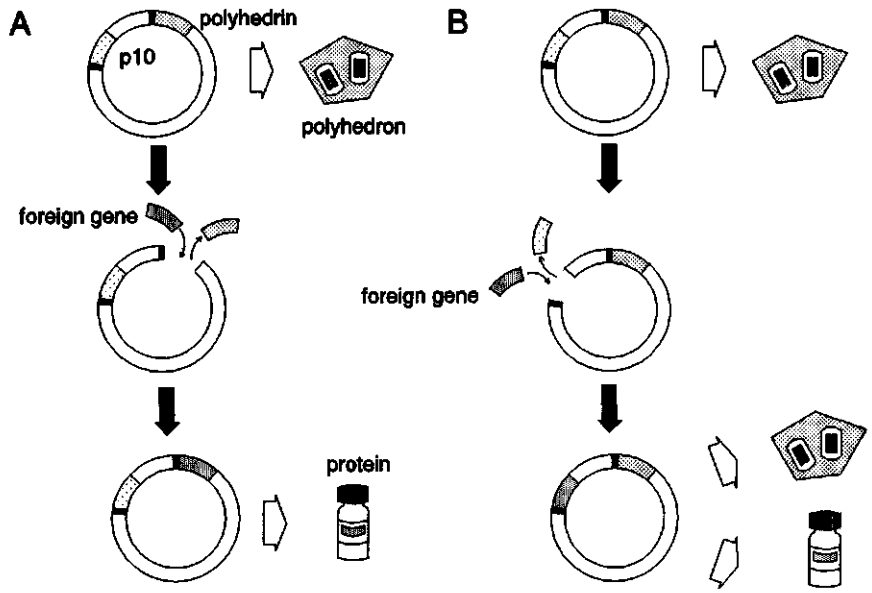

Figure 2.
Schematic diagram of the baculovirus expression vector. A: replacement of the polyhedrin gene, B: replacement of the p10 gene.

influenza virus haemagglutonin-oligosaccharide side chain to complex type. They used larvae of *Heliothis virescens* and cell cultures of *S. frugiperda* cells. In mosquito (*Aedes albopictus*) cells no large complex-type oligosaccharides are present (Hsieh and Robbins 1984). However, Sindbis virus grown in these cells show the same complex glycosylation as in vertebrate cells (Hsieh and Robbins 1984). Davidson and Castellino (1991a) reported that human plasma plasminogen (HPg) expressed in *S. frugiperda* and *Mamestra brassicae* consisted for 40% of complex-type saccharide identical to native HPg. In a further study (Davidson and Castellino (1991b) they state that insect cells contain most glycosyl transferases present in mammalian cells. These enzymes are activated under proper conditions, depending on time. However, this statement has been contradicted by Grabenhorst et al. (1992). They used Sf-21 cells to produce IFN-ß and

IL-2 proteins. Upon their findings they conclude that the baculovirus expression system cannot be considered as a suitable host for production of human glycoprotein therapeutics.

When the foreign DNA has to be expressed in insects, which is particulary relevant when genetic manipulations are aimed at improving the insecticidal action, the loss of the polyhedrin gene is not convenient because infection of larvae with non-occluded virus is not efficient. Here, the p10 gene can be used to insert the DNA (figure 2B). This gene codes for a protein with a molecular mass of 10 kDa. The function of this protein is unknown, but deletion of the gene does not interfere with virus replication (Vlak et al. 1988). The level of expression of the p10 gene is comparable to polyhedrin expression (Vlak et al. 1990), thus providing a good alternative site for insertion. The lack of a phenotypic marker for recombinant selection has been overcome by employing a colour screening system (Vlak et al. 1990).

3. Factors affecting replication

3.1. The cell

Several cell lines are available for infection with NPVs. Almost all data mentioned in this paper were obtained with cell lines from the fall armyworm (*Spodoptera frugiperda*) and the cabbage looper (*Trichoplusia ni*). The cell line IPLB-SF-21 (further denoted as Sf-21) was derived from ovarial tissue of *S. frugiperda* (Vaughn et al. 1977). A clonal isolate, Sf-9 is more or less the standard cell line for in vitro production of recombinant virus. This cell line is available from the American Tissue Culture Association (cat. no. CRL 1711). The *T. ni* cell line Tn-368 (Hink 1970) is also frequently used.

All three cell lines both grow on solid supports and in suspension. Insect cells grown on a surface do not attach as firmly as most vertebrate

cells. Gentle (mechanical) methods are sufficient to remove them from the surface. A cloned cell line from the Tn-386 cell line designated Tn-368A shows a higher degree of attachment compared to the parent cell line. Tn-368A was reported to give higher yields upon infection with NPV compared to the parent cell line (Bilimoria and Carpenter 1983). Another *T. ni* cell line, coded BTI-Tn-5B1-4, is anchorage dependent and produces 28-fold more secreted soluble tissue factor (sTF) than the Sf-9 cell line whereas the production of an Epstein-Barr viral atachment protein (EBV gp105) is similar for both cell lines although EBV gp105 is secreted more efficiently by the BTI-Tn-5B1-4 cell line (Wickham and Nemerow 1993). The above mentioned data indicate that an attached cell line is capable of higher production.

Recombinant protein yield can differ highly between several cell lines and a high yield of recombinant protein is no garantee for high yields when another baculovirus vector is used in the same cell line. This can be concluded from an extensive study of 23 insect cell lines and three recombinant viruses (Hink et al. 1991).

Infection is most efficient when cells are infected in the exponential growth phase (Volkman and Summers 1975). However, resuspending cells during various phases of growth into fresh medium can restore yields to almost maximum level (Wang et al. 1993, Reuveny et al. 1993a). The phase in the cell cycle has some influence. A higher fraction of infected cells is obtained when cells are infected in late or middle S phase as compared to cells infected during G2 phase (Lynn and Hink 1978). The amount of polyhedra and NOVs produced per cell, however, does not vary significantly between cells infected during different stages in cell cycle.

Cell density has great influence on virus production. The optimal cell density for maximum virus production per cell for *T. ni* (Tn-368) cells was reported to be $7.7x10^5$ cell.cm^{-3} (Hink et al. 1977). Maximal production per culture volume, however, was obtained with a cell density of $3.8x10^6$ cells.cm^{-3}. Insect cells can show a form of contact inhibition. Infected Tn-368 cells grown in a confluent monolayer ($5.7x10^5$ cells.cm^{-2}) and at a

density of $1.4x10^5$ cells.cm^{-2} showed a distinct difference in virus production. In the first case only about 0.1% of the cells contained polyhedra 36 hour p.i.; in the latter case this fraction was 90% (Wood et al. 1982).

The effect of cell density on production is not unambiguous. Wickham et al. (1990) tested four different cell lines at several cell densities for their production of ß-galactosidase upon infection with a recombinant baculovirus. The effect of cell density was little for Sf-9 and Sf-21 cells. Tn-368, however, showed significant lower levels of ß-galactosidase production at cell densities greater than about $1x10^5$ cells.cm^{-2}. Effects on another *T. ni* cell line designated Tn-5B1, were dramatic. ß-galactosidase production per cell dropped from 220 to 15 units per million cells when cell density was increased from $0.8x10^5$ to $3.2x10^5$ cells.cm^{-2}.

The above mentioned investigations on the influence of cell density on virus production were done with cells grown on solid supports. Cells grown in suspension also show different production levels at varying cell densities. Sf-9 cells infected with a recombinant virus expressing a bovine rotavirus nucleocapsid protein (VP6), showed a ten-fold decrease in production when cell density was increased from $2x10^6$ to $3x10^6$ cells.cm^{-3} (Caron et al. 1990). When medium was replaced in suspension cultures just after infection, VP6 protein production was restored to maximal production, suggesting depletion of essential medium components or accumulation of waste products. Lindsay and Betenbaugh (1992) conclude that the effect of cell density on recombinant protein production "may be caused, at least in part, by medium and oxygen supply limitations". This conclusion has been confirmed by other investigators (Ferrance et al. 1993, Reuveny et al. 1993a)

The initial multiplicity of infection (m.o.i.), which is the number of infectious viruses per cell, influences both the fraction of infected cells and the number of polyhedra per cell at the end of infection. When m.o.i. is 4 or less the number of polyhedra per AcNPV infected Tn-368 cell becomes

20, 72 hours p.i. (Brown and Faulkner 1975). This number increases when the m.o.i. increases until a maximum of about 60 polyhedra per cell is reached at an m.o.i. of 20-30. When the m.o.i. is increased further, the number of polyhedra per cell drops again. To infect 98% of the cells in a *T. ni* culture an m.o.i. of 5 is necessary (Dougherty et al. 1981). In a study with a recombinant baculovirus expressing ß-galactosidase Sf-9 cells were infected with m.o.i. values between 0 and 100 (Licari and Bailey 1991). Final ß-galactosidase activity increased with increasing m.o.i. when cells were infected in late exponential growth phase. When they were infected earlier in exponential growth phase the m.o.i. value had little influence on final ß-galactosidase activity. In both cases m.o.i. had a clear negative influence on viable cell density. In both studies mentioned the influence of m.o.i. was determined in a surface-attached cell culture.

In sparged cultures an m.o.i. between 0.5 and 10 makes no difference in protein yield upon infection of insect-cells with a recombinant AcNPV (Maiorella et al. 1988, Murhammer and Goochee 1988). In shake flasks no great difference in ß-galactosidase production was found upon infection of Sf-9 cells with an m.o.i. ranging between 1-20 (Neutra et al. 1992).

3.2. Media

Media for insect-cell culture are expensive and medium costs are the cost-determining factor in commercial production (Tramper and Vlak 1986, Pollard and Khosrovi 1987). Therefore the development of cheap and better defined media is one of the important areas in insect-cell culture research. For a more extensive review on this topic the reader is referred to a paper of Vaughn and Weiss (1991).

Information on the physiology of insect cells is scarce. Research has been mainly focused on metabolites traditionally monitored in animal-cell culture (glucose, glutamine, lactate and ammonia). A comprehensive study on the metabolism of Sf-9 cells was published by Ferrance et al. (1993).

They measured metabolite concentrations in a 1.2 dm^3 reactor culture and derived equations for the molecular flow of several metabolites.

Because insect-cell physiology is not yet completely understood, media are often complex. The most widely used media are based on the medium originally formulated by Grace (1962). They include TNM-FH (Hink 1970), BML-TC/10 (Gardiner and Stockdale 1975) and IPL-41 (Weiss et al. 1981a). These media are usually supplemented with more or less undefined components. Originally haemolymph (the body fluid of insects) was used, but this was soon replaced by mammalian sera, in particular foetal calf serum (FCS). In most cases 10% FCS is added. Optimal serum concentration for a *Spodoptera littoralis* cell line has been reported to be 15% (v/v) (Roberts 1984). *Spodoptera frugiperda* cells can be adapted to grow in medium with 1% serum with no significant reduction in doubling time or polyhedron production (Tramper and Vlak 1986). Broussard and Summers (1989) tested the effect of serum concentration on the production of polyhedra and baculovirus-expressed ß-galactosidase in Sf-9 cells. Compared to 10% (v/v) serum-containing Grace and TNM-FH medium, media supplemented with 0.5% (v/v) resulted in higher yields of polyhedra or ß-galactosidase. Even Grace medium without serum improved the yield of both products.

Serum is an undesired compound in media because: it is expensive, its composition is unknown and varies from batch to batch, it can contain adventitiuos agents and there are ethical concerns about the origin of serum. The function of serum is not completely understood, but some suggested functions are: supply of nutrients like lipids, and growth factors, protection of cells against toxic compounds and protection against mechanical forces (Maiorella et al. 1988). To develop a serum-free medium Maiorella et al. (1988) used IPL-41 medium (Weiss et al. 1981a) as a basis. This medium gave, supplemented with serum, the highest cell yield compared to other media tested. To replace the serum yeast extract was added for supply of nutrients and growth factors and protection against toxic components, Pluronic® was added as protectant against mechanical forces and lipids were

supplied as microemulsions. Similary Schlaeger et al. (1993) designed a low-cost serum-free medium.

Two serum replacements used in mammalian-cell culture (CSPR-1 and CSPR-3) have been successfully applied to culture of Sf-21 cells (Vaughn and Fan 1989). Both serum replacements also support the production of AcNPV in Sf-21 cells (Vaughn et al. 1991). At present serum-free media which contain a minimum amount of protein are commercially available, e.g. Ex-Cell 400™, Ex-Cell 401™ (both J.R. Scientific), Sf900 and Sf 900 II (both Gibco). These media support cell growth and virus replication.

Growth of *S. frugiperda* cells on completely defined medium was reported by Wilkie et al. (1980). Chemically-defined media will be a valuable tool in elucidating basic features of insect-cell metabolism.

For media used in production of proteins for diagnostic or theurapeutic applications different demands apply than for media used in biopesticide production. In the first applications mentioned emphasis will be put on a defined and reproducible composition, whereas in biopesticide application the price will be a more prominent factor.

For commercial development of baculoviruses as biopesticides, medium costs are still too high. To make biopesticide production economically possible in bioreactors of 1 m^3 or more, media costs have to be lowered to between \$2 and \$5 per dm^3 (Godwin et al. 1991). Weiss et al. (1992) presented such a low-cost medium for production of viral pesticides. Production with this medium gave similar results compared to standard serum-supplemented media.

3.3. Oxygen

Oxygen consumption rates of animal cells range from $1.4x10^{-17}$ moles.cell^{-1}.s^{-1} to $12.5x10^{-17}$ moles.cell^{-1}.s^{-1} (Fleischaker and Sinskey 1981). For insect cells oxygen consumption rates were reported for *T. ni* (Tn-368)

cells: $6.2x10^{-17}$ moles.cell^{-1}.s^{-1} (Streett and Hink 1978) and *S. frugiperda* (Sf-9) cells: $4.3x10^{-17}$ moles.cell^{-1}.s^{-1} (Maiorella et al. 1988), $5.6x10^{-17}$ moles.cell^{-1}.s^{-1} (Kamen et al. 1991) and even $1.2x10^{-16}$ moles.cell^{-1}.s^{-1} (Reuveny et al. 1993b). In our laboratory a value of $4.5x10^{-17}$ moles.cell^{-1}.s^{-1} was found for Sf-21 cells (R. Kompier, unpublished data). Upon infection an initial increase of oxygen consumption rate was found in *T. ni* cells, reaching a maximum of $12x10^{-17}$ moles.cell^{-1}.s^{-1} 14 hours p.i. (Streett and Hink 1978). Maiorella et al. (1988) reported that oxygen consumption only slightly increased when the Sf-9 cells were infected. They measured an oxygen consumption rate of $4.6x10^{-17}$ moles.cell^{-1}.s^{-1} 21 hours p.i. For the same cell line Kamen et al. (1991) observed a 30-40% increase and Reuveny et al. (1993b) a doubling of oxygen consumption upon infection. In our laboratory also a (slight) increase of oxygen consumption rate (to $5.4x10^{-17}$ moles.cell^{-1}.s^{-1}) was found upon infection for Sf-21 cells (R. Kompier, unpublished data). In a study of Schopf et al. (1990) respiratory activities of Sf-9 cells infected with wild-type (wt) and a recombinant AcNPV expressing ß-galactosidase were compared. Oxygen consumption rate in recombinant-virus infected cells showed a maximum rate of $8.5x10^{-17}$ moles.cell^{-1}.s^{-1} 16 hours p.i. Cells which were not infected consumed oxygen at a rate of $5.7x10^{-17}$ moles.cell^{-1}.s^{-1}. With wt AcNPV the difference between infected and non-infected cells was less significant. Measurement of dissolved oxygen (DO) levels in roller bottles with Sf-21 cells showed also different levels of oxygen consumption between infected and non-infected cells. When cells were infected 6 days after seeding DO was decreased 25% more compared to the non-infected control cultures (Weiss et al. 1982). Klöppinger et al. (1991) obtained highest growth rate and cell density in a bioreactor when the DO level was controlled at 40%. Polyhedra yield was greatly reduced when the DO level was held at 20 compared to levels of 40% and higher (Klöppinger et al. 1991). For the growth of Sf-21 cells an optimal DO level of 70% was reported (Jäger et al. 1991).

3.4. Physical factors

Most Lepidopteran cell lines grow at a temperature of 28-30°C. Infection of cells usually takes place at the same temperature. For infection of *S. frugiperda* cells with a *T. ni* NPV optimal NOV and polyhedra production per cell were found at an infection temperature of 28°C, i.e. equal to optimal growth temperature (Kelly 1981). Data obtained by Hink and Strauss (Hink and Strauss 1976a) and Knudson and Tinsley (1974) indicated that between 21° and 32°C the fraction of cells containing polyhedra upon infection was about the same. The level of 100% infection of *S. frugiperda* cells however was reached faster at temperatures of 27° and 30°C (Knudson and Tinsley 1974). Hink and Strauss (1976a) reported that at temperatures of 25° and 32°C higher amounts of polyhedra per infected *T. ni* cell were obtained compared to infection at 28°C, suggesting that optimal temperature for infection is not necessarily the same as optimal growth temperature. In a study of Reuveny et al. (1993b) a similar production of two different proteins in Sf-9 cells at 22, 25 and 27°C was found (figure 3). Production at 30°C was considerably lower.

The optimal pH for insect-cell culture is slightly acidic; usually the medium has a pH of 6.2 or 6.3. Optimal pH for infection of *T. ni* cells was reported to be between 6.0 and 6.25 (Hink and Strauss 1980).

Insect cells show a relative high tolerance to osmotic pressure (Agathos et al. 1990). Standard media vary in osmolarity from 315 to 375 mOsm/kg. *T. ni* cells were reported to be undamaged nor did they show an effect on susceptibility to infection when the osmotic pressure during 1 hour was varied from 50 to 450 mOsm/kg (Dougherty et al. 1981).

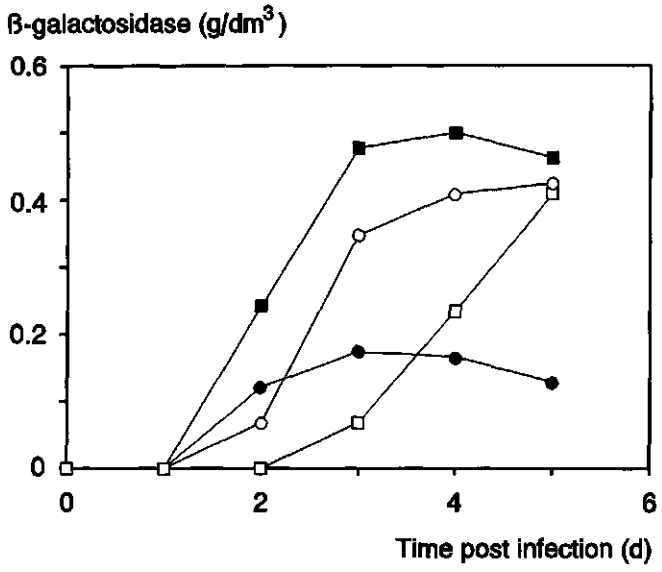

Figure 3.
The influence of temperature on baculovirus-expressed ß-galactosidase in Sf-9 cells (adapted from Reuveny et al. (1993b). □: 22°C, ○: 25°C, ■: 27°C, ●: 30°C.

4. Large-scale production

4.1. Design considerations

Like all animal cells, insect cells are sensitive to hydrodynamic forces. This implies that special measures must be taken when insect-cell cultures are scaled up. Tramper et al. (1992) wrote an extensive review on this topic.

To quantify the effect of laminar shear forces on insect cells rotaviscometers have been used. *S. frugiperda* (Sf-21) cells cultured in TNM-FH medium containing 0.1% methylcellulose, loose viability at laminar shear stresses of 1-4 $N.m^{-2}$ (Tramper et al. 1986). Significant cell

lysis occurs at shear stresses of 0.1 $N.m^{-2}$ and 0.59 $N.m^{-2}$ for Tn-368 and Sf-9 cells, respectively without the addition of methylcellulose (Goldblum et al. 1990). Differences between *T. ni* and *S. frugiperda* cells depend probably on differences in cell size. Quantitative data for shear sensitivity, however, are difficult to compare. Components of the medium can influence the strength of cells. In the mentioned experiments for example, media were supplemented with 10% foetal calf serum, which is known to protect cells against hydrodynamical forces (Kunas and Papoutsakis 1990).

In bioreactors cells are not only submitted to laminar hydrodynamical forces. Especially the introduction of air through sparging causes much stress to the cells. Growing hybridoma cells at different stirrer speeds and using head-space aeration, Oh et al. (1989) did not find significant changes in cell growth within the measured stirrer speed range (100-450 rpm). When the reactor was aerated by sparging a decrease of cell growth was measured within the same stirrer speed range. This effect enhanced with increasing stirrer speed.

Handa-Corrigan et al. (1989) showed that the most dramatic effect of air bubbles on mammalian cells occurs at the surface of the cell culture where bubbles disengage. This is in agreement with our findings with insect cells (Tramper et al. 1988). Murhammer and Goochee (1990) suggested a "killing zone" in the vicinity of the sparger. They base this hypothesis on a comparison of two airlift reactors differing only in sparger design. Tramper et al. (1988) developed a model which correlates the assumed first-order death rate of insect cells to the reactor parameters of a bubble column and to a so-called hypothetical killing volume. This volume is hypothesized to be associated with each air bubble during its lifetime. All viable cells within this killing volume will die. With this model, validated for bubble column reactors and to a large extent for airlift reactors (Tramper et al. 1990), it can be calculated that the height/diameter ratio is the most important design parameter for scale up. At low reactor volumes, this ratio necessary for net cell growth is unrealistic high. This, combined with the absence of sufficient shear protective agents, is probably the reason

why investigators initially failed to grow insect cells in lab-scale bubble column and airlift reactors (Tramper et al. 1986, Wu et al. 1989).

The hypothetical killing volume is not depending on bioreactor dimensions, but on cell characteristics, medium composition, physical parameters (pH, temperature) and air-bubble size. The hypothetical killing volume can be easily measured and gives a value which is indicative for cell strength under bioreactor conditions. For Sf-21 cells a value of $4x10^{-3}m^3.m^{-3}$ was found for air bubbles of 6 mm (Tramper et al. 1988). For a hybridoma cell line grown in medium containing 10% serum a value of $2x10^{-3}m^3.m^{-3}$ was found (Van der Pol et al. 1990). This indicates that the sensitivity of Sf-21 cells is of the same order of magnitude as of the hybridoma cell line used.

Bavarian et al. (1991) used microscopic, high-speed video equipment to visualize the interaction between insect cells and air bubbles. Among others they observed the adhesion of Sf-9 and Tn-386 cells to rising air bubbles. In a succeeding paper (Chalmers and Bavarian 1991) they suggest that bubble break up results in destruction of cells absorbed to the bubble film and destruction of cells in the boundary layer flow into the cavity which originates after a bubble bursts. They propose that the "killing volume" described by Tramper et al. (1988) consists of the medium and cells that make up the bubble film and the thin shell surrounding the cavity resulting from bubble bursting.

To minimize cell death in a bioreactor several components can be added to the culture medium. Addition of 0.5% methylcellulose decreases the rate of lysis of insect cells (based on LDH release) in a rotaviscometer 65-fold (Goldblum et al. 1990). Shear sensitivity of these cells also depended on the methylcellulose used: higher molecular weight methylcellulose gives better protection of cells. The addition of Pluronic® F-68 gave cells almost no extra protection against laminar shear (Goldblum et al. 1990). In bioreactors, however, Pluronic® F-68 proved to be a good protecting agent; Sf-9 cells could be successfully grown in sparged, stirred bioreactors and airlift bioreactors (Murhammer and Goochee 1988).

Pluronic L-35 which has a lower molecular mass compared to Pluronic F-68 provides, on a molar basis, a similar protection to Sf-9 cells cultured in an airlift bioreactor as Pluronic F-68 (Murhammer and Goochee 1990). Hink and Strauss (1980) used methylcellulose in concentrations of 0.1-0.3% (w/v) to enhance growth of Tn-368 cells in suspension.

4.2. Production

The first systems for large-scale cultivation of insect cells consisted of roller bottles (Vaughn 1976a). In order to make this system more suitable for large-scale production a semi-automated cultivation procedure was developed (Weiss et al. 1981a). Typical values for cell densities in roller bottles are in the order of $3x10^6$ cells.cm^{-3} medium (Weiss et al. 1981a, Vaughn 1976a). Polyhedra yield could be improved from $8.5x10^5$ polyhedra.cm^{-3} (Vaughn 1976a) to $9x10^7$ polyhedra.cm^{-3} (Weiss et al. 1981b).

Weiss and Vaughn (1986) used Dyna Cell Propagators to increase the available surface area. These vessels contain a spiral core which gives the 1.7 dm^3 bottle an internal surface of 9500 cm^2. The vessels allow continuous perfusion operation, thus preventing accumulation of waste products in the vessel. With a perfusion rate of 25 $cm^3.h^{-1}$ a yield of $2.3x10^7$ polyhedra.cm^{-3} was achieved.

Most, if not all, insect-cell lines are able to grow in suspension. Shake and spinner flasks are routinely used to grow insect cells in suspension. Shake flasks provide a simple and cost-effective way to produce small amounts of baculovirus-expressed protein. An optimized procedure for shake flask culture has been described (Neutra et al. 1992). Hink and Strauss (1976b) observed higher cell densities in spinner flasks when the head-space was actively aerated; cautious sparging of air resulted in even higher cell concentrations. To prevent cell clumping 0.1% methylcellulose was added. The pH increased during growth. Adjusting the

pH 48 hours after inoculation with 0.1 M H_3PO_4 allowed higher maximum cell densities ($3x10^6$ cells.cm^{-3}). Adjusting the pH with 0.1 M HCl resulted in cessation of growth. Hink and Strauss (1976b) also grew the cells in fermenters with 0.4 and 2 dm^3 volume. Here, similar densities were reached although the growth rate was lower. A reactor equipped with a vibromixer gave no satisfactory results.

In later work the same authors describe culture of Tn-368 cells in 2-3 dm^3 reactors and the subsequent production of virus in these cells (Hink and Strauss 1980, Hink 1982). The reactors used to culture the cells were stirred at speeds of 220 r.p.m. and aerated through sparging at 750 $cm^3.min^{-1}$. To reduce damage to the cells the medium was supplemented with 0.3% methylcellulose. The DO level was kept at 50% and the pH was kept constant.

Biospin perfusion bioreactors of 3 dm^3 were used by Weiss et al. (1989) to produce recombinant human ß-interferon (rHu ß-IFN). Sf-21 cells infected with the recombinant AcNPV at an m.o.i. of 10 were perfused at a rate of 50 $cm^3.h^{-1}$. About 10^6 units of rHu ß-IFN were produced per cm^3 culture. Caron et al. (1990) reached a high level of bovine rotavirus nucleocapsid protein (VP6) by infecting Sf-9 cells with a recombinant virus. In a 4 dm^3 surface-aerated bioreactor a production of 350 $mg.dm^{-3}$ VP6 was reached, which is about 25% of total cell protein. In a helical ribbon impeller (HRI) bioreactor 63 $mg.dm^{-3}$ of VP6 was produced (Kamen et al. 1991). The difference between production of VP6 in the HRI bioreactor and the conventional stirred reactor could be caused by the different media used (Caron et al. 1990). In the conventional-stirred reactor TNM-FH+10% FCS was used, while in the HRI reactor the IPL-41-based serum-free medium described by Maiorella et al. (1988) was used.

Most of the bioreactor work done with insect cells has been performed in stirred reactors of less than 5 dm^3. Head-space aeration becomes increasingly insufficient with increasing scale. Sparging of the reactor in combination with stirring introduces hydrodynamical forces resulting in increased cell death. However, this is not an obstacle for large-

scale cultivation. Jain et al. (1991) cultured and infected Sf-9 cells in a stirred, directly sparged reactor with a volume of 70 dm^3 and equipped with hydrofoil impellers. Both Barkhem et al. (1992) and Guillame et al. (1992) scaled up a process from a 2 dm^3 reactor without major problems. In the first case Sf-9 cells were cultured and infected in a 100 dm^3 reactor, in the second case a 150 dm^3 reactor with Sf-21 cells was used.

To circumvent problems resulting from hydrodynamical forces reactors can be designed which cause minimal shear stress or cells can be protected. Protection of cells against shear stress by immobilization in gel beads or capsules is effective but introduces a difficulty, when cells need to be infected. King et al. (1988) surpassed this problem by using a temperature-sensitive baculovirus recombinant. Viruses and cells were co-immobilized in alginate poly-lysine capsules. The infected cells were grown at the non-permissive temperature (33°C). To start replication the temperature was lowered to 27°C, the permissive temperature. In the capsules cell densities of $8x10^7$ $cells.cm^{-3}$ were reached and the virus titre was ten-fold higher than in conventional cultures. A major disadvantage of immobilization, however, is the lytic character of the infection. Cells are only used for a limited time, making immobilization relatively expensive.

The addition of protective polymers like Pluronic® F-68 makes it possible to culture insect cells in sparged systems (Murhammer and Goochee 1988). A 0.75 dm^3 airlift reactor and a sparged stirred tank reactor of 3 dm^3 were used to test the influence of Pluronic® on production of ß-galactosidase in infected Sf-9 cells. Although Pluronic® had good protective capabilities, ß-galactosidase production in head-space aerated systems (0.284 - 0.333 $mg.10^{-6}$ cells) was higher than in sparged systems (0.145 - 0.214 $mg.10^{-6}$ cells). Maiorella et al. (1988) proved that large-scale production with a baculovirus-expression system in an airlift reactor is possible (figure 4). In a 20 dm^3 airlift reactor they cultured Sf-9 cells in medium containing Pluronic® to densities above $5x10^6$ $cells.cm^{-3}$. For production of human macrophage colony stimulating factor (M-CSF) they infected the cells at a density of $2.3x10^6$ $cells.cm^{-3}$. The final concentration

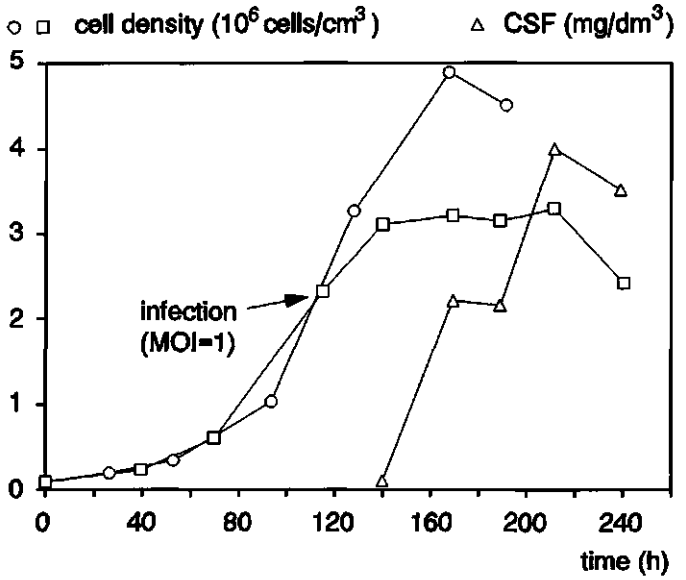

Figure 4.
Production of macrophage colony stimulating factor (M-CSF) in a 20 dm^3 airlift reactor (adapted from Maiorella et al. 1988). ○: cell density of uninfected control culture, □: cell density of infected culture, △: M-CSF concentration.

of M-CSF was 40 mg.dm^{-3} which corresponds to about 12.5 μg.10^{-6} cells. Growth of Sf-9 cells has also been demonstrated in a 10 dm^3 airlift bioreactor (Weiss et al. 1989). In Ex-Cell 400™ serum-free medium a cell concentration of 2.9x10^6 cells.cm^{-3} was established. It was not clear from the report if the Ex-Cell 400™ medium was supplemented with a protective agent. In a newly developed medium containing Pluronic®, Schlaeger et al. (1992) cultured Sf-9 cells in 23 and 60 dm^3 airlift reactors.

Bioreactors can be equipped with systems that allow aeration without bubble formation. Silicone tubing, which is permeable for oxygen, has been used for oxygenation of insect-cell cultures (Miltenburger and David 1980). *Mamestra brassicae* cells were cultured in a reactor filled up to 9.5 dm^3 with 15 m of silicone tube wound around the heating system. The cell

concentration was about $3x10^6$ cells.cm^{-3}. *S. frugiperda* cells grew in a similar reactor with 10 dm^3 medium and 15 m silicone tubing to densities of $1\text{-}5x10^6$ cells.cm^{-3} (Eberhard and Schügerl 1987, Akhnoukh et al. 1993).

Another aeration device consists of moving microporous polypropylene hollow fibre membranes (Lehmann et al. 1987). In a reactor equipped with this device several animal cell lines were cultured including three insect-cell lines (Jäger et al. 1990). Sf-21 cells grew in batch to a cell density of $1.8x10^6$ cells.cm^{-3} and with continuous perfusion to $1.2x10^7$ cells.cm^{-3}. For two *Drosophila* cell lines (Kc and Schneider-2) cell densities were, $6.2x10^6$ for batch culture and $1.2x10^7$ cells.cm^{-3} respectively and for perfusion culture $1.6x10^7$ and $3.1x10^7$ cells.cm^3, respectively.

Systems based on membrane aeration are potentially suitable for scale up. The aeration equipment, however, is expensive and occupies a large volume of the reactor (e.g. for silicone tubing about 15% of the working volume). The possibility of fouling of membranes is an additional disadvantage.

In packed-bed reactors high expression levels of recombinant baculoviruses have been obtained. In a reactor packed with 3 mm glass beads, ß-galactosidase has been produced using a recombinant AcNPV (Shuler et al. 1990). The cell line used was Tn 5B1-4, a strongly attaching strain. The top and bottom of the packed-bed reactor were connected to a bubble column, thus providing circulation of medium based on the airlift principle. Production was 89 units ß-galactosidase per 10^6 cells. When it is assumed that 1 mg of ß-galactosidase has an activity of 400 units (Kennel and Riezman 1977) this value correlates with 220 µg ß-galactosidase per 10^6 cells. In a packed-bed reactor packed with Fibra-cel carriers, a fibrous polyester carrier, ß-galactosidase was produced with Sf-21 cells (Kompier et al. 1990). The ß-galactosidase level here was around 300 µg per 10^6 cells.

Due to the lytic infection cycle of baculoviruses the production time of a batch-operated reactor is limited. To start a new production cycle the reactor has to be cleaned and sterilized because virus left in the reactor will

infect cells immediately and thus prevent cell growth. For a more or less continuous production process of baculovirus (or baculovirus-expressed proteins) separation of cell growth and infection is necessary.

A semi-continuous production process has been set up for production of viruses and cells (Hink and Strauss 1980, Hink 1982). Cells were cultured in a 3 dm^3 reactor and every 24 hours half of the reactor content was pumped into a settling vessel. From this vessel part of the medium was recycled into the reactor, which was then filled up to the original volume by adding fresh medium. The concentrated cell suspension, which remained in the settling vessel, was pumped into the first of a series of four spinner flasks, where cells were inoculated with virus. Every 24 hours this procedure was repeated whereby the contents of a spinner flask was pumped to the next one. Virus was harvested from the fourth spinner flask. In this cell suspension 89-97% of the cells were infected and virus yield was $4x10^7$-10^8 polyhedra.cm^{-3}. Yields of the fifth and sixth harvest were lower.

Another two-stage production process was studied by Klöppinger et al. (1990). They used a batch-operated bioreactor to grow the cells. Virus was produced in a second bioreactor which was also operated in a batch mode. To infect the virus-production reactor part of a previous batch was used. In a repeated batch mode $0.9x10^7$ polyhedra.cm^{-3} were produced. Polyhedra yield could be improved to $1.6x10^7$ polyhedra.cm^{-3} when prior to infection medium was changed and the virus reactor was perfused with medium whereby NOVs were washed out of the reactor. Production of baculovirus-expressed ß-galactosidase could only be improved from $0.52x10^6$ U.cm^{-3} to $1.28x10^6$ U.cm^{-3} when changing from batch to perfusion mode (Klöppinger et al. 1991).

The same system as used by Klöppinger et al. (1990, 1991) has been applied for production of bacterial chloroamphenicol acetyltransferase (CAT) in BmNPV-infected *Bombix mori* cells (Zhang et al. 1993). Four consecutive infections were done, each lasting 5 days. During these runs production was reproducible. In a long-term experiment ß-galactosidase

was produced in AcNPV-infected Sf-21 cells (van Lier et al. 1995). Here 8 consecutive runs of 5 days with a reproducible production could be performed whereafter production of ß-galactosidase declined and stayed low during the rest of in total 23 runs.

A fully continuous production process involving cell growth and virus production was employed by Kompier et al. (1988). Two head-space aerated stirred reactors with a working volume of 0.8 dm^3 were connected in series. Medium was continuously fed to the first reactor where SF-21 cells were cultured. The outlet of this reactor was connected to the second reactor where the infection of the cells took place. The polyhedra yield in this reactor was about 10^7 per cm^3. This production was maintained for about four weeks, then virus production decreased rapidly (figure 5). This effective production time was also found when in the same reactor system a recombinant virus expressing ß-galactosidase behind the polyhedrin promoter (van Lier et al. 1992, figure 5) or p10 promoter (van Lier et al. 1994) was used. In the two runs described by Kompier et al. (1988) only a small fraction of the cells (60% and 30% respectively) was infected. To increase the fraction of infected cells the infection reactor was replaced by two reactors each containing half the volume of the original reactor, thus creating a more plug-flow like infection system (van Lier et al. 1990). An increased fraction of infected cells up to 70% was obtained, but also a decreased time during which virus was produced.

The decrease of virus production was ascribed to a phenomenon known as passage effect (Tramper and Vlak 1986). When virus is propagated in vitro and serially passaged, the infectivity diminishes as evidenced by the production of fewer NOVs and polyhedra per cell and an increasing number of polyhedra with an abnormal morphology (MacKinnon et al. 1974, Potter et al. 1976). However, Faulkner and Henderson (1972) did not find significant changes in infectious virus titre after 12 virus passages of *T. ni* NPV. For *S. frugiperda* cells infected with AcNPV a decrease of virus titre was observed at the ninth passage (de Gooijer et al. 1990, 1992). Faulkner (1981) concluded that the effects of prolonged serial

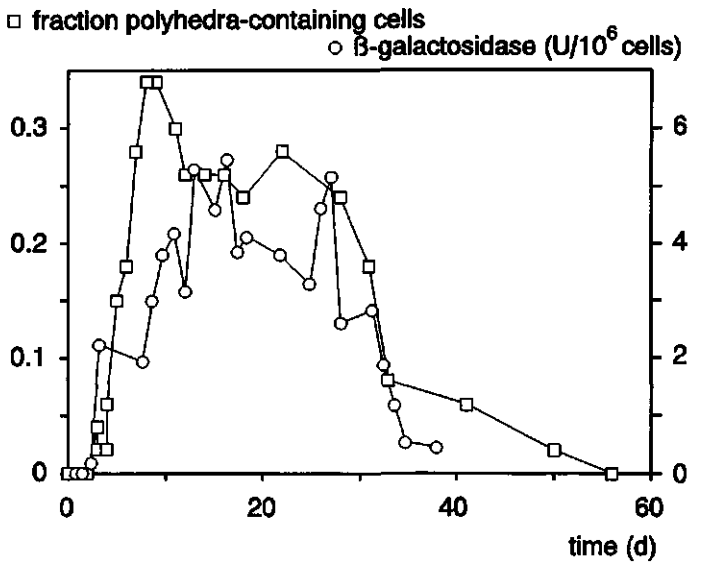

Figure 5.
Continuous production in a two stage bioreactor configuration. □: fraction of polyhedra-containing cells upon infection with wild-type virus (Kompier et al. 1988), ○: ß-galactosidase production per 10^6 cells upon infection with a lacZ recombinant virus (van Lier et al. 1992).

passage become significant after 10 passages and severe after 25 passages.

Samples obtained from continuous bioreactor systems showed that during the production time a mutant virus becomes dominant (Kool et al. 1991). This mutant lacks about 40% of the genome compared to standard virus. The deleted DNA contained the polyhedrin gene and also genes essential for virus replication. The replication of the mutant appeared to be dependent on an intact helper virus. The passage effect in the continuous system is thought to be the result of interference between the deletion mutant and standard virus (Kool et al. 1991, Wickham et al. 1991b).

5. Modelling

To gain insight in the influence of bioreactor parameters on the infection process, models are a valuable tool. Although much information on the basic parameters of the infection process is not (yet) available, some modelling has already been done. Shuler et al. (1990) made a descriptive model of the mechanism of virus entry in the cell. Based on binding studies Wickham et al. (1991b) proposed a model assuming three modes of cell entry by viruses. They conclude that AcNPV attaches to the cell via multivalent binding and that receptors are available in excess, thus allowing complete covering of the cells with virus without saturation of receptors. Based on their model they calculated that the number of receptors on Sf-9 cells ranges from 10^5 to 10^7 per cell. The affinity of the virus to the receptors was predicted to be in the range 10^4 - 10^5 M^{-1}.

The infection rate has been proposed to follow first-order kinetics with regard to viable-insect-cell concentration (de Gooijer et al. 1989, Wickham et al. 1990). With this assumption and using cell balances De Gooijer et al. (1989) calculated a first-order reaction constant in the order of $1.10^{-5}.s^{-1}$ using data from continuous bioreactor systems. The first-order kinetics approach was also followed by Power et al. (1992) to model cell growth and production of virus and recombinant protein upon infection. With this model experimental data from batch cultures could be described well.

A different approach to modeling is to reach a more structural description. For monolayer cultures a model has been developed that is based on a Poisson distribution to describe the infection of an insect cell with more than one virus (Licari and Bailey 1992). With the model cell density (both infected and uninfected cells), cell lysis, virus concentration and recombinant protein production can be calculated. The model predictions match ß-galactosidase levels experimentally obtained at different m.o.i. values.

Another model, containing the Poisson distribution was presented by

de Gooijer et al. (1990, 1992). To elucidate the mechanisms involved with the passage effect occurring in continuous bioreactor systems, they developed a model describing infection levels in continuous bioreactor systems. The model is based on the hypothesis that the limited running time of a series of continuously operated bioreactors is associated with the occurrence of a defective NOV with interfering properties as described by Kool et al. (1991). Furthermore it was assumed that cells have a limited number of virus entry sites and that there are, apart from infective and defective NOVs, NOVs incapable of infection although occupying entry sites. The model described experimental data obtained in continuously operated bioreactor systems (Kompier et al. 1988, van Lier 1990) and repeated (fed-)batch reactors (van Lier et al. 1995) well.

6. Conclusion

The baculovirus expression-vector system offers a powerful tool for recombinant protein production. Several cell culture systems can be used for large-scale production. The systems, however, need further optimization to take full advantage of their potential. The differential oxygen demand between infected and uninfected insect cells has to be taken into account. Media are, although serum free and hence better defined, still complex and expensive. A better knowledge of (infected) insect-cell physiology may lead to more simple, and hence probably less expensive, media.

On the biological level, the stability of virus upon serial passage is of concern when virus produced in vitro is used as inoculum (as is the case in continuous production). Protein processing also deserves attention. Although protein processing is similar in many aspects to mammalian cells, it is not always as efficient (Sissom and Ellis 1989). Different glycosylation patterns may also cause problems when biological activity of a recombinant protein is highly depending on correct addition of sugar groups.

For commercial application of baculoviruses as bio-insecticides

large-scale and low-cost production is necessary. Polyhedra production systems reported in large-scale cell cultures (Vaughn 1976b) should be dramatically improved to meet the commercial demands for a price-competitive product.

7. References

Agathos, S.N., Jeong, Y.-H. and Venkat, K. (1990) Growth kinetics of free and immobilized insect cell cultures. Proc. N.Y. Acad. Sci. **589**, 372-398.

Akhnoukh, R., Kretzmer, G. and Schügerl, K. (1993) Investigation of the cultivation of Spodoptera frugiperda in spinner flasks and in a 10 l bioreactor with bubble-free aeration. Bioproc. Eng. **8**, 229-233.

Bavarian, F., Fan, L.S., Chalmers, J.J. (1991) Microscopic visualization of insect cell-bubble interactions. I: Rising bubbles, air-medium interface, an the foam layer. Biotechnol. Prog. **7**, 140-150.

Barkhem, T., Carlsson, B., Danielsson, A., Norinder, U., Frieberg, H. and Öhman, L. (1992) Production in a 100 liter stirred tank reactor of functional full length human thyroid receptor $ß_1$ in Sf-9 insect cells using a recombinant baculovirus. In: Vlak, J.M., Schlaeger, E.-J. and Bernard, A.R. (eds) Baculovirus and recombinant protein production processes. Editiones Roche, Basel, pp. 235-246.

Bilimoria, S.L. and Carpenter, W.M. (1983) TN-368A: an attached strain of Hink's *Trichoplusia ni* (TN-368) cell line. In Vitro **19**, 870-874.

Bishop, D.H.L. (1989) Genetically engineered viral insecticides - A progress report 1986-1989. Pestic. Sci. **27**, 173-189.

Bishop, D.H.L., Hill-Perkins, M., Jones, I.M., Kitts, P., Lopez-Ferber, M., Clarke, A.T., Possee, R.D., Pullen, J. and Weyer, U. (1992) Construction of baculovirus expression vectors. In: Vlak, J.M., Schlaeger, E.-J. and Bernard, A.R. (eds) Baculovirus and recombinant protein production processes. Editiones Roche, Basel, pp. 27-49.

Broussard, D.R. and Summers, M.D. (1989) Effects of serum concentration and media composition on the level of polyhedrin and foreign gene expression by baculovirus vectors. J. Invertebr. Pathol. **54**, 144-150.

Brown, M. and Faulkner, P. (1975) Factors affecting the yield of virus in a cloned cell line of *Trichoplusia ni* infected with a nuclear polyhedrosis virus. J. Invertebr. Pathol. **26**, 251-257.

Caron, A.W., Archambault, J. and Massie, B. (1990) High-level recombinant protein production in bioreactors using the baculovirus-insect cell expression system. Biotechnol. Bioeng. **36**, 1133-1140.

Chalmers, J.J. and Bavarian, F. (1991) Microscopic visualization of insect cell-bubble interactions. II: The bubble film and bubble rupture. Biotechnol. Prog. **7**, 151-158.

Davidson, D.J. and Castellino, F.J. (1991a) Oligosaccharide processing in the expression of human plasminogen cDNA in insect cells. In Vitro Cell. Dev. Biol. **27**, 56A.

Davidson, J.J. and Castellino, F.J. (1991b) Asparagine-linked oligosaccharide processing in Lepidopteran insect cells. Temporal dependence of the nature of the oligosaccharides assembled on Asparagine-289 of recombinant human plasminogen produced in baculovirus vector infected *Spodoptera frugiperda* (IPLB-SF-21AE) cells. Biochem. **30**, 6167-6174.

Davies, A.H. (1994) Current methods for manipulating baculoviruses. Bio/Technol. **12**, 47-50.

De Gooijer, C.D., van Lier, F.L.J., van den End, E.J., Vlak, J.M. and Tramper, J. (1989) A model for baculovirus production with continuous insect cell cultures. Appl. Microbiol. Biotechnol. **30**, 497-501.

De Gooijer, C.D., Koken, R.H.M., van Lier, F.L.J., Kool, M., Vlak, J.M. and Tramper, J. (1990) Dynamic modelling of the baculovirus infection process in continuous insect-cell reactors. Presented at the Annual Meeting of the American Institute of Chemical Engineers, Chicago, November, 1990.

De Gooijer, C.D., Koken, R.H.M., van Lier, F.L.J., Kool, M., Vlak, J.M. and Tramper, J. (1992) A structured dynamic model for the baculovirus infection process in insect-cell reactor configurations. Biotechnol. Bioeng. Accepted.

Dougherty, E.M., Weiner, R.M., Vaughn, J.L. and Reichelderfer, C.F. (1981) Physical factors that affect in vitro *Autographa californica* nuclear polyhedrosis virus infection. Appl. Environ. Microbiol. **41**, 1168-1172.

Eberhard, U. and Schügerl, K. (1987) Investigations of reactors for insect cell culture. Develop. Biol. Standard. **66**, 325-330.

Faulkner, P. (1981) Baculoviruses. In: Pathogenesis of invertebrate microbial diseases (E.A. Davidson ed), pp 3-37. Osmun & Co., Allanheld.

Faulkner, P. and Henderson, J.F. (1972) Serial passage of a nuclear polyhedrosis virus of the cabbage looper (*Trichoplusia ni*) in a continuous tissue culture cell line. Virology **50**, 920-924.

Federici, B.A. (1986) Ultrastructure of baculoviruses. In: The biology of baculoviruses. Vol. I. Biological properties and molecular biology (R.R. Granados and B.A. Federici eds.), pp 61-88. CRC Press, Inc., Boca Raton.

Ferrance, J.P., Goel, A. and Ataai, M.M. (1993) Utilization of glucose and amino acids in insect cell cultures: quantifying the metabolic flows within the primary pathways and medium development. Biotechnol. Bioeng. **42**, 697-707.

Fleischaker, R.J. and Sinskey, A.J. (1981) Oxygen demand and supply in cell culture. Eur. J. Appl. Microbiol. Biotechnol. **12**, 193-197.

Francki, R.I.B., Fauquet, C.M., Knudson, D.L. and Brown, F. (eds.) (1991) Classification and nomenclature of viruses. Springer-Verlag, New York.

Gardiner, G.R. and Stockdale, H. (1975) Two tissue culture media for production of lepidopteran cells and nuclear polyhedrosis viruses. J. Invertebr. Pathol. **25**, 363-370.

Godwin, G., Gorfien, S., Tilkins, M.L. and Weiss, S. (1991) Development of a low cost serum-free medium for the large-scale production of viral pesticides in insect cell culture. In: Fraser, M.J. (ed.) Proceedings of the eight international conference on invertebrate and fish tissue culture. Tissue Culture Association, Columbia, 102-110.

Goldblum, S.D., Bae, Y.-K., Hink, W.F. and Chalmers, J. (1990) The protective effect of methylcellulose on suspended insect cells subjected to laminar shear stress. Biotechnol. Progr. **6**, 383-390.

Grabbenhorst, E., Karger, C., Reis, U., Collins, J., Lindenmaier, W., Rollwage, K., Jäger, V. and Conradt, H.S. (1992) Processing of recombinant proteins: comparison of N- and O-glycosylation of recombinant human glycoproteins in heterologous mammalian cells and baculovirus-infected Sf 21 cella. In: Vlak, J.M., Schlaeger, E.-J. and Bernard, A.R. (eds) Baculovirus and recombinant protein production processes. Editiones Roche, Basel, pp. 145-156.

Grace, T.D.C. (1962) Establishment of four strains of cells from insect tissue grown in vitro. Nature **195**, 788-789.

Granados, R.R. (1980) Infectivity and mode of action of baculoviruses. Biotechnol. Bioeng. **22**, 1377-1405.

Gröner, A. (1986) Specificity and safety of baculoviruses. In: The biology of baculoviruses. Vol. I. Biological properties and molecular biology (R.R. Granados and B.A. Federici, eds.), pp 177-202. CRC Press, Inc., Boca Raton.

Guillame, J.M., Soria, H.M., Couteault, N., Hurwitz, D.R., Multon, M.C. and Crespo, A. (1992) Insect cell fermentation scale-up for recombinant protein production. In: Vlak, J.M., Schlaeger, E.-J. and Bernard, A.R. (eds) Baculovirus and recombinant protein production processes. Editiones Roche, Basel, pp. 255-261.

Handa-Corrigan, A., Emery, A.N. and Spier, R.E. (1989) Effect of gas-liquid interfaces on the growth of suspended mammalian cells: mechanisms of cell damage by bubbles. Enzyme Microb. Technol. **11**, 230-235.

Hink, W.F. (1970) Established cell line from the cabbage looper, *Trichoplusia ni*. Nature **226**, 466-467.

Hink, W.F. (1982) Production of *Autographa californica* nuclear polyhedrosis virus in cells from large-scale suspension cultures. In: Microbial and viral pesticides (E. Kurstak, ed.), pp 493-506. Marcel Dekker, Inc., New York.

Hink, W.F. and Strauss, E.M. (1976a) Replication of alfalfa looper nuclear polyhedrosis virus in the *Trichoplusia ni* (TN-368) cell line. In: Invertebrate tissue culture - Applications in medicine, biology and agriculture (E. Kurstak and K. Maramorosch, eds.), pp 369-374. Academic Press, Inc., New York.

Hink, W.F. and Strauss, E.M. (1976b) Growth of *Trichoplusia ni* (TN-368) cells in suspension culture. In: Invertebrate tissue culture - Applications in medicine, biology and agriculture (E. Kurstak and K. Maramorosch, eds.), pp 297-300. Academic Press, Inc., New York.

Hink, W.F., Strauss, E.M. and Ramoska, W.A. (1977) Propagation of *Autographa californica* nuclear polyhedrosis virus in cell culture: methods for infecting cells. J. Invertebr. Pathol. **30**, 185-191.

Hink, W.F. and Strauss, E.M. (1980) Semi-continuous culture of the TN-368 cell line in fermenters with virus production in harvested cells. In: Invertebrate systems in vitro (E. Kurstak, K. Maramorosch and A. Dübendorfer eds.), pp 27-33. Elsevier North-Holland Biomedical Press, Amsterdam.

Hink, W.F., Thomsen, D.R., Davidson, D.J., Meyer, A.L. and Castellino, F.J. (1991) Expression of three recombinant proteins using baculovirus vectors in 23 insect cell lines. Biotechnol. Progr. **7**, 9-14.

Hsieh, P. and Robbins, P.W. (1984) Regulation of asparagine-linked oligosaccharide processing. J. Biol. Chem. **259**, 2375-2382.

Huber, J. (1986) Use of baculovirus in pest management programs. In: The biology of baculoviruses. Vol. II. Practical applications for insect control (R.R. Granados and B.A. Federici eds.), pp 177-202. CRC Press, Inc., Boca Raton.

Jäger, V., Blasey, H.D., Claus, J., Kratje, R.B., Lucki-Lange, M., Marx, U., Ryll, T., Schütz, C. and Wagner, R. (1990) Evaluation of the efficiency of membrane aerated and perfused animal cell bioreactors for several bioprocesses. In: Biologicals from recombinant microorganisms and animal cells - Production and recovery (M.D. White, S. Reuveny and A. Shafferman eds.) VCH publishers, Weinheim, 155-158.

Jäger, V., Deutschmann, S. and Lind, W. (1991) High density perfusion culture of insect cells in stirred bioreactors for the production of recombinant HIV-1 gag-particles (p55). ESACT meeting september 1991 Abstract Book, 80.

Jain, D., Ramasubramanyan, Gould, S., Lenny, A., Candelore, M., Tota, M., Strader, C., Alves, K., Cuca, G., Tung, J.S., Hunt, G., Junker, B., Buckland, B.C. and Silberklang, M. (1991) Large-scale recombinant protein production using the insect cell-baculovirus expression vector system: antistasin and ß-adrenergic receptor. In: Spier ,R.E., Griffiths, J.B. and Meignier, B. (eds.) Production of biologicals from animal cells in culture. Butterworth-Heinemann Ltd., Oxford, 345-351.

Kamen, A.A., Tom, R.L., Caron, A.W., Chavarie, C., Massie, B. and Archambault, J. (1991) Culture of insect cells in a helicon ribbon impeller bioreactor. Biotechnol. Bioeng. **38**, 619-628.

Kelly, D.C. (1981) Baculovirus replication: electron microscopy of the sequence of infection *Trichoplusia ni* nuclear polyhedrosis virus in *Spodoptera frugiperda* cells. J. Gen. Virol. **52**, 209-219.

Kennel, D. and Riezman, H. (1977) Transcription and translation initiation frequencies of the *Escherichia coli* lac operon. J. Mol. Biol. **114**, 1-21.

King, G.A., Daugulis, A.J., Faulkner, P., Bayly, D. and Goosen, M.F.A. (1988) Growth of baculovirus-infected insect cells in microcapsules to a high cell and virus density. Biotechnol. Letters **10**, 683-688.

Klöppinger, M., Fertig, G., Fraune, E. and Miltenburger, H.G. (1990) Multistage production of *Autographa californica* nuclear polyhedrosis virus in insect cell cultures. Cytotechnol. **4**, 271-278.

Klöppinger, M., Fertig, G., Fraune, E. and Miltenburger, H.G. (1991) High cell density perfusion culture of insect cells for production of baculovirus and recombinant protein. In: Spier ,R.E., Griffiths, J.B. and Meignier, B. (eds.) Production of biologicals from animal cells in culture. Butterworth-Heinemann Ltd., Oxford, 470-476.

Knudson, D.L. and Tinsley, T.W. (1974) Replication of a nuclear polyhedrosis virus in a continuous cell culture of *Spodoptera frugiperda*: purification, assay of infectivity, and growth characteristics of the virus. J. Virol. **14**, 934-944.

Kompier, R., Tramper, J. and Vlak, J.M. (1988) A continuous process for production of baculovirus using insect-cell cultures. Biotechnol. Letters **10**, 849-854.

Kompier, R., Kislev, N., Segal, I. and Kadouri, A. (1990) The use of a non-woven fabric carrier for the production of ß-galactosidase from insect cells. In: Biologicals from recombinant microorganisms and animal cells - Production and recovery (M.D. White, S. Reuveny and A. Shafferman eds.) VCH publishers, Weinheim, 221-233.

Kool, M., Voncken, J.W. van Lier, F.L.J., Tramper, J. and Vlak, J.M. (1991) Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with defective interfering properties. Virology **183**, 739-746.

Kunas, K.T. and Papoutsakis, E.T. (1990) The protective effect of serum against hydrodynamic damage of hybridoma cells in agitated and surface-aerated bioreactors. J. Biotechnol. **15**, 57-70.

Kuroda, K., Gröner, A., Frese, K., Drenckhahn, D., Hauser, Ch., Rott, R., Doerfler, W. and Klenk, H.-D. (1989) Synthesis of biologically active influenza virus haemagglutonin in insect larvae. J. Virol. **63**, 1677-1685.

Laird, M., Lacey, L.A. and Davidson, E.W. (eds.) (1990) Safety of microbial insecticides. CRC Press, Inc., Boca Raton.

Lehmann, J., Piehl, G.W. and Schulz, R. (1987) Bubble free cell culture aeration with porous moving membranes. Develop. Biol. Standard. **66**, 227-240.

Licari, P. and Bailey, J.E. (1991) Factors influencing recombinant protein yields in an insect cell-baculovirus expression system: multiplicity of infection and intracellular protein degradation. Biotechnol. Bioeng. **37**, 238-246.

Licari, P. and Bailey, J.E. (1992) Modeling the population dynamics of baculovirus-infected insect cells: optimizing strategies for enhanced recombinant protein yields. Biotechnol. Bioeng. **39**, 432-441.

Lindsay, D.A. and Betenbaugh, M.J. (1992) Quantification of cell culture factors affecting recombinant protein yields in baculovirus-infected insect cells. Biotechnol. Bioeng. **39**, 614-618.

Luckow, V.E. (1991) Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In: Recombinant DNA technology and applications (A. Prokop, R.K. Bajpai and C.S. Ho eds.) McGraw-Hill, New York.

Luckow, V.E. and Summers, M.D. (1988) Trends in the development of baculovirus expression vectors. Bio/Technol **6**, 47-55.

Lynn, D.E. and Hink, W.F. (1978) Infection of synchronized TN-368 cell cultures with alfalfa looper nuclear polyhedrosis virus. J. Invertebr. Pathol. **32**, 1-5.

MacKinnon, E.A., Henderson, J.F., Stolz, D.B. and Faulkner, P. (1974) Morphogenesis of nuclear polyhedrosis virus under conditions of prolonged virus passage in vitro. J. Ultrastruct. Res. **49**, 419-435.

Maiorella, B., Inlow, D., Shauger, A. and Harano, D. (1988) Large-scale insect cell-culture for recombinant protein production. Bio/Technol. **6**, 1406-1410.

Miller, L.K. (1989) Insect baculoviruses: Powerful gene expression vectors. BioAssays **11**, 91-95.

Miltenburger, H.G. and David, P. (1980) Mass production of insect cells in suspension. Develop. Biol. Standard. **46**, 183-186.

Murhammer, D.W. and Goochee, C.F. (1988) Scaleup of insect cell cultures: protective effects of Pluronic F-68. Bio/Technol. **6**, 1411-1418.

Murhammer, D.W. and Goochee, C.F. (1990) Sparged animal cell bioreactors: mechanism of cell damage and Pluronic F-68 protection. Biotechnol. Prog. **6**, 391-397.

Oh, S.K.W., Nienow, A.W., Al-Rubeai, M. and Emery, A.N. (1989) The effects of agitation intensity with and without continuous sparging on the growth and antibody production of hybridoma cells. J. Biotechnol. **12**, 45-62.

Potter, K.N., Faulkner, P. and MacKinnon, E.A. (1976) Strain selection during serial passage of *Trichoplusia ni* nuclear polyhedrosis virus. J. Virol. **18**, 1040-1050.

Pollard, R. and Khosrovi, B. (1978) Reactor design for fermentation of fragile tissue cells. Process Biochem. **13**, 31-37.

Power, J., Greenfield, P.F., Nielsen, L. and Reid, S. (1992) Modelling the growth and protein production by insect cells following infection by a recombinant baculovirus in suspension culture. Cytotechnol. **9**, 149-155.

Redfield, R.R., Birx, D.L., Ketter, N., Tramont, E., Polonis, V., Davis, D., Brundage, J.F., Smith, G., Johnson, S., Fowler, A., Wierzba, T., Shafferman, A., Volvovitz, F., Oster, C. and Burke, D.S. (1991) A phase I evaluation of the safety and immunogenicity of vaccination with recombinant gp160 in patients with early human immunodeficiency virus infection. N. Engl. J. Med. **324**, 1677-1684.

Reuveny, S., Kim, Y.J., Kemp, C.W. and Shiloach, J. (1993a) Production of recombinant proteins in high-density insect cell cultures. Biotechnol. Bioeng. **42**, 235-239.

Reuveny, S., Kim, Y.J., Kemp, C.W. and Shiloach, J. (1993b) Effect of temperature and oxygen on cell growth and recombinant protein production in insect cell cultures. Appl. Microbiol. Biotechnol. **38**, 619-623.

Roberts, P.L. (1984) Growth of insect cells in recycled medium and the use of various serum supplements. Biotechnol. Lett. **6**, 633-638.

Schlaeger, E.-J., Foggetta, M., Vonach, J.M. and Christensen, K. (1993) SF-1. a low cost culture medium for the production of recombinant proteins in baculovirus infected insect cells. Biotechnol. Techniques **7**, 183-188.

Schlaeger, E.-J., Loetscher, H. and Gentz, R. (1992) Fermentation scale up: production of production soluble human TNF receptors. In: Vlak, J.M., Schlaeger, E.-J. and Bernard, A.R. (eds) Baculovirus and recombinant protein production processes. Editiones Roche, Basel, pp. 201-208.

Schopf, B., Howaldt, M.W. and Bailey, J.E. (1990) DNA distribution and respiratory activity of *Spodoptera frugiperda* populations infected with wild-type and recombinant *Autographa californica* nuclear polyhedrosis virus. J. Biotechnol. **15**, 169-186.

Shieh, T.R. and Bohmfalk, G.T. (1980) Production and efficacy of baculoviruses. Biotechnol. Bioeng. **22**, 1357-1375.

Shuler, M.L., Cho, T., Wickham, T., Ogonah, O., Kool, M., Hammer, D.A., Granados, R.R. and Wood, H.A. (1990) Bioreactor development for production of viral pesticides or heterologous proteins in insect cell cultures. Ann. N.Y. Acad. Sci. **589**, 399-422.

Sissom, J. and Ellis, L. (1989) Secretion of the extracelluar domain of the human insulin receptor from insect cells by use of a baculovirus vector. Biochem. J. **261**, 119-126.

Smith, G.E., Fraser, M.J. and Summers, M.D. (1983) Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: deletion mutants within the polyhedrin gene. J. Virol. **46**, 584-593.

Streett, D.A. and Hink, W.F. (1978) Oxygen consumption of *Trichoplusia ni* (TN-368) insect cell line infected with *Autographa californica* nuclear polyhedrosis virus. J. Invertebr. Pathol. **32**, 112-113.

Tramper, J. and Vlak, J.M. (1986) Some engineering and economic aspects of continuous cultivation of insect cells for the production of baculoviruses. Ann. N.Y. Acad. Sci. **469**, 279-288.

Tramper, J., Williams, J.B., Joustra, D. and Vlak, J.M. (1986) Shear sensitivity of insect cells in suspension. Enzyme Microb. Technol. **8**, 33-36.

Tramper, J., Smit, D., Straatman, J. and Vlak, J.M. (1988) Bubble column design for growth of fragile insect cells. Bioproc. Eng. **3**, 37-41.

Tramper, J., van den End, E.J., de Gooijer, C.D., Kompier, R., van Lier, F.L.J., Usmany, M. and Vlak, J.M. (1990) Production of baculovirus in a continuous insect-cell culture. Bioreactor design, operation and modelling. Ann. N.Y. Acad. Sci. **589**, 423-430.

Tramper, J., de Gooijer, C.D. and Vlak, J.M. (1992) Scale-up considerations and bioreactor development for animal cell cultivation with special emphasis on insect cells. In: Goosen, M.F.A., Daugulis, A. and Faulkner, P. (eds.) Insect cell culture engineering. Marcel Dekker, Inc., New York, 139-177.

Van Brunt, J. (1987) First hurdle in AIDS vaccine cleared. Bio/Technol. **5**, 1118.

Van der Pol, L., Zijlstra, G., Thalen, M. and Tramper, J. (1990) Effect of serum concentration on production of monoclonal antibodies and on shear sensitivity of a hybridoma. Bioproc. Eng. **5**, 241-245.

Van Lier, F.L.J., van den End, E.J., de Gooijer, C.D., Vlak, J.M. and Tramper, J. (1990) Continuous production of baculovirus in a cascade of insect-cell reactors. Appl. Microbiol. Biotechnol. **33**, 43-47.

Van Lier, F.L.J., van der Meijs, W.C.J., Grobben, N.A., Olie, R.A., Vlak, J.M. and Tramper, J. (1991) Continuous ß-galactosidase production with a recombinant baculovirus insect-cell system in bioreactors. J. Biotechnol. **22**, 291-298.

Van Lier, F.L.J., van Duijnhoven, G.C.F., de Vaan, M.M.J.A.C.M., Vlak, J.M. and Tramper, J. (1994) Continuous ß-galactosidase production in insect cells with a p10 gene based baculovirus vector in a two-stage bioreactor system. Biotechnol. Progr. **10**, 60-64.

Van Lier, F.L.J., van den Hombergh, J.P.T.W., de Gooijer, C.D., den Boer, M.M., Vlak, J.M. and Tramper, J. (1995) Long-term semi-continuous production of recombinant baculovirus protein in a repeated (fed-)batch two-stage reactor system. Enzyme Microb. Technol. In press.

Vaughn, J.L. (1976a) The production of nuclear polyhedrosis virus in large-volume cell cultures. J. Invertebr. Pathol. **28**, 233-237.

Vaughn, J.L. (1976b) The production of viruses for insect control in large scale cultures of insect cells. In: Invertebrate tissue culture - Research applications (K. Maramorosch ed.), pp 295-303. Academic Press, New York.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J. and McCawley, P. (1977) The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro **13**, 213-217.

Vaughn, J.L. and Fan, F. (1989) Use of serum replacements for the culture of insect cells. In Vitro Cell. Develop. Biol. **25**, 143-145.

Vaughn, J.L., Fan, F., Dougherty, E.M., Adams, J.R., Guzo, D. and McClintock, J.T. (1991) The use of commercial serum replacements in media for the in vitro replication of nuclear polyhedrosis virus. J. Invertebr. Pathol. **58**, 297-304.

Vaughn, J.L. and Weiss, S.A. (1991) Formulating media for the culture of insect cells. BioPharm **4**, 16-19.

Vialard, J., Lalumière, M., Vernet, T., Briedis, D., Alkhatib, G., Henning, D., Levin, D. and Richardson, C. (1990) Synthesis of the membrane fusion and haemagglutonin proteins of measles virus, using a novel baculovirus vector containing the ß-galactosidase gene. J. Virol. **64**, 37-50.

Vlak, J.M. (1990) Genetic engineering of baculovirus insecticides. In: Molecular approaches to pure and applied entomology (M.J. Whitten and J.G. Oakesholt eds.) In press. Springer, New York.

Vlak, J.M. and Keus, J.A.R. (1990) The baculovirus expression vector system for production of viral vaccines. Adv. Biotechnol. Process **14**, 91-127.

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klinge-Roode, E.C., Geervliet, J.B.F., Roosien, J. and van Lent, J.W.M. (1988) Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-ß-galactosidase fusion gene. J. Gen. Virol. **69**, 765-776.

Vlak, J.M., Schouten, A., Usmany, M., Belsham, G.J., Klinge-Roode, E.C., Maule, A.J., van Lent, J.W.M. and Zuidema, D. (1990) Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. Virology **179**, 312-320.

Volkman, L.E. and Summers, M.D. (1975) Nuclear polyhedrosis virus detection: relative capabilities of clones developed from *Trichoplusia ni* ovarium cell line TN-368 to serve as indicator cells in a plaque assay. J. Virol. **16**, 1630-1637.

Wang, M.-Y., Vakharia, V. and Bentley, W.E. (1993) Expression of epoxide hydrolase in insect cells: a focus on the infected cell. Biotechnol. Bioeng. **42**, 240-246.

Weiss, S.A., Orr, T., Smith, G.C., Kalter, S.S., Vaughn, J.L. and Dougherty, E.M. (1982) Quantitative measurement of oxygen consumption in insect cell culture infected with polyhedrosis virus. Biotechnol. Bioeng. **24**, 1145-1154.

Weiss, S.A., Smith, G.C., Kalter, S.S. and Vaughn, J.L. (1981a) Improved method for the production of insect cell cultures in large volume. In Vitro **17**, 495-502.

Weiss, S.A., Smith, G.C., Kalter, S.S., Vaughn, J.L. and Dougherty, E. (1981b) Improved replication of *Autographa californica* nuclear polyhedrosis virus in roller bottles: characterization of the progeny virus. Intervirology **15**, 213-222.

Weiss, S.A. and Vaughn, J.L. (1986) Cell culture methods for large scale propagation of baculoviruses. In: The biology of baculoviruses. Vol. II. Practical applications for insect control (R.R. Granados and B.A. Federici, eds.), pp 63-87. CRC Press, Inc., Boca Raton.

Weiss, S.A., Belisle, B.W., DeGiovanni, A., Godwin, G., Kohler, J. and Summers, M.D. (1989) Insect cells as substrates for biologicals. Develop. Biol. Standard. **70**, 271-279.

Weiss, S.A., Godwin, G.P., Whitford, W.G., Gorfien, S.F. and Dougherty, E.M. (1992) Viral pesticides: in vitro process development. In: Proceedings 10th Australian biotechnology conference. Melbourne.

Wickham, T.J., Hammer, D.A., Granados, R.R., Wood, H.A. and Shuler, M.L. (1990) Evaluation of cell lines and infection conditions for recombinant protein production using baculovirus. Presented at the Annual Meeting of the American Institute of Chemical Engineers, Chicago, November, 1990.

Wickham, T.J., Davids, T., Granados, R.R., Hammer, D.A., Shuler, M.L. and Wood, H.A. (1991a) Baculovirus defective interfering particles are responsible for variations in recombinant protein production as a function ofmultiplicity of infection. Biotechnol. Lett. **13**, 483-488.

Wickham, T.J., Granados, R.R., Wood, H.A., Hammer, D.A. and Shuler, M.L. (1991b) General analysis of receptor-mediated viral attachment to cell surfaces. Submitted.

Wickham, T.J. an Nemerow, G.R. (1993) Optimization of growth methods and recombinant protein production in BTI-Tn-5B1-4 insect cells using the baculovirus expression system. Biotechnol. Progr. **9**, 25-30.

Wilkie, G.E.I., Stockdale, H. and Pirt, S.V. (1980) Chemically-defined media for production of insect cells in vitro. Develop. Biol. Standard. **46**, 29-37.

Wood, H.A., Johnston, L.B. and Burand, J.P. (1982) Inhibition of *Autographa californica* nuclear polyhedrosis virus replication in high-density *Trichoplusia ni* cell cultures. Virology **119**, 245-254.

Wu, J., King, G., Daugulis, A.J., Faulkner, P., Bone, D.H. and Goosen, M.F.A. (1989) Engineering aspects of insect cell suspension culture: a review. Appl. Microbiol. Biotechnol. **32**, 249-255.

Zhang, J., Kalogerakis, N., Behie, L.A. and Iatrou, K. (1993) A two-stage bioreactor system for the production of recombinant proteins using a genetically engineered baculovirus/insect cell system. Biotechnol. Bioeng. **42**, 357-366.

Zuidema, D., Schouten, A., Usmany, M., Maule, A.J., Belsham, G.J., Roosien, J., Klinge-Roode, van Lent, J.W.M. and Vlak, J.M. (1990) Expression of cauliflower mosaic virus gene I in insect cells using a novel polyhedrin-based baculovirus expression vector. J. Gen. Virol. **71**, 2201-2209.

Chapter 2 has been published as: van Lier, F.L.J., van den End, E.J., de Gooijer, C.D., Vlak, J.M. and Tramper, J. (1990) Continuous production of baculovirus in a cascade of insect-cell reactors. Appl. Microbiol. Biotechnol. **33**, 43-47.

2

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

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.

Chapter 2

1. 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 possibilty to produce 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 (figure 1) in which baculoviruses or recombinant proteins are produced (Tramper et al. 1990).

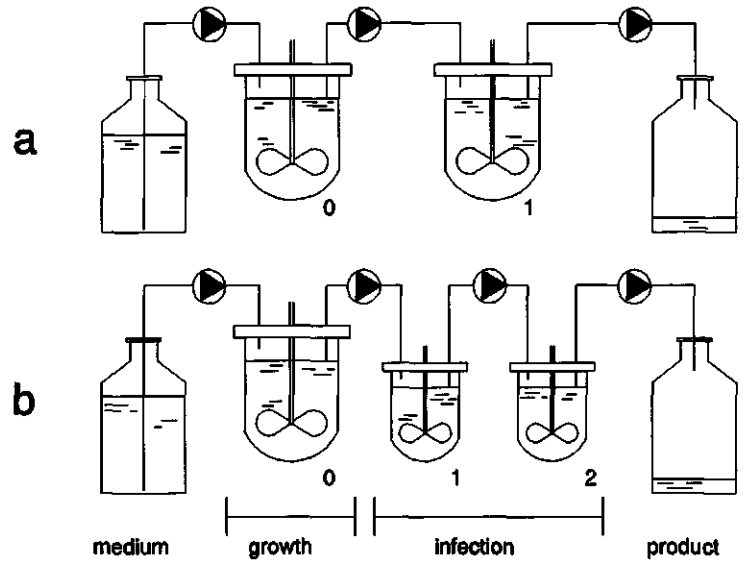

Figure 1.
Reactor configurations for continuous baculovirus production. a) Configuration described by Kompier et al. (1988). b) Configuration described in the present study.

The run-time of the system will be limited due to 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 an 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 (figure 1a) for about one 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 will occur 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 (figure 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 *Autographa 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 which contained 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. 1990) we concluded that the passage effect was enhanced by the change in residence time distribution in the virus production part.

2. 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) to culture the insect cells (reactor 0 in figure 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 figure 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 $dm^3.h^{-1}$. 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 $cm^3.h^{-1}$. Consequently, the mean residence time in each of both virus producing reactors 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 were 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 $2.5x10^{-2}$ 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 successively 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 a 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 400x). To determine the infectivity of NOVs a volume of 4-cm^3 aliquots of the samples were centrifuged (1500 g, for 15 min) and 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.

3. 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 figure 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 four days after infection in all three experiments until it reached a constant value (table 1). After about eighteen days the non-viable cell fraction slowly decreased in both reactors.

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*	14	11.5-9.0*	38

* No stable value was reached: cell concentration declined slowly.

To assess how efficient the cells were infected, we determined the fraction of cells containing polyhedra with time. In figure 3a this fraction is shown for the first infection reactor and in figure 3b for the second infection reactor, for all three experiments. If it is assumed that cells coming into the reactor are immediatly 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

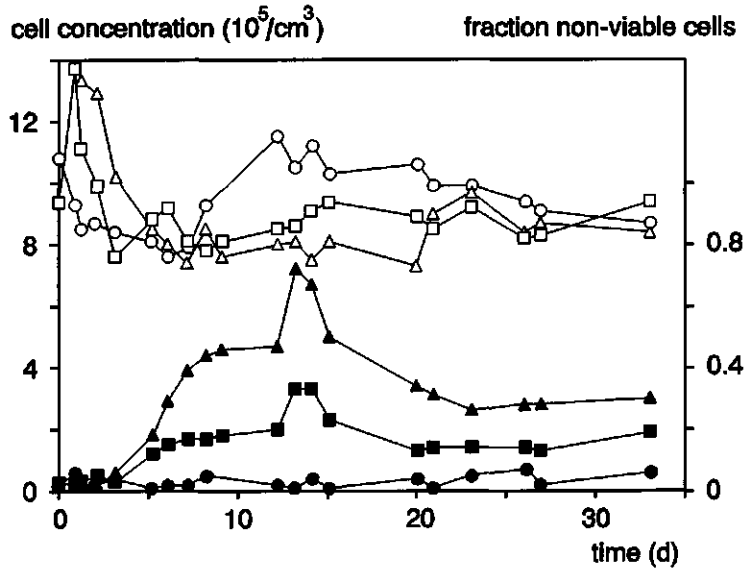

Figure 2.
Cell growth in all three reactors of the cascade for experiment 1. Open symbols: total cell concentrations, closed symbols: fraction of non-viable cells. □, ■: cell-growth reactor, △, ▲: first infection reactor, ○, ●: second infection reactor.

based on an ideal situation, the values of 30-35% and 50-70% which we found in respectively the first and second infection reactor, 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 plug-flow like. To assess if the decrease of cells containing polyhedra was due to a passage effect a computer model (de Gooijer et al. 1990) 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

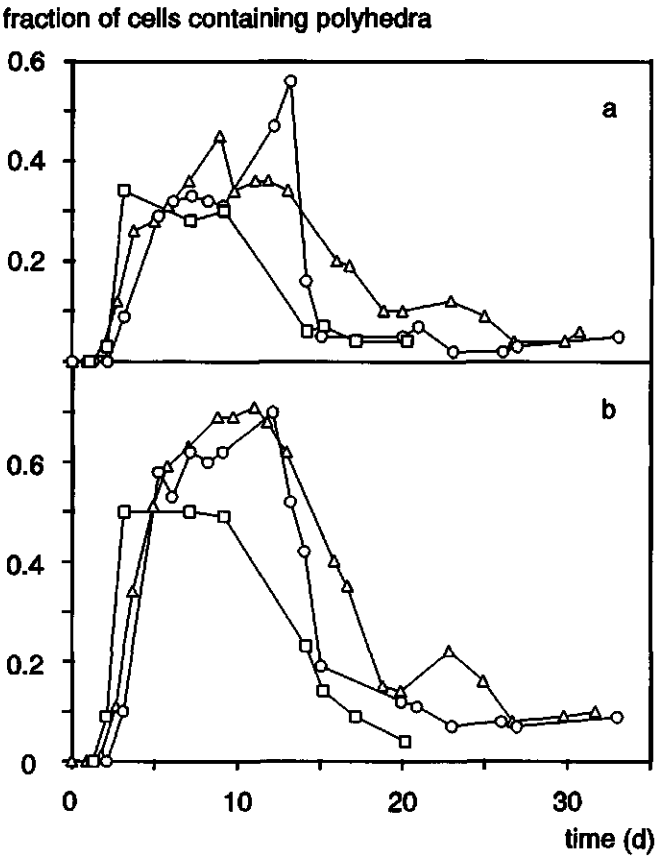

Figure 3.
Fraction of cells which contained polyhedra. a) First infection reactor. b) Second infection reactor. □: experiment 1, △: experiment 2, ○: experiment 3.

passage distribution in a system with a single infection reactor (figure 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.

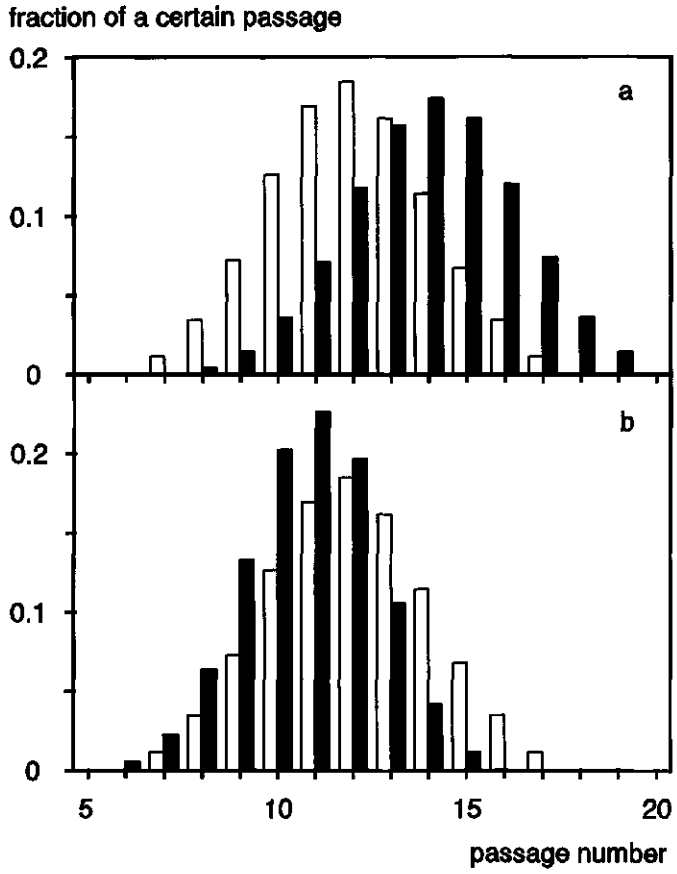

Figure 4.
Comparison of the virus passage distribution between a reactor configuration with one infection reactor (open bars) and a configuration with two infection reactors (solid bars). a) Both configurations 25 days after infection. b) Configuration with one infection reactor 25 days after infection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

A comparison between the predicted virus passage distributions in both reactor configurations when the decrease of infection occured was also made and is shown in figure 4b. Both distributions showed a good similarity, considering the simplicity of the model.

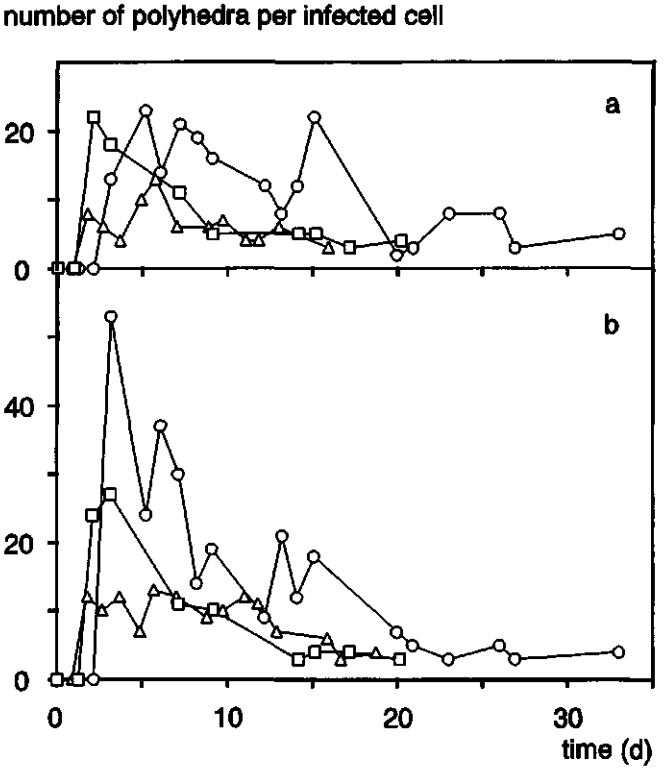

Figure 5.
Number of polyhedra per cell containing polyhedra. a) first innfection reactor. b) Second infection reactor. □: experiment 1, △: experiment 2, ○: experiment 3.

In figure 5 the number of polyhedra per polyhedra-containing cell is shown for the first (figure 5a) and the second (figure 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 concomittant 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 (figure 4). This strongly indicates the occurrence of a passage effect.

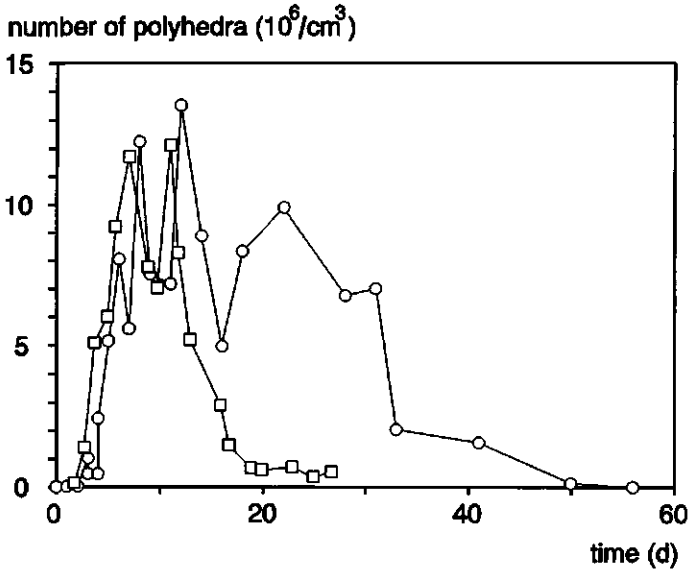

Figure 6.
Production of polyhedra in the second infection reactor compared to production in a reactor configuration with a single infection reactor (Kompier et al. 1988). ○: one infection reactor, □: two infection reactors.

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 per 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.

4. Conclusions

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 which 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 occurence of a passage effect. This decrease occured 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.

5. Acknowledgements

The authors thank Mrs. Magda Usmany for technical assistance and Mr. Marcel Kool for reading the manuscript. This project was partly financed by the Dutch Programme Commitee for Agricultural Biotechnology.

6. References

De Gooijer, C.D., van Lier, F.L.J., van den End, E.J., Vlak, J.M. and Tramper, J. (1989) A model for baculovirus production with continuous insect cell cultures. Appl. Microbiol. Biotechnol. **30**, 497-501.

De Gooijer, C.D., Koken, R.H.M., van Lier, F.L.J., Kool, M., Vlak, J.M. and Tramper, J. (1990) Dynamic modeling of the baculovirus infection process in continuous insect-cell reactors. Presented at the Annual Meeting of the American Institute of Chemical Engineers, Chicago, November, 1990.

Faulkner, P. (1981) Baculoviruses. In: Davidson, E.A. (ed.) Pathogenesis of invertebrate microbial diseases. Osmun & Co., Allenheld, 3-37.

Hink, W.F. (1970) Established cell line from the cabbage looper, *Trichoplusia ni*. Nature **226**, 466-467.

Kompier, R., Tramper, J. and Vlak, J.M. (1988) A continuous process for the production of baculovirus using insect-cell cultures. Biotechnol. Lett. **10**, 849-854.

Levenspiel, O. (1972) Chemical reaction engineering. J. Wiley & Sons, New York, 253-296

Luckow, V.A. and Summers, M.D. (1988) Trends in the development of baculovirus expression vectors. Bio/Technol. **6**, 47-55.

MacKinnon, E.A., Henderson, J.F., Stolz, D.B. and Faulkner, P. (1974) Morphogenesis of nuclear polyhedrosis virus under conditions of prolonged passage *in vitro*. J. Ultrastruct. Res. **49**, 419-435.

Martignoni, M.E. (1984) Baculoviruses: an attractive alternative. In: Garner, W.Y. and Harvey, J. (eds.) Chemical and biological controls in forestry. American Chemical Society, Washington DC, 55-67.

Shapiro, M. (1986) In vivo production of baculoviruses. In: Granados, R.R., and Federici, B.A. (eds.) The biology of baculoviruses, vol II. CRC Press, Inc., Boca Raton, 31-61.

Smith, G.E. and Summers, M.D. (1978) Analysis of baculovirus genomes with restriction endonucleases. Virology **89**, 517-527.

Tramper, J. and Vlak, J.M. (1986) Some engineering and economic aspects of continuous cultivation of insect cells for the production of baculoviruses. Ann. N.Y. Acad. Sci. **469**, 279-288.

Tramper, J., van den End, E.J., de Gooijer, C.D., Kompier, K., van Lier, F.L.J., Usmany, M. and Vlak, J.M. (1990) Production of baculovirus in a continuous insect-cell culture: bioreactor design, operation and modelling. Ann. N.Y. Acad. Sci. **589**, 423-430.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J. and McCawley, P. (1977) The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro **13**, 213-217.

Vlak, J.M. (1979) The proteins of nonoccluded *Autographa californica* nuclear polyhedrosis virus produced in an established cell line of *Spodoptera frugiperda*. J. Invertebr. Pathol. **34**, 110-118.

Weiss, S.A. and Vaughn, J.L. (1986) Cell culture methods for large-scale propagation of baculoviruses. In: Granados, R.R., Federici, B.A. (eds.) The biology of baculoviruses, vol II. CRC Press, Inc., Boca Raton, 63-87.

Chapter 3 has been published as: van Lier, F.L.J., van der Meijs, W.C.J., Grobben, N.G., Olie, R.A., Vlak, J.M. and Tramper, J. (1992) Continuous ß-galactosidase production with a recombinant baculovirus insect-cell system in bioreactors. J. Biotechnol. **22**, 291-298.

3

Continuous ß-galactosidase production with a recombinant baculovirus insect-cell system in bioreactors

Summary

Insect cells were exploited to produce bacterial ß-galactosidase by infecting them with a recombinant nuclear polyhedrosis virus (baculovirus) of *Autographa californica*. The insect cells were cultured in a continuous stirred tank reactor (CSTR) and led to a second CSTR where they were infected with a recombinant virus in which the lacZ gene from *Escherichia coli* was inserted. In the effluent of the production reactor maximum activities of 15 units ß-galactosidase per 10^6 cells were measured. For about 25 d ß-galactosidase production remained constant, but then rapidly declined. This drop was due to a decrease in production of active ß-galactosidase rather than to inactivation of this enzyme. It was concluded that the reduced production was due to reduced polyhedrin promoter-driven synthesis.

1. Introduction

Baculoviruses are specific pathogens of insects, which cause fatal disease. Due to their specificity and efficacy baculoviruses are attractive control agents of insect pests (Martignoni 1984). As a result of a number of unique properties these viruses can also be exploited as vectors for the high-level expression of foreign genes (Luckow and Summers 1988, Maeda 1989, Miller 1989, Luckow 1991). Viruses or expressed proteins can be produced in insect larvae, but insect-cell cultures are more suitable for large-scale production (Hink et al. 1977, Hink 1982, Tramper and Vlak 1986).

Baculoviruses have a characteristic bi-phasic replication cycle (figure 1). In the initial phase infected insect cells produce and release many new virus particles, called non-occluded viruses (NOVs), into the medium. In the second phase virus particles are occluded into large proteinaceous capsules, called polyhedra. The major constituent of polyhedra is the polyhedrin protein (Vlak and Rohrmann 1985). The released NOVs cause the spread of the infection in the insect or insect-cell culture. The polyhedra are the infectious form for insects.

Polyhedrin is expressed in large quantities in infected insect cells. Because of this high expression and since polyhedrin is not essential for NOV replication (Smith et al. 1983), the polyhedrin gene can be replaced by a foreign gene. Many foreign genes have been expressed in insect cells, mainly using the *Autographa californica* nuclear polyhedrosis virus (AcNPV) as expression vector (Luckow and Summers 1988, Miller 1989, Vlak and Keus 1990, Luckow 1991). The recombinant proteins are usually immunologically, antigenically and functionally similar to the authentic proteins (Luckow and Summers 1988).

For many commercial applications large-scale insect-cell culture is essential. Most of the reported production systems of recombinant proteins or polyhedra, have been either batch wise or semi-continuous processes (van Lier et al. 1992). Due to the release of NOVs from infected cells

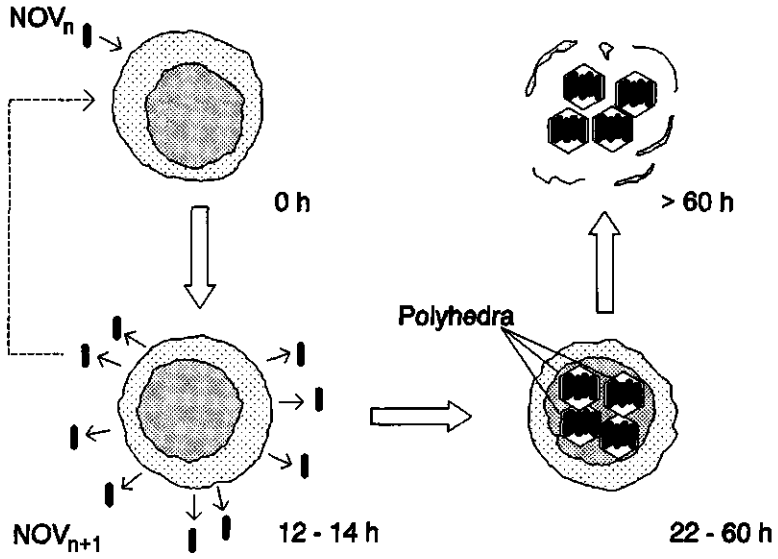

Figure 1.
Infection cycle of a nuclear polyhedrosis virus. NOV: non-occluded virus, the subscript denotes the passage number.

during infection, a continuous process is, in principle, possible (Tramper and Vlak 1986, Tramper et al. 1990). AcNPV polyhedra were produced in a continuously-operated multistage reactor configuration, consisting of one bioreactor to grow insect cells and one (Kompier et al. 1988) or two coupled (van Lier et al. 1990) bioreactors to produce polyhedra. Production of polyhedra and NOVs in both reactor systems was maintained for a limited time (about 25 and 14 d respectively) followed by a rapid decline. This phenomenon was attributed to the passage effect, a decreasing infectivity of the virus upon serial passage (Tramper and Vlak 1986, Faulkner 1981). This effect is characterized by lower polyhedra production per cell and by polyhedra with abnormal morphology (MacKinnon et al. 1974, Potter et al. 1976). The passage effect becomes significant after ten virus passages and severe when the number of passages exceeds twenty-

five (Faulkner 1981).

To gain more insight in the phenomenon of decreasing productivity in the multistage reactorsystem, a recombinant in stead of wild-type AcNPV was used in this study. Using wild-type AcNPV, the measurement of production is dependent on microscopic inspection for the presence or absence of polyhedra (Kompier et al. 1988, van Lier et al. 1990). Polyhedron formation is the result of expression of many genes and is, therefore, an inadequate parameter for the expression of polyhedrin. The latter is most relevant for the use of baculoviruses as expression vectors. In the recombinant used in this study the polyhedrin gene was replaced by the lacZ gene of *Escherichia coli* coding for ß-galactosidase (Summers and Smith 1987). This allowed the study of polyhedrin promoter-driven expression alone in the continuous two-stage reactorsystem described by Kompier et al. (1988), independent of factors affecting polyhedron formation. It appeared that the decline in ß-galactosidase production in this system followed a similar pattern as the decrease in production of wild-type polyhedra.

2. Materials and methods

In this study the *Spodoptera frugiperda* cell line Sf-AE-21 (Vaughn et al. 1977) was used. The cells were maintained in tissue culture flasks (Greiner) with TNM-FH medium (Hink 1970) supplemented with 10% (v/v) fetal calf serum. When the cells were grown in suspension, 0.1% (w/v) methylcellulose was added to the medium. The NOV form of a recombinant AcNPV, in which the polyhedrin gene was replaced with the lac Z gene from *E. coli* (Summers and Smith 1987), was used.

To monitor the infection in batch suspension cultures, insect cells were grown in conical flasks of 100 cm^3 containing 20 cm^3 cell suspension. The conical flasks were placed on a rotary shaker (120 rpm) inside an incubator which was kept at 28°C. Samples of the cell suspension were

treated as described below for samples from the production reactor.

The continuous reactor system consisted of one 1 dm^3 round-bottom reactor (Applikon) with a working volume of 0.8 dm^3 to grow insect cells connected to a similar reactor to produce ß-galactosidase. This system was operated as described by Kompier et al. (1988). To monitor the continuous process, samples of about 1 cm^3 from the growth reactor and of about 5 cm^3 from the production reactor were taken on a daily basis. The number of cells and cell viability, as indicated by the exclusion of trypan blue 0.2% (w/v), were determined using a Neubauer hemocytometer. Four cm^3 of the samples from the production reactor were centrifuged (5 min, 1500 x g). The sedimented cells were washed with 2.5 cm^3 washing buffer (10 mM Tris/HCl, pH 7.4, 0.15 M NaCl). After resedimentation, the cells were lysed by resuspension in 2.5 cm^3 water (milliQ). The activity and the amount of ß-galactosidase were determined in the supernatant from both centrifugation steps and in the lysed cells. The activity of ß-galactosidase was measured following hydrolysis of 2-nitrophenyl-galactopyranoside (ONPG) in a phosphate buffer pH 7.0 by spectroscopy at 420 nm ($\varepsilon = 4.5 \times 10^3$ $M^{-1}.m^{-1}$) and 28°C. To determine the physical amount of ß-galactosidase, infected-cell proteins were separated in a 12.5% polyacrylamide gel as described by Laemmli (1970).

The supernatant of the first centrifuge step was sterilized through a 45 µm filter. The concentration of infectious NOVs was measured using an end point dilution method (Vlak 1979) and expressed as tissue culture infective dose 50% ($TCID_{50}$) units. To assess if cells were infected, 10 mm^3 of a 0.4 g dm^{-3} solution of 5-bromo-4-chloro-3-indolyl-ß-D-galacto-pyranoside (X-gal) was added to each well and the test plate was incubated for 1 d. Blue wells were counted positive.

3. Results and discussion

In figure 2 ß-galactosidase production in insect cells grown in suspension in conical flasks is shown. Production started about 20 h after infection of cells. Initially, the ß-galactosidase produced stayed inside the cells. After about 50 h ß-galactosidase was also found in the medium, probably due to lysis of infected cells. For experiments with the continuous two-stage bioreactor system we chose a mean residence time of 60 h. As a result a maximum amount of ß-galactosidase is present in the cells, as was estimated by calculating the residence time distribution and taking into account the production per cell (figure 2). This residence time made these experiments also comparable to analogous experiments with wild-type AcNPV (Kompier et al. 1988).

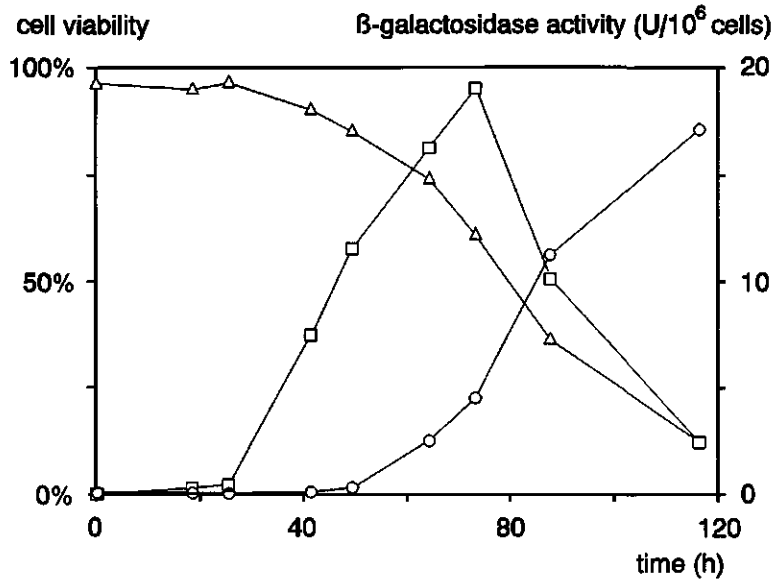

Figure 2.
Batch ß-galactosidase production in insect cells, cultured in shaken conical flasks. Cell density: $1x10^6$ cells.cm^{-3}; multiplicity of infection: 10. □: ß-galactosidase activity in the cells, ○: ß-galactosidase activity in the medium, △: cell viability.

During continuous culturing cell concentration in the cell-growth reactor stayed relatively constant during each experiment and varied between the experiments from $8x10^5$ to $1x10^6$ cm^{-3}. Cell viabilty in all experiments was 95% or higher. Cell concentrations in the production reactors were slightly higher than in the upstream cell-growth reactor, indicating that not all cells were immediatly infected because growth of infected cells is arrested upon infection (Faulkner 1981). Active ß-galactosidase was produced at a relative constant rate during about 25 d. Figure 3 shows the total ß-galactosidase activity over time during the three separate experiments. The first experiment gave initial activities of about 15 U per 10^6 cells. The other two experiments resulted in activities of about 5 U per 10^6 cells. These production levels on a per cell basis are low as compared to yields of ß-galactosidase in bioreactors described in other reports. Murhammer et al. (1988) produced ß-galactosidase in 0.75 dm^3 airlift reactors and in 3 dm^3 head- space aerated and sparged stirred tank reactors. In head-space aerated systems 0.284 - 0.33 mg per 10^6 cells was produced and 0.145 - 0.214 mg per 10^6 cells in sparged systems. When it is assumed that 1 mg ß-galactosidase has an activity of 400 U (Kennel and Riezman 1977) these values become 114 - 133 U per 10^6 cells and 58 - 86 U per 10^6 cells, respectively. The bioreactors used were batch reactors. A lower production per cell is expected in a continuous bioreactor system due to a distribution in residence time. The system described here has not yet been fully optimized. By appropriate adjustments of parameters, such as medium composition and cell density, production can be most likely improved (Caron et al. 1990). Also, by running it in a mode which is not fully continuous the productivity may be improved (Klöppinger et al. 1990).

The effective production time of ß-galactosidase (about 25 d) was similar in each experiment although the ß-galactosidase level differed. The period of 25 d was also found to be the effective production time of polyhedra in a two-stage reactor system (Kompier et al. 1988). The decrease in production coincided with the decline of infectious virus (figure 4). To assess if the decrease in ß-galactosidase activity was the result of a

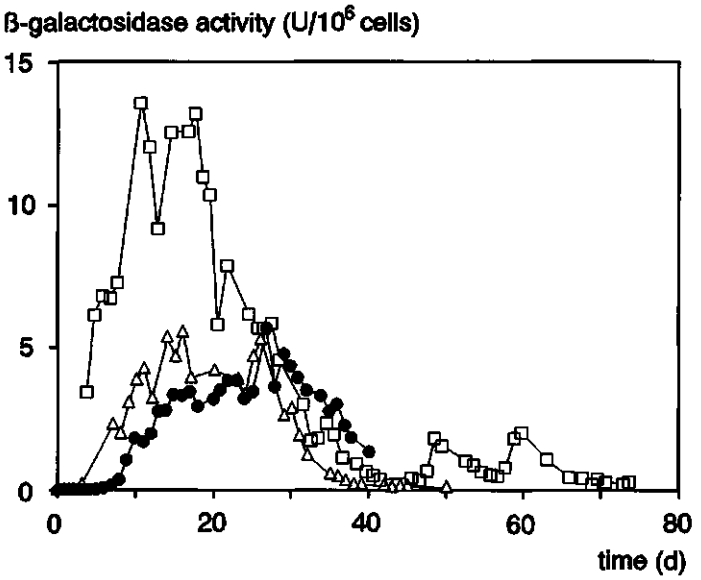

Figure 3.
Total ß-galactosidase production (units per 10^6 cells) in a two-stage reactor system for three separate experiments. □, ● en △: first, second and third experiment, respectively.

decrease in ß-galactosidase production or due to inactivation of the enzyme, SDS/polyacrylamide gel electrophoresis of lysed cells was carried out (figure 5). The total amount of ß-galactosidase decreased with time. The production of most viral proteins (arrows in figure 5), remained constant and served as internal control. These results also indicate that the decrease in production of polyhedra (Kompier et al. 1988) is caused by a reduction of polyhedrin synthesis and not an effect of other factors affecting polyhedron morphogenesis. Recently, this decline was attributed to the presence of defective interfering viruses. These viruses lacked the polyhedrin gene (Kool et al. 1991).

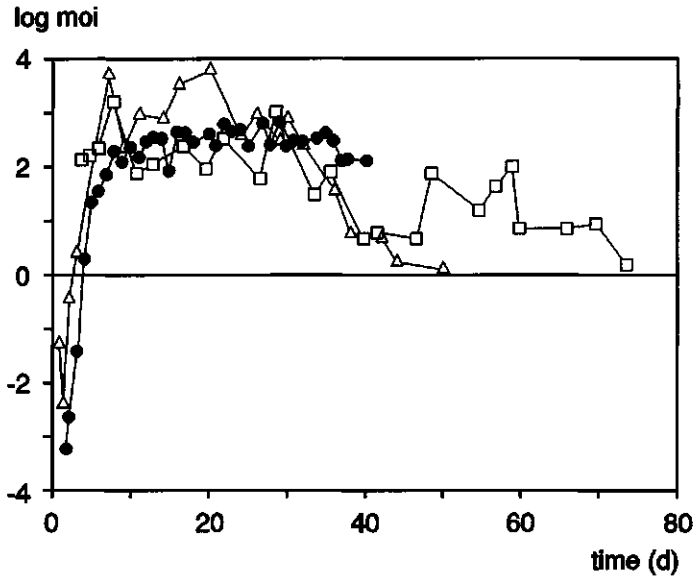

Figure 4.
Multiplicity of infection (logarithmic) in the infection reactor of a two-stage reactor system during three separate experiments. □, ●, en △: first, second and third experiment, respectively.

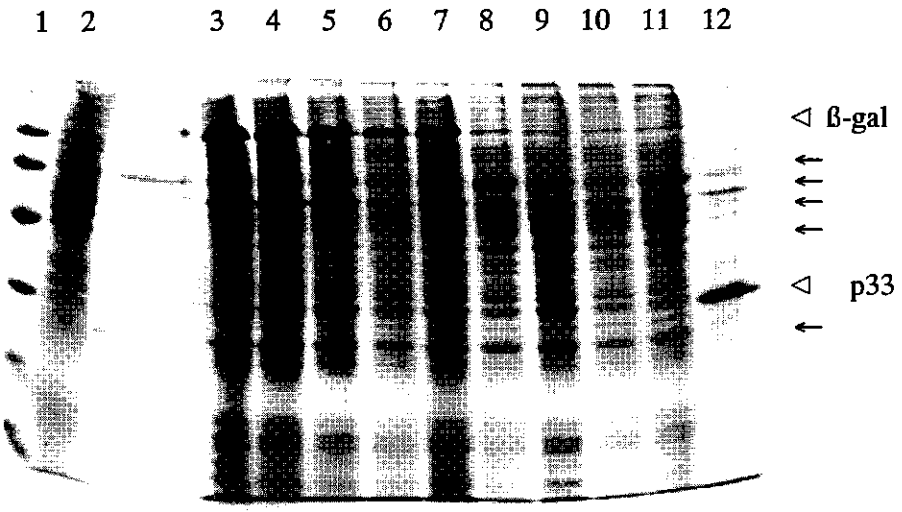

Figure 5.
Polyacrylamide gel electrophoresis of cell samples from the third continuous experiment. Lane 1: marker proteins (14, 20, 30, 43, 67 and 94kDa); lane 2: uninfected Sf cells, lane 3-11: samples from the infection bioreactor 13, 17, 24, 27, 31, 36, 39, 44 and 46 days, respectively, after initiation of the infection; lane 12: wild-type AcNPV-infected cells. ß-gal: ß-galactosidase; p33: polyhedrin. The arrows indicate the position of various infected-cell specific proteins.

4. Conclusions

Beta-galactosidase was produced continuously in insect cells in a two-stage reactor system. Production levels during steady state were about 5-15 U per 10^6 cells and were maintained for about 25 d, in a similar fashion as the production of polyhedra (Kompier et al. 1988). The decline of ß-galactosidase activity measured appeared to be due to a decrease in the amount of ß-galactosidase rather than to inactivation of the enzyme. The reduced production appears to be a general phenomenon associated

with baculovirus replication in continuously-operated two-stage bioreactor systems.

5. Acknowledgements

The authors thank mr. E.J. van den End, mr. C.D. de Gooijer and mr. M. Kool for their helpful discussions, ms. M. Usmany for technical support and ms. M.M.J.A.C.M. de Vaan for assistance with the polyacrylamide gel electrophoreses. This work was partly financed by the Dutch Programme Committee for Agricultural Biotechnology.

6. References

Caron, A.W., Archambault, J. and Massie, B. (1990) High-level recombinant protein production in bioreactors using the baculovirus-insect cell expression system. Biotechnol. Bioeng. **36**, 1133-1140.

Faulkner, P. (1981) Baculoviruses. In: Davidson, E.A. (Ed.) Pathogenesis of invertebrate microbial diseases. Osmun & Co., Allenheld, 3-37.

Hink, W.F. (1970) Established insect cell line from the cabbage looper, *Trichoplusia ni*. Nature **226**, 466-467.

Hink, W.F. (1982) Production of *Autographa californica* nuclear polyhedrosis virus in cells from large-scale suspension cultures. In: Kurstak, E. (Ed.) Microbial and viral pesticides. Marcel Dekker, New York, 493-506.

Kennel, D. and Riezman, H. (1977) Transcription and translation initiation frequencies of the *Escherichia coli* lac operon. J. Mol. Biol. **114**, 1-21.

Klöppinger, M., Fertig, G., Fraune, E. and Miltenburger, H.G. 1990 Multistage production of *Autographa californica* nuclear polyhedrosis virus in insect cell cultures. Cytotechnol. **4**, 271-278.

Kompier, R., Tramper, J. and Vlak, J.M. (1988) A continuous process for the production of baculovirus using insect-cell cultures. Biotechnol. Lett. **10**, 849-854.

Kool, M., Voncken, J.W., van Lier, F.L.J., Tramper, J. and Vlak, J.M. (1991) Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with interfering properties. Virology **183**, 739-746.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature **227**, 680-685.

Luckow, V.E. (1991) Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In: Prokop, A., Bajpaj, R.K. and Ho, C.S. (Eds.) Recombinant DNA technology and applications, McGraw-Hill, New York, 97-152.

Luckow, V.A. and Summers, M.D. (1988) Trends in the development of baculovirus expression vectors. Bio/Technol. **6**, 47-55.

MacKinnon, E.A., Henderson, J.F., Stolz, D.B. and Faulkner, P. (1974) Morphogenesis of nuclear polyhedrosis virus under conditions of prolonged passage *in vitro*. J. Ultrastruct. Res. **49**, 419-435.

Maeda, S. (1989) Expression of foreign genes in insects using baculovirus vectors. Ann. Rev. Entomol. **34**, 351-372.

Martignoni, M.E. (1984) Baculoviruses: an attractive biological alternative. In: Garner, W.Y. and Harvey, J. (Eds.) Chemical and biological controls in forestry. American Chemical Society, Washington, 55-67.

Miller, L.K. (1989) Insect baculoviruses: powerful gene expression vectors. BioAssays **11**, 91-95.

Murhammer, D.W. and Goochee, C.F. (1988) Scaleup of insect cell cultures: protective effects of pluronic F-68. Bio/technol. **6**, 1411-1418.

Potter, K.N., Faulkner, P. and MacKinnon, E.A. (1976) Strain selection during serial passage of *Trichoplusia ni* nuclear polyhedrosis virus. J. Virol. **18**, 1040-1050.

Smith, G.E., Fraser, M.J. and Summers, M.D. (1983) Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: deletion mutations within the polyhedrin gene. J. Virol. **46**, 584-593.

Summers, M.D. and Smith, G.E. (1987) A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricult. Exp. Stn. Bull. No. 1555.

Tramper, J. and Vlak, J.M. (1986) Some engineering and economic aspects of continuous cultivation of insect cells for the production of baculoviruses. Ann. N.Y. Acad. Sci. **469**, 279-288.

Tramper, J., van den End, E.J., de Gooijer, C.D., Kompier, R., van Lier, F.L.J., Usmany, M. and Vlak, J.M. (1990) Production of baculovirus in a continuous insect-cell culture: bioreactor design, operation and modelling. Ann. N.Y. Acad. Sci. **589**, 423-430.

Van Lier, F.L.J., van den End, E.J., de Gooijer, C.D., Tramper, J. and Vlak, J.M. (1990) Continuous production of baculovirus in a cascade of insect-cell reactors. Appl. Microbiol. Biotechnol. **33**, 43-47

Van Lier, F.L.J., Vlak, J.M. and Tramper, J. (1992) Production of baculovirus-expressed proteins from suspension cultures of insect cells. In: Spier, R.E. and Griffiths, J.B. (Eds.) Animal cell biotechnology - volume V. Academic Press, London, In press.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J. and McCawley, P. (1977) The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro **13**, 213-217.

Vlak, J.M. (1979) The proteins of nonoccluded *Autographa californica* nuclear polyhedrosis virus produced in an established cell line of *Spodoptera frugiperda*. J. Invertebr. Pathol. **34**, 110-118.

Vlak, J.M. and Keus, J.A.R. (1990) The baculovirus expression vector system for production of viral vaccines. Adv. Biotechnol. Processes **14**, 91-127.

Vlak, J.M. and Rohrmann, G.F. (1985) The nature of polyhedrins. In: Maramorosch, K. and Sherman, K.E. (Eds.) Viral insecticides for biological control. Academic Press, New York, 489-542.

Chapter 4 has been published as: van Lier, F.L.J., van Duijnhoven, G.C.F., de Vaan, M.M.J.A.C.M., Vlak, J.M. and Tramper, J. (1994) Continuous ß-galactosidase production in insect cells with a baculovirus vector based on the p10 gene in a two-stage bioreactor system. Biotechnol. Progr. **10**, 60-64.

4

Continuous ß-galactosidase production in insect cells with a p10-gene based baculovirus vector in a two-stage bioreactor system

Summary

Continuous production of polyhedra or baculovirus-expressed proteins in insect-cell cultures is limited to about four weeks. The decrease in production has been ascribed to interference of defective deletion mutants with wild-type baculoviruses. The deleted genome sequences include the polyhedrin gene (or the heterologous gene of interest): in the remaining part the major late p10 gene is always maintained. In the present study the productivity of a recombinant baculovirus with the lacZ gene from *Escherichia coli* cloned downstream of the p10 promoter at the p10 locus was investigated. It was hypothesized that this p10-promoter-driven gene is preserved over a longer period of time in a continuously operated two-stage bioreactor system than foreign genes behind the polyhedrin promoter at the polyhedrin locus. In two separate runs, ß-galactosidase production with the p10-lacZ recombinant reached quasi steady-state levels of 30 and 60 U per cm^3. Polyhedron production was about $3x10^6$ and $6x10^6$ polyhedra per cm^3, respectively. However, both polyhedron and ß-galactosidase production decreased after about 30 days of relative constant production. In the infection reactor deletion mutants of the virus, which contained both the polyhedrin and lacZ gene, were predominant. Therefore, the presence of the polyhedrin or p10 gene alone in deletion mutants is not sufficient for prolonged expression; other genes involved in major late gene expression and not present in the deleted virus are probably necessary.

1 Introduction

Baculoviruses are insect pathogens, specific to one or only a few related insect species. The baculovirus most studied is the nuclear polyhedrosis virus isolated from the alfalfa looper *Autographa californica* (AcMNPV). Once ingested by larva polyhedra dissolve in the insect midgut and occluded virus particles are released. In midgut cells and other, secondary infected, tissues a non-occluded virus (NOV) form of the virus is produced which is infectious to cultured insect cells. About 10-14 hours after infection of a cell NOVs are released from the cell. After about 24 hours virus particles are packed into polyhedra. A few days after infection the cell lyses, thus releasing the polyhedra.

The polyhedrin protein is the major constituent of a polyhedron. Because an infected cell produces polyhedrin in large quantities the polyhedrin promoter can be used to drive foreign genes (Luckow and Summers 1988). This forms the basis of the baculovirus expression system.

The release of NOVs during virus replication allows the design and evaluation of a continuous system for the production of virus in insect-cell cultures. A continuously operated production system was proposed where cell growth was physically separated from virus production by using two reactors in series (Tramper and Vlak 1986). Production of wild-type AcMNPV is feasible in such a system (Kompier et al. 1988). The production of polyhedra is maintained for about four weeks after which production rapidly decreases. When a recombinant AcMNPV expressing the lacZ gene from *Escherichia coli* instead of the polyhedrin gene is used in the two-stage reactor configuration, production of ß-galactosidase also lasts about four weeks (van Lier et al. 1992).

The decrease in production in the continuous bioreactor system was ascribed to defective interference caused by the occurrence of deletion mutants of the virus in the infection reactor (Kool et al. 1991). These mutants lack up to 40% of the viral genome (figure 1). The deleted segment includes the polyhedrin gene and at least one gene (DNA

polymerase) necessary for replication of virus. The mutant virus thus needs an intact virus to provide the necessary gene products for virus replication. It is hypothesized that upon co-infection of a cell with defective and intact virus, the defective virus is replicated faster than the intact NOV, competing for replication factors and thus interfering with intact virus replication (Kool et al. 1991, Wickham et al. 1991).

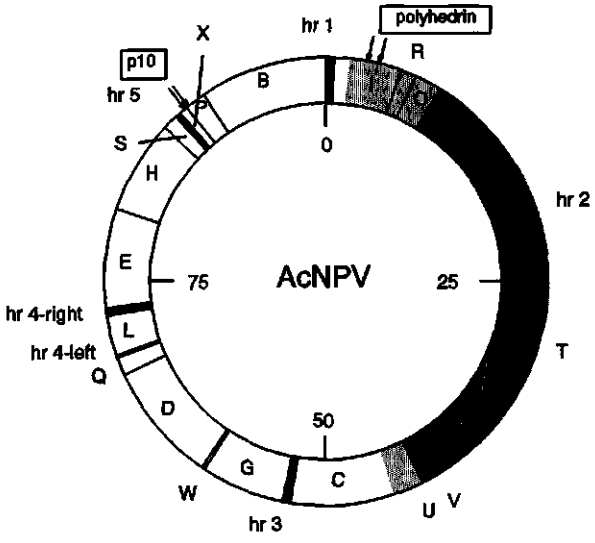

Figure 1.
Physical map of *Eco*R1 sites in AcMNPV DNA with the segment that is disappearing with time in the infection reactor. Highly-repetitive (hr) regions are indicated as black areas, the locations of the p10 and polyhedrin gene are indicated outside the circle with arrows. Light-shaded part: deleted part as found by Kool et al. (1991) in continuous reactor systems with wild type and polyhedrin-based recombinants. Dark shaded part: deletion found in this study.

Both the polyhedrin gene (Kompier et al. 1988) and the lacZ gene (van Lier et al. 1992) at the same location are deleted in the defective

mutant, resulting in the low-level expression of these genes. In contrast, another highly-expressed gene, the p10 gene, is maintained in the defective AcMNPV genome. Like the polyhedrin gene, it has a strong promoter and is not necessary for NOV replication (Vlak et al. 1988). The p10 gene codes for a 10 kDa protein with unknown function. Expression vectors based on replacement of the p10 gene and exploiting the p10 promoter have been designed (Vlak et al. 1990). Since the p10 gene is preserved in defective mutants it is hypothesized that this gene is expressed over a longer period in a continuously operated two-stage bioreactor system. To test this hypothesis, a recombinant AcMNPV with the lacZ gene of *E. coli* behind the p10 promoter (van Oers et al. 1992) was used. This recombinant virus thus produced upon infection two easy to analyze marker products: polyhedrin for the deleted and ß-galactosidase for the undeleted segment of the virus genome.

The amount of polyhedra produced in a continuous two-stage bioreactor system with the p10-based AcMNPV-lacZ was comparable to production of polyhedra using wild-type AcMNPV. The production of ß-galactosidase with such a recombinant was higher than obtained with a polyhedrin-based recombinant AcMNPV. For about 30 days both polyhedra and ß-galactosidase were produced on a relatively constant level, but then production decreased rapidly. In samples taken from the infection reactor defective virus particles were found which were slightly larger in size than those found in bioreactors with a polyhedrin-based AcMNPV-lacZ recombinant and contained the lacZ gene.

2. Materials and methods

Cells, virus, medium

A cell line of the fall army worm *Spodoptera frugiperda*, originally isolated by Vaughn et al. (1977) and denoted IPLB-Sf-21 was used. The recombinant *Autographa californica* multiple-nucleocapsid nuclear

polyhedrosis virus (AcMNPV) containing the lacZ gene from *Escherichia coli* instead of the p10 gene (van Oers et al. 1992) was coded AcMNPV-RK1. Cells were grown in TNM-FH medium (Hink 1970) supplemented with 10% (v/v) fetal calf serum. When cells were grown in suspension 0.1% (w/v) methylcellulose was added to the medium to prevent cell damage. Cells were maintained in 25 cm^2 tissue culture flasks (Greiner).

Bioreactor system

The bioreactor system (figure 2), consisted of two round-bottomed fermentors each with a working volume of 0.8 dm^3 and equipped with a marine impeller. Temperature was kept at 28°C with a refrigerated water bath. The reactors were head-space aerated through filters with a pore size of 0.2 μm. The air flow was 10 $dm^3.h^{-1}$. To transport liquid, silicone tubing and peristaltic pumps were used. Medium was continuously added via a drop former to the first reactor at a rate of 13.3 $cm^3.h^{-1}$. Samples from the bioreactors were taken via a pipe in the culture fluid. The samples were led to the sample flasks by pressurizing the reactor. The sample system was cleaned by blowing sterile air through it.

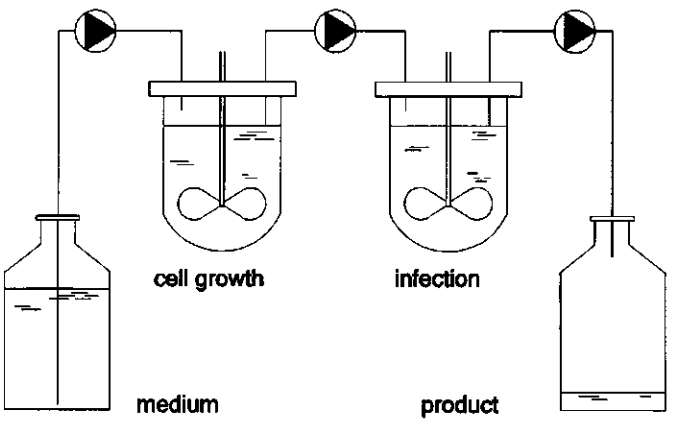

Figure 2.
Schematic drawing of the continuous reactor system.

From the cell-culture reactor and the virus-production reactor samples of about 1 cm^3 and 5 cm^3 were taken, respectively. From the latter 4 cm^3 was centrifuged (1500 x g, 5 min), washed in 4 cm^3 phosphate buffered saline (PBS) and again centrifuged (1500 x g, 5 min). The pellet was resuspended in 1 cm^3 PBS. Half of the first supernatant fraction was stored at 4°C. The other half of the first supernatant fraction, the supernatant fraction of the washing step and the resuspended pellet were stored at -20°C.

Cell and polyhedron counts

In samples from both reactors the cell number was determined using a bright-line Neubauer haemacytometer. The trypan blue exclusion method was used to determine cell viability. In samples from the virus-production reactor the fraction of cells containing polyhedra was determined using the same method.

To count the number of polyhedra, part of the resuspended cell pellet was lysed by adding 0.1% (w/v) sodium dodecyl sulphate (SDS) and incubating for 45 min at 45°C. After appropriate dilution, polyhedra were counted using a Neubauer haemacytometer.

ß-galactosidase assay

A sample of the resuspended cell pellet was lysed by sonification (3 times for 30 s with a 30 s interval). The lysed cell pellet and both supernatant fractions stored at -20°C were used in the ß-galactosidase assay (Miller 1972) at 37°C. Samples obtained during the second experiment were assayed at both 37°C and 28°C, the latter for comparison with previously obtained data (van Lier et al. 1992).

Virus titer

The number of infectious NOVs was determined in the supernatant fraction stored at 4°C. The end point dilution method used here was described by Vlak (1979).

Characterization of mutant AcMNPV-RK1

The length of NOVs was measured with a Philips CM12 electron microscope. Extracellular virus was centrifugated from the culture medium (Summers and Smith 1987) and were stained negatively with 2% (w/v) phosphotungstic acid, pH 6.8 for 1 min.

Viral DNA was isolated from several supernatant samples from the bioreactor and was digested with restriction enzyme *Eco*R1 and separated in a 0.8% agarose gel. The DNA was transferred to a nitrocellulose filter according to the method of Southern (1975) and hybridised with AcMNPV-RK1 DNA which was radio-actively labelled by nick translation (Rigby et al. 1977).

3. Results and discussion

To test the hypothesis if the expression of the p10 gene in a continuous bioreactor system is maintained longer than the expression of the polyhedrin gene, two experiments were performed using a p10-lacZ AcMNPV recombinant. Conditions were kept the same in both experiments, except that during the initial part of the first experiment medium with serum from a different batch was used. Cell density and viability (figure 3) in the infection reactor remained constant in both experiments. The difference in cell density between the two experiments may be attributed to a difference in serum batch. Cell density in the cell-growth bioreactor showed a distinct increase after changing the medium (data not shown). Cell density in the infection reactor increased at the end of the experiment, but this could be due to a decreased concentration of infectious virus which allowed uninfected cells to divide.

After 30 days the number of polyhedra per cell dropped. It was also observed that the fraction of polyhedra with an abnormal morphology increased during the course of the experiments and especially after the 30 days of more or less constant production. In figure 4 the production of

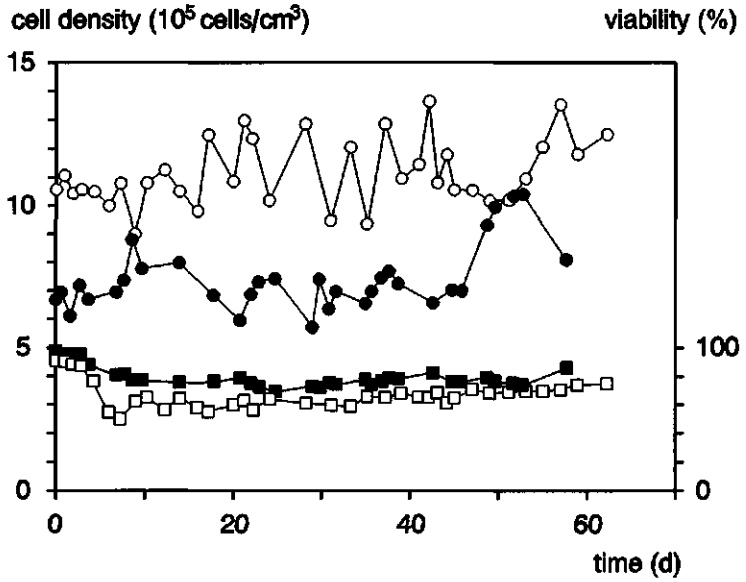

Figure 3.
Total cell density (●: experiment 1, ○: experiment 2) and viability (■: experiment 1, □: experiment 2) in the infection reactor during two independent experiments.

polyhedra (figure 4a) and ß-galactosidase (figure 4b) per cm^3 are shown. In previous experiments in continuous two-stage bioreactor systems (Kompier et al. 1988, van Lier et al. 1992) the decrease in production was found to be associated with the occurrence of defective interfering NOVs (Kool et al. 1991). These defective NOVs lacked the polyhedrin gene (or the lacZ gene) but still contained the p10 gene. In the present experiment gene products from both genes decreased at the same time with the same rate. This indicates that the presence of the lacZ gene in a defective NOV, in the retained DNA segment per se is not sufficient for prolonged expression. It is possible that factors involved in both polyhedrin and p10 gene expression are limiting. The initiation of the drop in virus titer (figure 4c) occurred at about the same time as the decrease of polyhedra and

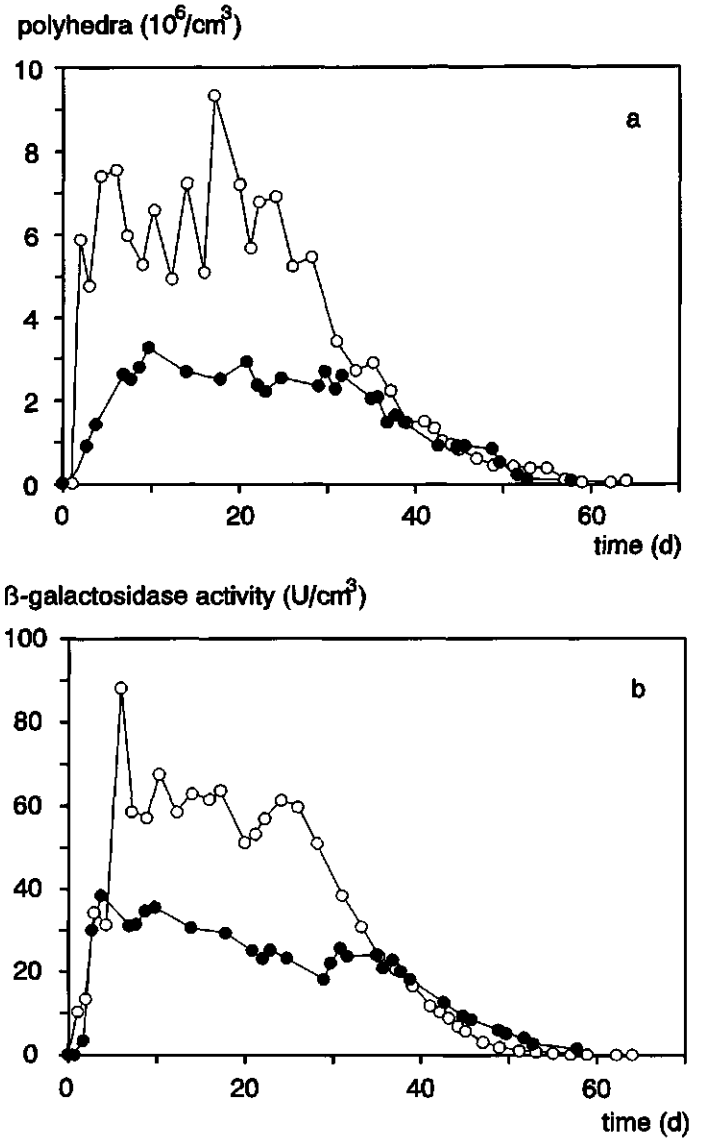

Figure 4.
Production per cm^3. a) polyhedra. b) ß-galactosidase. ●: experiment 1, ○: experiment 2.

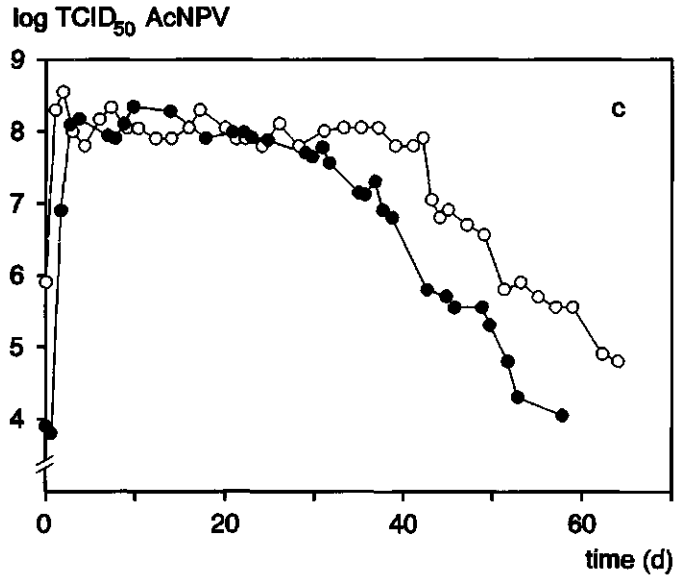

Figure 4.
Production per cm^3. c) infectious NOV. ●: experiment 1, ○: experiment 2.

ß-galactosidase production. Despite the drop in virus titer, cell viability in the infection reactor does not increase. This is probably due to the presence of virus mutants which have a cytopathic effect but do not produce polyhedra or ß-galactosidase and are therefore not detectable in the virus assay.

Due to the higher cell number, production of polyhedra and ß-galactosidase was higher during the second experiment. The fraction of cells containing polyhedra, however, was during the first experiment higher than during the second (figure 5). The increase in the level of polyhedra-containing cells 36 days after the start of the production reactor coincided with the introduction of medium supplemented with a different batch of serum. The fraction of polyhedra-containing cells (62% and 47%) was high considering the residence time distribution in the reactor and considering the fact that polyhedra become visible by light microscopy only

after about 24 hours. With a mean residence time of 60 hours the maximum fraction of polyhedra-containing cells can be calculated to be 68% (Levenspiel 1972).

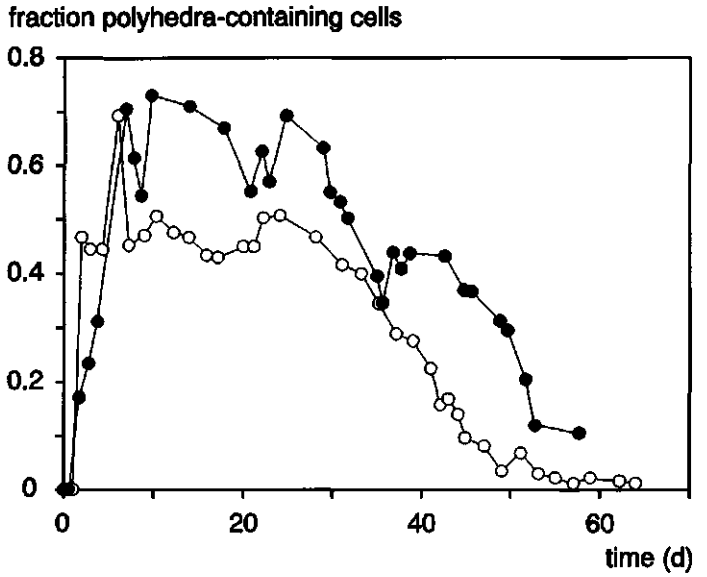

Figure 5.
Fraction of cells which contained polyhedra in the infection reactor. ●: experiment 1, ○: experiment 2.

The average number of polyhedra per cell during pseudo steady state was 7.7 in experiment 1 and 12.3 in experiment 2. This polyhedra production per cell is low when compared to the average values of 15 and 25 found by Kompier et al. (1988) in a similar reactor system with wild-type AcMNPV. The polyhedra production during the second experiment expressed per volume culture medium, however, was comparable to the experiments of Kompier et al. (1988).

The ß-galactosidase activity during experiment 2 was also measured at 28°C. Activity at 28°C was 61% of the activity at 37°C, resulting in an

average ß-galactosidase activity of 15 and 33 U per 10^6 cells at 28°C for experiment 1 and 2 respectively. These values are high as compared to earlier experiments where a polyhedrin-based recombinant AcMNPV producing ß-galactosidase was used (van Lier et al. 1992) and a ß-galactosidase activity at 28°C of 5-15 U/10^6 cells was measured.

The production of ß-galactosidase was in the same order of magnitude as found in other insect cell systems. In a 0.75 dm^3 airlift reactor and a 3 dm^3 stirred and sparged reactor Murhammer and Goochee (1988) produced 44-64 U ß-galactosidase per 10^6 cells. When the stirred reactor was head-space aerated, 74-99 U per 10^6 cells was produced. These values are high compared to our values but were obtained in batch-operated reactors where there is no residence time distribution. However, comparison of data is very difficult. As discussed by Wickham et al. (1992) different ß-galactosidase vectors, different cell densities and differences between cell lines as a result of passaging may result in different expression levels. Licari and Bailey (1991) showed that ß-galactosidase activity can vary considerably depending on MOI and cell density. They obtained activities ranging between almost 0 and 150 U/10^6 cells (values adjusted to 28°C).

The length of virus particles from infected-cell supernatant samples, taken 41 days after starting the production reactor was measured using an electron microscope and compared to particles present in the initial inoculum. In the microscopic samples two lengths of NOVs could be identified. These NOVs had an average length of 230 and 342 nm. The average length of NOVs in inoculum is about 330 nm. The defective interfering viruses as found by Kool et al. (1991) were about 190 nm in length. Wickham et al. (1991) found defective interfering particles of 200 nm upon serial passage of AcMNPV.

NOV DNA was also digested with *Eco*R1 and separated in an agarose gel (data not shown). Using nucleic acid hybridization of the bands with radio-actively labelled RK1-AcMNPV DNA it was found that fragments A, J, K, N, M, T, U and V (Figure 1) were present in less than equimolar amounts in extra-cellular virus. The virus DNA present in

equimolar amounts contained both the polyhedrin and the lacZ gene, but missed about 35% of the genome including the DNA polymerase gene. This indicates that the deletion mutants originating during our experiments with p10-lacZ AcMNPV differ slightly from the defective interfering particles found during experiments with the polyhedrin-based ß-galactosidase recombinant (Kool et al. 1991). Further investigation by restriction endonuclease analysis of the viral DNA is needed to locate and analyze these differences.

The polyhedrin and p10 promoters are very similar sharing the transcription initiation signal TAAG as part of the dodecanucleotide consensus sequence (Vlak and Rohrmann 1985). The polyhedrin and p10 genes are, however, independently regulated. Deletion of the polyhedrin promoter does not affect the expression of the p10 (van Oers et al. 1992), whereas deletion of the p10 promoter even upregulates polyhedrin production (Chaabihi et al. 1993). The strength of the AcMNPV polyhedrin and p10 promoters is of the same order of magnitude, the polyhedrin promoter being about 30% stronger than the p10 promoter (Roelvink et al. 1992). Even when both genes are retained in the defective virus, as is the case here, their expression drops considerably suggesting that the presence of the coding sequence alone is not sufficient. It is likely that the reduced level of gene expression is caused by the lack of a sufficient amount of transcription/translation factors coded for by the deleted segment of the genome, such as the putative RNA polymerase (subunit), the *etl* gen product (Crawford and Miller 1988) and the late essential factor *lef-1* (Paserelli and Miller 1993).

4. Conclusions

Continuous production of ß-galactosidase in a two-stage bioreactor system with a baculovirus (AcMNPV) expression vector based on the p10 gene at the p10 locus was maintained for about four weeks. After this

period polyhedron and p10-promoter-based ß-galactosidase production as well as production of infectious extracellular virus particles rapidly declined. In the infection reactor defective virus particles were observed which contained both the lacZ and the polyhedrin gene but lacked large segments of the genome. These defective particles are most likely responsible for the decrease in recombinant protein production through interference with the wild-type virus.

The hypothesis that a foreign gene behind the p10 promoter at the p10 locus will be expressed in a continuously operated two-stage bioreactor system for a longer time than when placed downstream of the polyhedrin promoter at the polyhedrin locus proved incorrect. This implies that the site of insertion of a foreign gene into a baculovirus expression vector is irrelevant in the present continuous production set-up. A possible way to maintain the production for a longer period of time is to control the number of virus passages. This may be achieved by running the infection reactor in a fed batch mode (van Lier et al., in preparation).

5. Acknowledgements

The authors thank Marcel Kool for assistance with the characterization of the virus and for helpful discussions. Fred van den End and Kees de Gooijer are also acknowledged for their helpful comments. We thank Magda Usmany for technical assistance and Joop Groenewegen for assistance with electron microscopy. This work was partly financed by the Dutch Programme Committee for Agricultural Biotechnology.

6. References

Chaabihi, H., Ogliastro, M.-H., Martin, M., Giraud, C., Devauchelle, G. and Cerutti, M. (1993) Competition between baculovirus polyhedrin and p10 gene expression during infection of insect cells. J. Virol. **67**, 2664-2671.

Crawford, A.M. and Miller, L.K. (1988) Characterization of an early gene accelerating expression of late genes of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J. Virol. **62**, 2773-2781.

Hink, W.F. (1970) Established cell line from the cabbage looper, *Trichoplusia ni*. Nature **226**, 466-467.

Kompier, R., Tramper, J. and Vlak, J.M. (1988) A continuous process for production of baculovirus using insect-cell cultures. Biotechnol. Lett. **10**, 849-854.

Kool, M., Voncken, J.W., van Lier, F.L.J., Tramper, J. and Vlak, J.M. (1991) Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with defective interfering properties. Virology **183**, 739-746.

Licari, P. and Bailey, J.E. (1991) Factors influencing recombinant protein yields in an insect cell-baculovirus expression system: multiplicity of infection and intracellular protein degradation. Biotechnol. Bioeng. **37**, 238-246.

Levenspiel, O. (1972) Chemical reaction engineering. J. Wiley & Sons, New York, pp 253-296.

Luckow, V.E. and Summers, M.D. (1988) Trends in the development of baculovirus expression vectors. Bio/Technol. **6**, 47-55.

Miller, J.H. (1972) Assay of ß-galactosidase. In: Experiments in Molecular Genetics, Cold Spring Harbor laboratory, pp 352-355.

Murhammer, D.W. and Goochee, C.F. (1988) Scaleup of insect cell cultures: protective effects of pluronic F-68. Bio/Technol. **6**, 1411-1418.

Passarelli, A.L. and Miller, L.K. (1993) Identification and characterization of lef-1, a baculovirus gene involved in late and very late gene expression. J. Virol. **67**, 3481-3488.

Roelvink, P.W., van Meer, M.M.M., de Kort, C.A.D., Possee, R.D., Hammock, B.D. and Vlak, J.M. (1992) Dissimilar expression of *Autographa californica* nuclear polyhedrosis virus polyhedrin and p10 genes. J. Gen. Virol. **73**, 1481-1489.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**, 503-517.

Rigby, P.W.J., Dieckman, M., Rhodes, C. and Berg, P. (1977) Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**, 237.

Summers, M.D. and Smith, G.E. (1987) A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agric. Exp. Stn. Bull. No. 1555.

Tramper, J. and Vlak, J.M. (1986) Some engineering and economic aspects of continuous cultivation of insect cells for the production of baculoviruses. Ann. N.Y. Acad. Sci. **469**, 279-288.

Van Lier, F.L.J., van der Meijs, W.C.J., Grobben, N.G., Olie, R.A., Vlak, J.M. and Tramper, J. (1992) Continuous ß-galactosidase production with a recombinant baculovirus insect-cell system in bioreactors. J. Biotechnol. **22**, 291-298.

Van Oers, M.M., Malarme, D., Jore, J.M.P. and Vlak, J.M. (1992) Expression of the *Autographa californica* nuclear polyhedrosis virus p10 gene: effect of polyhedrin gene expression. Arch. Virol. **123**, 1-11.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J. and McCawley, P. (1977) The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro **13**, 213-217.

Vlak, J.M. (1979) The proteins of nonoccluded nuclear polyhedrosis virus produced in an established cell line of *Spodoptera frugiperda*. J. Invertebr. Pathol. **34**, 110-118.

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klinge-Roode, E.C., Geervliet, J.B.F, Roosien, J. and van Lent, J.W.M. (1988) Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-ß-galactosidase fusion gene. J. Gen. Virol. **69**, 765-776.

Vlak, J.M. and Rohrmann, G.F. (1985) The structure of baculovirus polyhedrins. In: Maramorosh, K. and Sherman, K.E. (eds.) Viral insecticides for biological control, 489-542.

Vlak, J.M., Schouten, A., Usmany, M., Belsham, G.J., Klinge-Roode, E.C., Maule, A.J., van Lent, J.W.M. and Zuidema, D. (1990) Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. Virology **179**, 312-320.

Wickham, T.J., Davis, T., Granados, R.R., Hammer, D.A., Shuler, M.L. and Wood, H.A. (1991) Baculovirus defective interfering particles are responsible for variations in recombinant protein production as a function of multiplicity of infection. Biotechnol. Lett. **13**, 483-488.

Wickham, T.J., Davis, T., Granados, R.R., Shuler, M.L. and Wood, H.A. (1992) Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. Biotechnol. Progr. **8**, 391-396.

Chapter 5 has been published as: de Gooijer, C.D., Koken, R.H.M., van Lier, F.L.J., Kool, M., Vlak, J.M. and Tramper, J. (1992) A structured dynamic model for the baculovirus infection process in insect cell reactor configurations. Biotechnol. Bioeng. **40**, 537-548.

5

A structured dynamic model for the baculovirus infection process in insect-cell reactor configurations

Summary

A mathematical description of the infection of insect cells with baculovirus in a continuously-operated reactor configuration is presented. The reactor configuration consists of one bioreactor in which insect cells (*Spodoptera frugiperda*) are grown, followed by one (Kompier et al. 1988) or two (van Lier et al. 1990) bioreactors in which cells are infected by a baculovirus (*Autographa californica* nuclear polyhedrosis virus). It was demonstrated that the so-called passage effect is responsible for the observed difference in run time between a configuration with one or with two infection vessels. Furthermore, a model is presented based on the hypothesis that the limited run time of series of continuously-operated bioreactors is associated with the occurrence of a virus particle (so-called virion) that is defective and has interfering properties (Kool et al. 1991). With the assumption that not all non-occluded virions are capable of establishing a correct infection leading to new virus production, infection levels in continuously-operated reactor configurations could be described well with the model.

1. Introduction

Baculoviruses are attractive biological agents for control of insect pests in agriculture. In addition, these viruses can be reprogrammed for the production of recombinant proteins by genetic engineering (Luckow and Summers 1988, Vlak and Keus 1989). To obtain commercially-attractive levels of productivity and at the same time meet regulatory requirements, the production of baculoviruses or recombinant proteins can best be achieved in insect-cell bioreactors, preferably operated in a continuous fashion (Tramper and Vlak 1986). Two phenotypically different forms of a baculovirus exist. The occluded form (polyhedra) is infectious for insect larvae, whereas the non-occluded virus form (NOV) is infectious for insect cells in culture. Kompier et al. (1988) and Van Lier et al. (1990) showed that polyhedra of *Autographa californica* nuclear polyhedrosis virus (AcNPV) could be produced over long periods of time in a system consisting of an upstream bioreactor in which insect cells are cultured, followed by one or two bioreactors where infection with the non-occluded form of AcNPV takes place (figure 1). The continuous production could be maintained up to about four weeks, after which production declined.

The level of infection could be increased by increasing the number of infection reactors as shown by Van Lier et al. (1990) for reactor configuration A. However, the time that this system could be operated at this enhanced level of infection decreased significantly to less than three weeks (figure 2).

Kool et al. (1990, 1991) showed that the reduction in productivity was due to the occurrence of mutant virions present in the infection reactor(s) that interfered with the replication of intact virus and affected productivity. This defective interference phenomenon has been confirmed by Wickham et al. (1991).

This paper presents a structured model for the infection of insect cells with NOV particles, capable of dynamically describing the infection process in continuous, batch and fed-batch bioreactors. This model forms the theo-

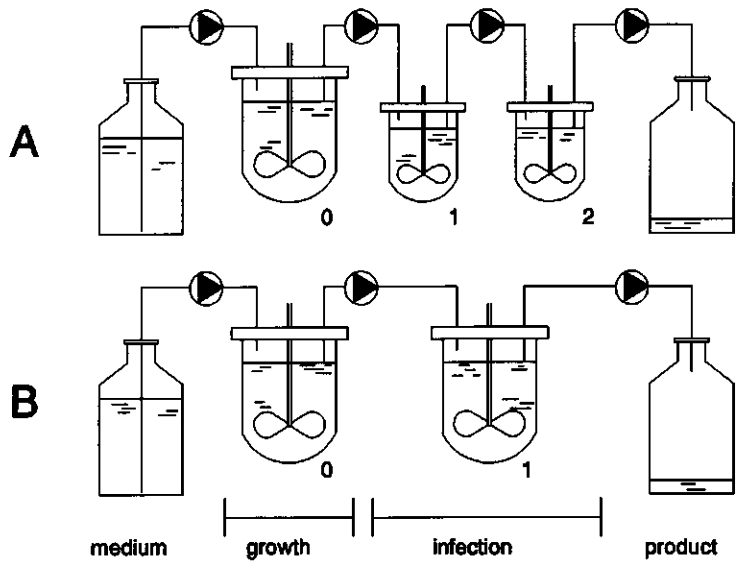

Figure 1.
Schematic representation of the experimental continuously-operated reactor configurations as used by Van Lier et al. (1990) (series A) and Kompier et al. (1988) (series B). The total residence time in both configurations is the same.

retical foundation explaining that the decrease in the levels of polyhedra-producing cells in continuously-operated vessels is the result of the so-called passage effect, and that this decrease can be quantitatively explained by the occurrence of a high number of so-called defective interfering virions (D-NOV).

2. Theory

The passage effect, as described by Faulkner (1981), manifests itself as a decrease in the production of polyhedra at higher passages of non-occluded baculovirus preparations. This effect is also reflected by a de-

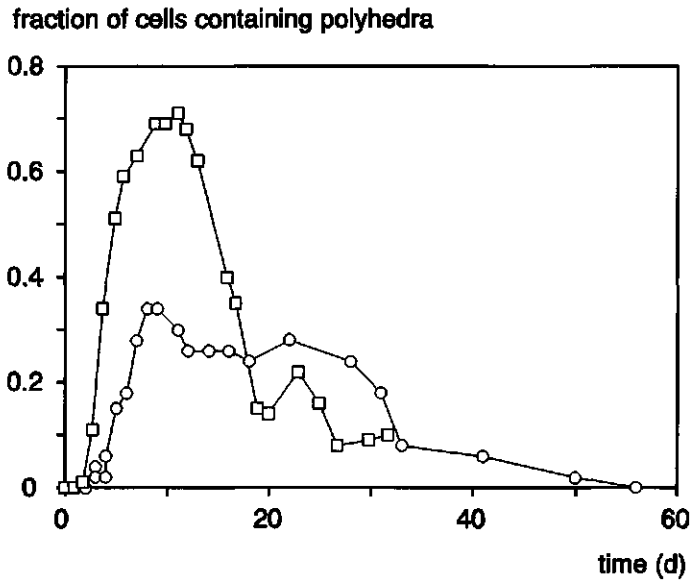

Figure 2
Percentage of cells visibly containing polyhedra present in reactor 2 of configuration A (□) or in reactor 1 of configuration B (○) versus time. Pseudo-steady-state levels of infection in the continuously-operated infection vessels of about 68% and 26% were reached, respectively.

crease in the number of infectious NOVs (I-NOVs) that is produced per insect cell. One passage is defined as the process of an I-NOV entering an insect cell, transport of the genetic information into the cell nucleus, production of new I-NOVs, transport to and budding through the cell membrane, and secretion of the virions to the extracellular fluid. After completion of this process the passage number is increased by one. Schematically this is illustrated in figure 3.

Upon entry in the infection vessel, a cell will be infected with a virion of a certain passage p. After some time the cell will start to produce virions of passage $p + 1$ (figure 3). These virions in turn will maintain the infection process in the continuously-operated infection vessels. Apart from

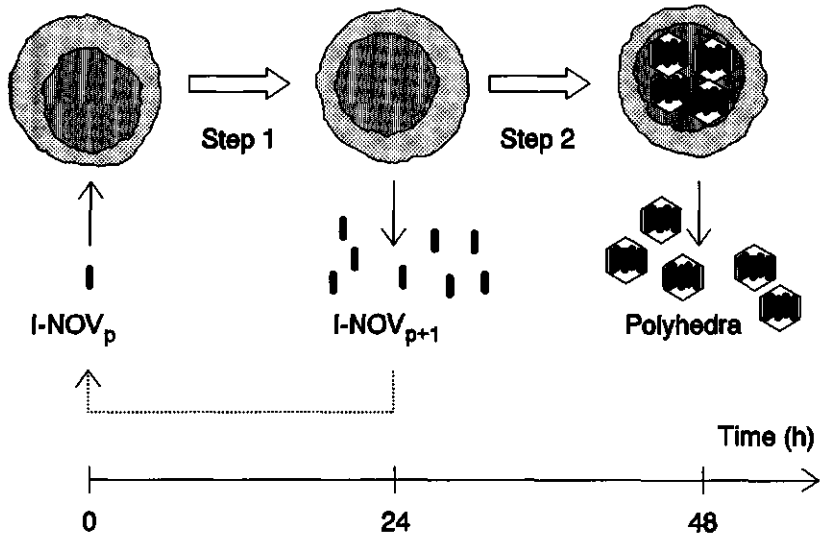

Figure 3
Schematic representation of the replication cycle of baculoviruses in insect cells (adapted from de Gooijer et al. (1989)). *p* and *p+1* denote passage numbers.

this infectious I-NOV, two additional types of virions are introduced in our model. The first additional type of virion was described by Kool et al. (1990, 1991). They showed the existence and established some of the properties of a defective interfering virion, present in the medium of the reactor during continuous runs. This D-NOV lacked about 44% of the viral genome, among which the polyhedrin gene and the gene coding for DNA-polymerase. Due to this deletion the D-NOV needs the intracellular presence of an intact I-NOV as a helper for multiplication. Since their genome is smaller, D-NOVs are likely to be reproduced faster than I-NOVs.

For both reactor configurations the theoretical level of infection can be calculated from a residence-time distribution of the insect cells in the infection vessels. Considering the fact that cells were denoted as visibly

infected when polyhedra were observed, only the cells with a residence time of more than 24 hours in the bioreactor with infected cells are of interest (figure 3). For our experiments, with a total residence time of 60 hours, it could be calculated that 68% of the cells had a residence time of more than 24 hours and therefore should have been visibly infected in the case of one infection reactor, whereas this is 82% in the second reactor for two infection vessels with the same total residence time (Levenspiel 1972) (Configurations B and A in figure 1, respectively). This theoretical value, however, could never be reached in our laboratory (figure 2). As we also did not reach a 100% infection in batch cultures (this paper), we assume that a second additional virion type is present which is characterized by its inability to complete a replication cycle as discussed above. These virions are of normal size and for example may attach to the cell surface but fail to form an endosome, or may attach, form an endosome, but fail to leave the endosome (Seth et al. 1985). In these mechanisms no reproduction takes place, whilst cell receptors are being occupied. To handle this assumption mathematically, we therefore included a NOV leading to an abortive infection (A-NOV) in our model. An A-NOV does occupy an entry site, but will not lead to any reproduction. Volkman et al. (1976) reported a ratio I-NOV/A-NOV of less than 1% for AcNPV produced in *Trichoplusia ni* cells. In our laboratory, approximately 1 g of viral DNA could be isolated from a sample containing 10^8 median tissue culture infective dose ($TCID_{50}$) units of extracellular AcNPV. With a molecular weight of the baculovirus of about 10^8 ($g.mol^{-1}$) and Avogadro's number of $6x10^{23}$ ($molecules.mol^{-1}$) a ratio of 1 I-NOV to 60 A-NOVs could be calculated.

Reactor model

A general mass balance of virions or cells over one reactor vessel can be described as:

$$\frac{dx}{dt} = (r_{x,\,in} - r_{x,\,out}) + r_{x,\,prod} - r_{x,\,use} \qquad (1)$$

where t : time (h)
x : compound (number of cells or virions)
$r_{x,in}$, $r_{x,out}$: in and outgoing flows of x (h^{-1})
$r_{x,prod}$: production rate of the compound (h^{-1})
$r_{x,use}$: consumption rate of x (h^{-1}).

This mass balance was applied to virions and insect cells.

Virions

Within the general mass balance for virions a simple production term $r_{x,prod}$ can be defined by the assumptions that enough virions are available to infect all insect cells, that all cells entering the infection reactor are infected immediately, and that after 16 hours the cells start to produce new virions. As we are interested in the passage effect this is done for each passage p. Hence,

$$r_{x,\,prod,\,p} = a * f_{p-1,\,t-16} \qquad (2)$$

with f : fraction of I-NOV of a certain passage, that is the total amount of $I\text{-}NOV_p$ divided by the total amount of all I-NOV
a : production constant formed by the number of I-NOVs produced per cell, multiplied by the number of cells flowing into the reactor per unit of time (h^{-1})
p : passage number.

The fraction $f_{p,t}$ of a certain passage at a certain time is determined not only by the fractions of the other passages at that time, but also by the fractions of the passages before that time. These passages determined how many insect cells are infected with a certain passage and with that how many virions of a higher passage will be produced 16 hours later. With increasing time, the number of passages increases, and with that the set of equations arising from equation (1) that has to be solved augments also. As with increasing time this exercise will become quite complicated, equation

(1) is made discrete:

$$x_{t+\Delta t}=x_t+((r_{x,\,in}-r_{x,\,out})+r_{x,\,prod}-r_{x,\,use})\Delta t \qquad (3)$$

with Δt : one time step (h).

Equation (3) can be applied to all types of NOV as well as insect cells. For the I-NOV this can be done for each virion passage. This leads to:

$$v_{i,\,m,\,p,\,t+\Delta t}=(1-D_m\Delta t)*(v_{i,\,m,\,p,\,t}+v_{i,\,in,\,m,\,p,\,t})+(R_{i,\,m,\,p}-I_{i,\,m,\,p})\Delta t \qquad (4)$$

where
- D : dilution rate in a reactor (h^{-1})
- I : virion consumption rate used for infection (h^{-1})
- R : rate of virion release by the cells (h^{-1})
- v : amount of virions
- m : reactor number in the series
- i : infectious I-NOV.

For D-NOVs and A-NOVs it is not necessary to calculate with separate passages, so equation (3) is simplified to:

$$v_{(dVa),\,m,\,t,\,\Delta t}=(1-D_m\Delta t)*(v_{dVa),\,m,\,t}+v_{dVa),\,\in,\,m,\,t})+(R_{dVa),\,m}-I_{(dVa),\,m})\Delta t \qquad (5)$$

where
- d : defective interfering virion (D-NOV)
- a : virion leading to an abortive infection (A-NOV)

The dilution rate D_m in reactor m will be zero as long as this reactor is in batch or fed-batch mode, which will be the case in the start-up phase of the reactor configuration. Due to the fact that in the cell growth vessel (m=0) in the series no virion production takes place, v_0 is equal to zero for all NOV types.

The total amount of I-NOV in vessel m can be calculated from:

$$V_{i,\,m,\,tot,\,t+\Delta t} = \sum_{p=0}^{p=p_{max}} V_{i,\,m,\,p,\,t+\Delta t} \qquad (6)$$

where p_{max} : maximum number of passages at time $t+\Delta t$
tot : total number of I-NOVs

The amount of virion release by the insect cells is controlled by the number of infected cells some time before. As shown previously (de Gooijer et al. 1989), the virions are not released at once. Therefore, a time distribution of the I-NOV and A-NOV release is introduced in the model, as illustrated in table 1.

Table 1
Distribution of virion release from infected insect cells (adapted from De Gooijer et al. (1989)).

Time (h)	% Release	% Release (total)
16	7	7
18	13	20
20	20	40
22	26	66
24	17	83
26	15	98
28	2	100

Kool et al. (1990, 1991) showed that D-NOVs have a 44% smaller genome than I-NOVs. If D-NOV DNA and I-NOV DNA are replicated at the same speed, the replication cycle of a D-NOV will be finished

sooner. Since the process of transport to and from the cell nucleus may also be of importance, the D-NOVs are arbitrarily assumed to have the same time distribution but moved forward in time by two hours.

To facilitate the calculation of the I-NOV balance for each passage, $R_{i,m,p} \cdot \Delta t$ is calculated in advance, that is as the number of virions that will be released by the cells after some time. Therefore the number of infected cells and $I_{i,m,p} \cdot \Delta t$ are evaluated in each time step. In the software these data are then stored in an array. Each time step the index of the array is increased, and in this way the virion amounts are available for calculation of $R_{i,m,p} \cdot \Delta t$ at the appropriate time.

Insect cells

Equation (3) can also be applied to viable insect cells. With a similar derivation as for equation (4), for the first infection vessel in the series, equation (3) turns into:

$$C_{m,\,t+\Delta t}=(1-D_m\Delta t)*(C_{m,\,t}+D_{m-1}\Delta t C_{m-1,\,t})+k_g(C_{m,\,t}-G_m)\Delta t \qquad (7)$$

where C : number of non-infected insect cells

G : number of cells that are infected in one time step

k_g : first-order cell growth constant (h^{-1})

Note that C_0 is the number of cells in the growth vessel. Cells that are not infected can grow in infection vessels, with the same speed as in the cell growth vessel. Infection of insect cells with baculovirus will annihilate cell division (Faulkner 1981).

Infection model

With the three virion types (I-NOV, A-NOV, and D-NOV), three important modes of infection can be distinguished, as illustrated in figure 4.

The first mode is formed by correct infections, being an insect cell

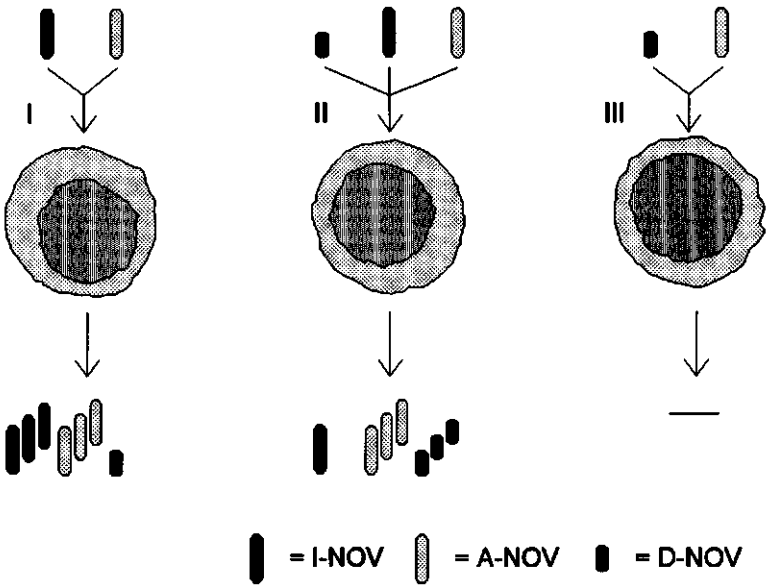

Figure 4
Schematic representation of three important modes of infection.

infected with at least one I-NOV, and not infected by any D-NOV. Such cells will produce I-NOV, A-NOV, and a small amount of D-NOVs. The second mode consists of defective infections, where an insect cell is infected with at least one I-NOV and at least one D-NOV. Such an insect cell will produce I-NOV, A-NOV, and a large quantity of D-NOVs. The third mode is formed by an abortive infection, defined by an insect cell that is infected with an A-NOV and/or D-NOV, but without an infection with infectious I-NOV. Insect cells infected this way will not produce any virions. For both the first and second mode of infection the ratio of the numbers of virions produced is constant with time.

To be able to calculate the fractions of each mode of infection, the following assumptions were made :

1) Each insect cell has an equal amount of 'entry sites' for NOVs. This number is much less than the number of receptors. Wickham et al.

(1990) reported a number of 10^5 AcNPV receptors per cell in the case of *Spodoptera frugiperda* cells. As production expressed as number of NOVs per cell is considerably less than this amount (this paper), it would be difficult to perform continuous runs if all NOVs would attach to cells and each virion - receptor interaction leads to an infection. Moreover, it is most likely that the entry of 10^5 NOVs in an insect cell will lead to a considerable overload of the cellular machinery, or cause cytotoxic effects.

2) The number of entry sites is constant in time for one experimental run.
3) All entry sites are equal.
4) All NOVs have identical binding sites. Hence, the probability that an NOV attaches is the same for all types of NOV.
5) Attachment of an NOV to a cell is irreversible.
6) The three NOV types cannot change into alternate types spontaneously.
7) Binding of NOVs to cells will take place within one time step.

With these assumptions the fractions of the different modes of infection can be calculated, for each time step, if the probabilities for the different modes of infection are known.

This problem, where a known number of three types of virions can occupy a known number of entry sites, is the same as the problem where red, white and blue balls are to be dispended over a number of small boxes. It is known from the latter that the probability of a box remaining empty has a Poisson distribution. Therefore, it is assumed that the probability (P_o) of an entry site remaining empty is having a Poisson distribution:

$$P_0 = e^{(\frac{V_{tot}}{BC})} \tag{8}$$

where B : number of entry sites on a cell
tot : total number of all virions.

From that, the probability (P_i) of an entry site being infected with an I-NOV is:

$$P_i = \frac{v_i(1-P_0)}{v_{tot}} \tag{9}$$

and the probability (P_d) that an entry site is infected with a D-NOV:

$$P_d = \frac{v_d(1-P_0)}{v_{tot}} \tag{10}$$

and finally the probability (P_a) that an entry site is infected with an A-NOV:

$$P_a = \frac{v_a(1-P_0)}{v_{tot}} \tag{11}$$

The sum of these probabilities equals unity:

$$P_0+P_i+P_d+P_a=1 \tag{12}$$

With these probabilities the fractions of the various modes of infection of the cells can be calculated. The fraction of the cells that are not infected (F_o) then is:

$$F_0=F(B_a=0 \wedge B_i=0 \wedge B_d=0)=P_0^B \tag{13}$$

The fraction of the cells that are abortively infected (F_a) then is:

$$F_a=F(B_i=0 \wedge (B_a+B_d>0))=\sum_{\alpha\geq 0,\ (\beta+\lambda)>0}^{\alpha+\beta+\lambda=B} \frac{B!}{\alpha!\beta!\lambda!} P_0^{\alpha} P_a^{\beta} P_d^{\lambda} \tag{14}$$

where
- α : number of entry sites per cell that are not occupied by any virion
- ß : number of entry sites per cell that are occupied by A-NOVs

λ : number of entry sites per cell that are occupied by D-NOVs.

The fraction of cells that have a correct infection (F_i) is calculated from:

$$F_i=F(B_i>0 \wedge B_d=0)=\sum_{\kappa>0,\alpha,\beta 30}^{\alpha+\beta+\kappa=B} \frac{B!}{\alpha!\beta!\kappa!} P_0^{\alpha} P_a^{\beta} P_i^{\kappa} \qquad (15)$$

where κ : number of entry sites per cell that is occupied by I-NOVs.

The fraction of cells with a defective infection (F_d) is calculated from :

$$F_d=F(B_i>0 \wedge B_d>0)=\sum_{\kappa,\ \lambda>0,\ \alpha,\ \beta\geq 0}^{\alpha+\beta+\kappa+\lambda=B} \frac{B!}{\alpha!\beta!\kappa!\lambda!} P_0^{\alpha} P_a^{\beta} P_i^{\kappa} P_d^{\lambda} \qquad (16)$$

As the sum of all fractions is equal to 1,

$$F_0+F_i+F_d+F_a=1 \qquad (17)$$

it is also possible to calculate F_d with equation (17). The actual amounts of cells and virions, as required in the equations (4), (5), and (7) can easily be calculated from these fractions. Note that the implementation of equations (14), (15), and (16) also limit the theoretical number of entry sites that can be handled in the model for numerical reasons. The maximum number in our software is 1546. With the above equations the concentration in time for each of the virion types can be calculated. With these concentrations the decline in infection level in continuously-operated bioreactors can be described.

3. Materials and methods

Cells and viruses

Spodoptera frugiperda cells (Vaughn et al. 1977) were maintained in TNM-FH medium (Hink 1970) without egg ultrafiltrate, but supplemented

with 10% fetal calf serum. For suspension cultures 0.1% (w/v) methylcellulose was added. The E2-strain of *Autographa californica* nuclear polyhedrosis virus (AcNPV) (Smith & Summers 1978) was used. The stock solution of the virus contained 10^8 $TCID_{50}$ units per cm^3.

Reactor configuration

The continuously-operated reactor configuration with one infection vessel has been described by Kompier et al. (1988), and the configuration with two infection vessels by van Lier et al. (1990).

Assays

In order to determine the infectivity of the virus, 4-cm^3 aliquots of the sample were centrifuged (1600 *g* for 15 min). The supernatant was filtered (0.45 μm), and the infectivity was measured using the end-point dilution method as described by Vlak (1979). The presence of polyhedra in the cell nucleus was determined using an inverted microscope (magnification 400x). The cell concentration was measured using a Neubauer hemocytometer.

Batch infection

Cells from a continuous culture were pumped into a round-bottom bioreactor (working volume: 0.6 dm^3, Applikon, Schiedam, the Netherlands). The cells were infected with 15 cm^3 containing 10^8 $TCID_{50}$ units of third passage AcNPV per cm^3, resulting in a multiplicity of infection of 2 and 3 $TCID_{50}$ units per cell in two separate experiments. Samples were taken to assess the number of infected cells and the cell viability with time.

Serial passages

Cells were grown in 100 cm^3 Erlenmeyer flasks in 20 cm^3 of medium until the cell density exceeded $1.5x10^6$ cells.cm^{-3}. To maintain the cells in an exponential growth phase, cells were diluted to a concentration of $5x10^4$

cells.cm^{-3} and further incubated at 27 °C. From these suspensions a volume equivalent to $2x10^6$ cells was taken and centrifuged (225 g, 5 min). A volume containing $2x10^7$ $TCID_{50}$ units of a single passage *p* was added to the cell pellet, thus resulting in a Multiplicity of Infection of 10. Medium was added to a volume of 20 cm^3 giving a cell concentration of 10^5 cells.cm^{-3} and a multiplicity of infection of 10 $TCID_{50}$ units per cell. The Erlenmeyer flask was then further incubated for three hours at 27 °C. The medium, and, with that, the non-bound I-NOVs were removed by centrifugation (225 g, 5 min). After resuspension in 20 cm^3 fresh medium the cells were incubated for 21 hours at 27 °C. The extracellular fluid of this sample then contained almost exclusively I-NOVs of a higher passage *p + 1*. After centrifugation the infectivity of the supernatant was assayed as described before. Hereafter, a new experiment was started.

4. Results and discussion

Passage-time distribution of I-NOVS

With the model discussed above, the effects of different reactor configurations were studied. For one set of parameters, the total residence time in the series was kept constant by varying the reactor volumes for each reactor at a constant flow. The fractions of infectious NOV of each passage in time were calculated for reactor configurations with one, two and three infection reactors, with the infection reactor volume being $1.2x10^{-3}$, $0.6x10^{-3}$, and $0.4x10^{-3}$ m^3, respectively. Results are shown in figure 5.

This figure clearly shows that higher passages occur sooner if the number of vessels in the series of infection reactors is increased, i.e. if plug flow is more closely approximated.

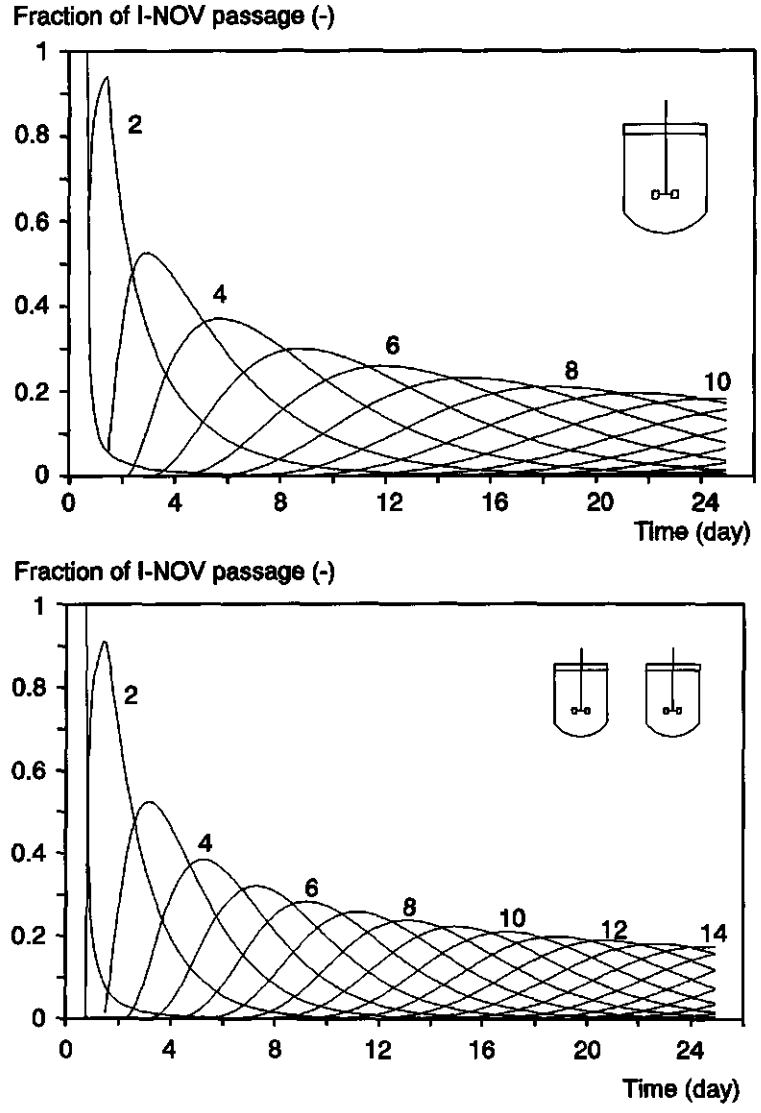

Figure 5.

Passage distribution of I-NOV, depicted as a fraction ($I\text{-}NOV_p$ / $I\text{-}NOV_{tot}$) occurring in time in the last vessel for three different continuously-operated reactor configurations with one, two and three infection vessels. The total residence time is kept constant for all configurations.

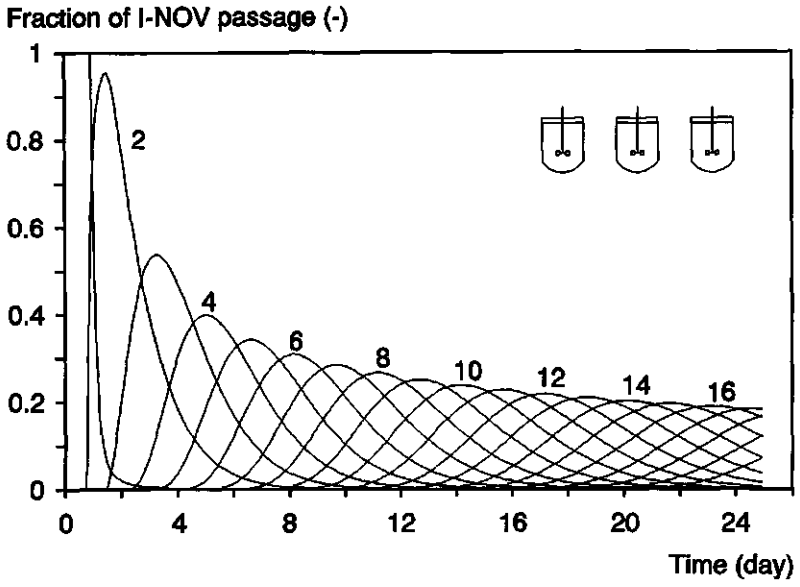

Figure 5 (continued).

The model was used to test the hypothesis whether the passage effect is a viable explanation for the decrease in the number of cells producing polyhedra in the experiments with different reactor configurations as mentioned in the introduction section. Therefore, at the calculated time ($t_{1/2}$)where the level of infection was only half of the pseudo-steady-state level as shown in figure 2, the distribution of the virion passages was calculated with the program. Results for the configurations with one and two infection vessels are shown in figure 6.

As both passage distributions in figure 6 showed a remarkably good resemblance, it can be concluded that the decline of polyhedra production after a certain time may indeed be attributed to the passage effect.

To assess this in more detail, virions were serially passaged in two independent series of batch experiments and the amount of NOV produced in each passage was determined. The results are shown in figure 7.

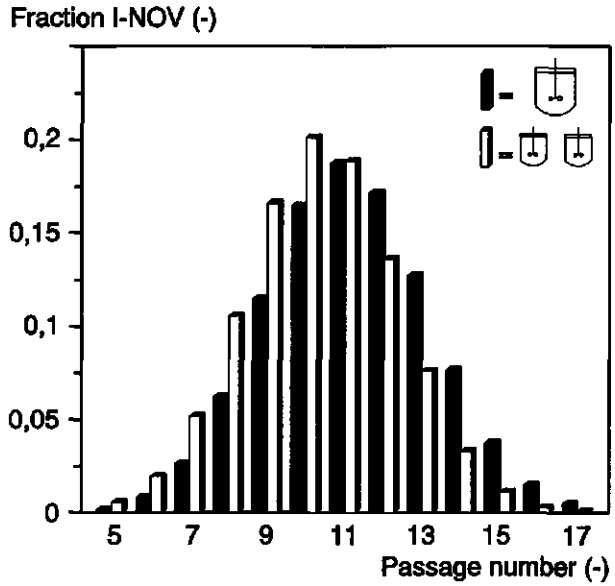

Figure 6.
Calculated passage distributions of I-NOV for two continuously-operated reactor configurations at the time the infection level is half of the pseudo-steady-state level. The I-NOV fraction of a certain passage ($I\text{-}NOV_p/I\text{-}NOV_{tot}$) versus the passage number p. The distribution for one infection reactor is in black, and for the second reactor of a series of two infection reactors the bars are white.

At the ninth passage a sharp drop in the number of I-NOVs produced per cell was observed. At higher passages, a further decrease in the number of polyhedra per cell was found. Furthermore, the polyhedra showed morphological aberrations, which is a known consequence of the passage effect (Faulkner, 1981).

The combination of figures 6 and 7 shows that at the time at which the infection level is half of the pseudo-steady-state level, the preponderance of the virions present in the two reactor configurations has a passage number higher than 8. Moreover, with virions of these passage numbers not enough virions is produced to maintain the continuous in-

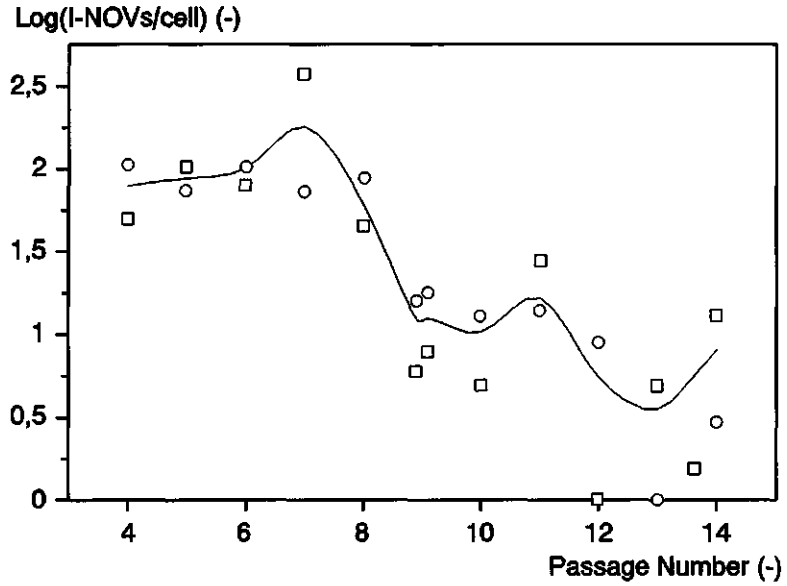

Figure 7.
Prolonged passages of I-NOV: the logarithm of the averaged number of produced I-NOVs per cell as a function of the passage number. (□, ○) are the results of two duplicate experimental series.

fection process at the pseudo steady-state level.

To further elucidate the phenomenon of the passage effect two other virion types A-NOV and D-NOV as described before were introduced into the infection model.

Infection process

The assumption of the A-NOV type facilitated the description of our data on batch infections, as shown in figure 8. Here, two independent runs were fitted by the model.

The fit of the model was reached on the basis of visibly infected cells. We assumed that of the synchronously-infected cells, the first ones become visibly infected after 20 hours, and the last after 44 hours. Visibly

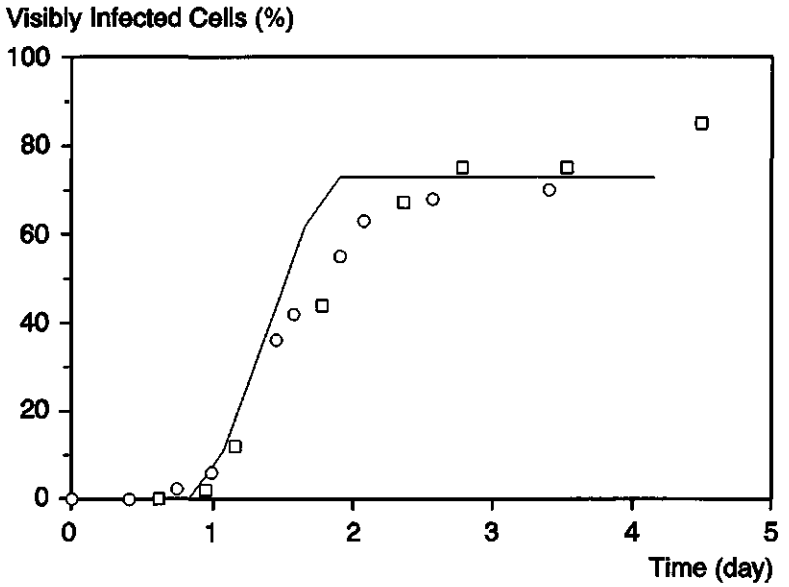

Figure 8.
Fit (——) of two batch infection runs (□, ○) with the model. The percentage of visibly infected cells versus time. Parameters are as in table 2 under A.

infected cells remain visible for 60 hours, then they lyse.

After including the three NOV types into the program all our experimental continuous runs could easily be described with the model. Input variables are given in table 2.

Parameters in table 2 that were either measured or set by experimental conditions were the reactor working and start-up volume, the flow through the series, the number of I-NOVs at start-up, the cell concentration in the growth vessel, the number of I-NOVs produced per cell, and the time step. Fitted parameters were the ratio between I-NOVs and A-NOVs both at start-up and with virion production, the number of entry sites, the D-NOV production rate at correct infections, and the number and ratio of virions produced at a defective infection. The ratio of I-NOV to A-NOV was in the same range as discussed earlier.

Table 2.
Input variables for the infection model. Values *A*,...,*E* are used in figures 8-12, respectively. Values under *D* are also used in table 3.

Variable	*A*	*B*
Reactor working volume	600	800
Reactor startup volume	600	400
Flow	0	1.33×10^{-2}
Number of I-NOVs at startup	1.5×10^{9}	1.6×10^{3}
Number of A-NOVs at startup	2.9×10^{10}	6.2×10^{4}
Number of D-NOVs at startup	0	0
Cell concentration in growth vessel (C_0)	1.0×10^{6}	9.0×10^{5}
Time step (Δt))	2	2
Number of entry sites *B*	33	33
Number of virions produced at a correct infection	3340	4000
of which A-NOVs	95	97.5
of which D-NOVs	10^{-6}	10^{-6}
Number of virions produced at a defective infection	1600	1600
of which A-NOVs	71	77
of which D-NOVs	25.5	22.9

C	*D*	*E*	Unit
2 x 600	2 x 600	800	cm^3
2 x 300	2 x 300	200	cm^3
$2x10^{-2}$	$2x10^{-2}$	$8.3x10^{-4}$	$dm^3\ h^{-1}$
$7.1x10^{6}$	$1.5x10^{9}$	$8.0x10^{7}$	-
$1.1x10^{8}$	$2.9x10^{10}$	$1.3x10^{9}$	-
0	0	0	-
$9.5x10^{5}$	$1.0x10^{6}$	$8.0x10^{5}$	cm^{-3}
2	2	1	h
40	33	33	$cell^{-1}$
3340	3340	3340	$cell^{-1}$
94	95	94	%
10^{-6}	10^{-6}	10^{-6}	%
1600	2000	1600	$cell^{-1}$
71	53	71	%
25.5	46.95	25.5	%

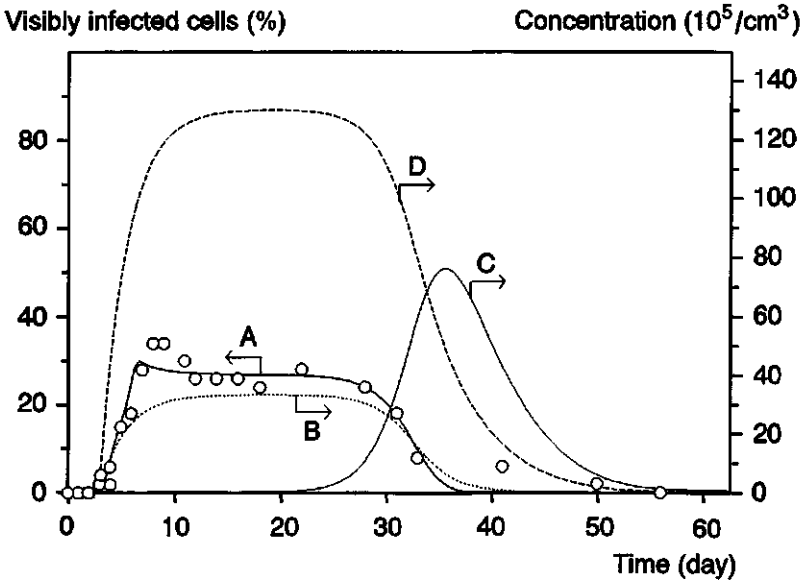

Figure 9.
Fit of the model on the percentage of visibly infected cells in one continuously-operated infection reactor. Parameters are in table 2 under B. ○: experimental data, line A: model description of the percentage of visibly infected cells, line B and C: model descriptions of the I-NOV and D-NOV concentration, respectively, line D: A-NOV concentration divided by 10.

Two examples of fits on the data of Kompier et al. (1988) and van Lier et al. (1990) are given in figure 9 and 10.

From these figures it can be seen that at the time at which the decrease in I-NOV concentration occurred, the number of D-NOVs increased. The model behaved this way with all experimental runs analyzed so far. Note that in figure 10, the concentration of virions does not decrease to zero. This was experimentally observed, and can be predicted by the model assuming that the cells that are either infected with I-NOV and D-NOV and/or A-NOV will still produce some I-NOVs.
Kool et al. (1990, 1991) showed with a recombinant virus that D-NOVs are

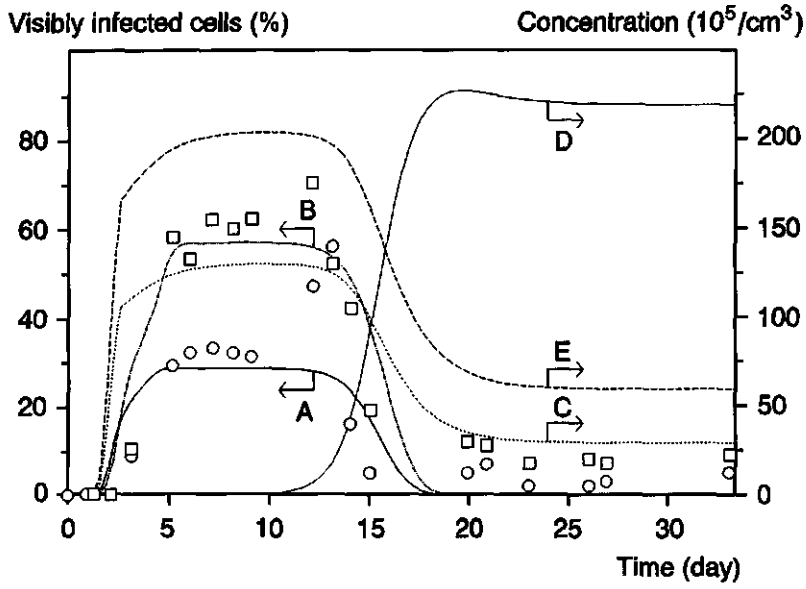

Figure 10.
Fit of the model on the experimental (○, □) percentage of visibly infected cells in the first (line A) and second (line B) reactor of a series of two continuously-operated infection reactors with the model. Lines C and D: I-NOV and D-NOV concentrations in the second vessel, respectively, line E: A-NOV concentration divided by 10. Parameters are in table 2 under C.

generated during continuous runs at each point in time, with a clear increase at the end of the run. Apparently, after a period of low occurrence, D-NOVs are produced in large amounts due to their faster rate of synthesis. After the exponential increase in the amount of D-NOVs, there are too few correct infections to produce enough I-NOVs to support D-NOV reproduction (helper function), and the infection process ceases. This seems a valid explanation for the passage effect.

As reported by Von Magnus (1959), prolonging the serial passages will eventually result in an increase in the number of I-NOVs again. Since D-NOVs need I-NOV to be reproduced, the number of D-NOVs will drop

when there is too little I-NOV. After this phase, at low multiplicities of infection of the three virion types the chance increases that correct infections will occur, thereby starting the I-NOV production again. In a continuous culture high passages occur soon, and eventually D-NOV production will decrease and subsequently the D-NOVs will be washed out of the reactor. Then, a low multiplicity of infection is reached and if not all I-NOVs are washed out, I-NOV reproduction starts again since the chance that insect cells become correctly infected increases in that situation. This reactor behaviour could also be simulated with the model, as illustrated in figure 11. This phenomenon has been observed in our bioreactor system (van Lier et al. 1992).

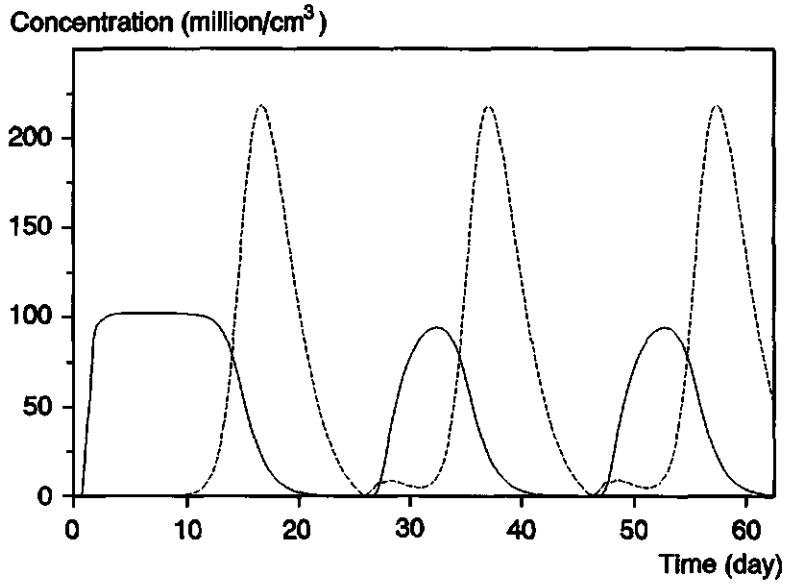

Figure 11.
The von Magnus effect in a continuously-operated infection vessel as simulated by the model. The concentration of I-NOV (———) and D-NOV (- - - -) versus time. Parameters are in table 2 under D.

Sensitivity analysis

In order to assess the significance of each parameter a sensitivity analysis was made. Parameter values were varied along the settings as under D in table 2. The influence of parameter variations on the pseudo-steady-state infection level L and on the time ($t_{1/2}$) at which the infection level is only half the pseudo-steady-state level were investigated. Results are given in table 3.

The sensitivity analysis presented in Table 3 shows some interesting features. The time lap between infection and D-NOV release obviously only influences $t_{1/2}$. If this time lap is smaller the D-NOVs will occur sooner, and hence $t_{1/2}$ will decrease. The influence of the time lap between infection and I-NOV/A-NOV release is much less pronounced. As the I-NOV/A-NOV ratio is not altered, the L will not be changed, and with a decreasing time lap I-NOVs and A-NOVs are produced sooner, and therefore D-NOVs have a smaller chance to infect cells. In that case the $t_{1/2}$ will occur later. The parameters that describe the composition of the virion mixture at startup are hard to determine but have no influence on $t_{1/2}$ and L. This can be explained by the large amount of virions that will be produced in a continuous culture shortly after startup (figure 9 and 10). This also explains the greater importance of the amounts of the three virion types produced at a correct, and to a lesser extent, defective infection. These amounts control the numbers of virions produced in pseudo-steady state, and with that both L and $t_{1/2}$. A higher number of entry sites per cell will lead to more virions infecting cells. At the pseudo-steady-state level of infection the majority of the infections will consist of correct and abortive infections. The NOV mixture then consists of mainly A-NOV with little I-NOV (figures 9 and 10), and if more virions can infect cells the chance that an I-NOV infects a cell increases. With that, an increase in the fraction of correctly infected cells can be expected.

Table 3.
Sensitivity analysis of the parameters. dL/dP and $dt_{1/2}/dP$ are the effects of varying the parameter value on the pseudo-steady-state infection level (L) and on the time at which the infectionlevel is half the pseudo-steady-state level ($t_{1/2}$), respectively. The first and second sign denote the effect of a respectively smaller and larger parameter (P) value than given under D in table 2, under *ceteris paribus* conditions. +: d/dP is positive, 0: there is hardly any influence, -: d/dP is negative.

Parameter	dL/dP	$dt_{1/2}/dP$
Number of virions produced at a correct infection	++	++
Number of virions produced at a defective infection	00	-0
Cell concentration	++	00
Number of entry sites	++	-0
Number of I-NOVs at startup	00	00
Number of A-NOVs at startup	00	00
Number of D-NOVs at startup	0-	0-
Time lapse between infection and I-NOV/A-NOV release	00	--
Time lapse between infection and D-NOV release	00	++
Fraction of A-NOVs produced at a correct infection	0-	0+
Fraction of D-NOVs produced at a correct infection	+0	+0
Fraction of A-NOVs produced at a defective infection	00	0+
Fraction of D-NOVs produced at a defective infection	00	00

Suggested mode of operation

Figures 9 and 10 showed that a low concentration of D-NOVs is not detrimental to the infection process. This observation provides a key to a promising strategy for reactor operation, especially for semi-continuous cultures (Klöppinger et al. 1990). Figure 12 depicts a simulation of a repeated batch. Cells are grown in a fed-batch-operated growth vessel, and the infection reactor is operated batchwise. After each cycle, the volume of the infection reactor was lowered to 3%. The virions remaining in this volume will start the infection of new cells that are then pumped into the infection vessel from the cell growth vessel.

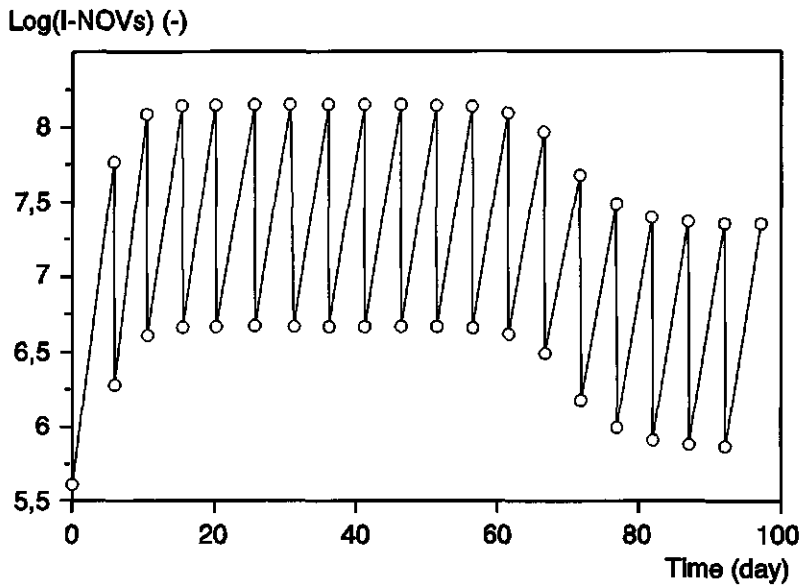

Figure 12.
Simulation of a repeated batch infection process. The concentration of I-NOV versus time. Parameters as in table 2 under E.

From this figure it is clear that production of I-NOVs in this system is maintained significantly longer than in a continuous culture. This can

easily be explained by the repeated dilution of the D-NOVs. With that, a very low multiplicity of infection will occur, and from that, the chance that cells are infected with both D-NOV and I-NOV will be very low. Hence, I-NOV will be produced before D-NOVs are available in significant quantities, and the infection process will continue for a longer period of time.

In a repeated fed-batch mode of operation of the infection vessel, however, insect cells are continuously grown and pumped into the infection vessel in a fed-batch mode until the reactor is completely filled. Thereafter the medium volume of the infection reactor is decreased to 3%. The remaining cells and virions start the infection process again in the next run. In a repeated fed batch the repeated dilution will be less significant, as more passages occur during the run. The run time must therefore approach a fully continuous culture, which indeed can be simulated by the model. These alternative modes of operation of reactor configurations are now experimentally tested to validate the model.

5. Conclusions

In the case of a continuously-operated reactor configuration, an increased number of infection reactors with the same total residence time resulted in an increased pseudo-steady-state level of infection, but a decreased run time. The observed decrease in infection level in continuously-operated series of reactors may be attributed to the passage effect. With the concept of the defective interfering virion and the non-infectious non-occluded virion, experimental data from the bioreactors can be described well with the proposed dynamic model, thereby giving an accurate description of the passage effect. Our current research is focused on the determination of the actual numbers of the three types of NOVs occurring in the continuously-operated reactor series, in order to validate the model presented here. Furthermore, an experimental confirmation of the

suggested repeated batch operation of the infection vessel in the reactor series is described in chapter 6.

6. Acknowledgements

The authors thank Nicole Grobben and Hans van den Hombergh for their experimental contributions. Rik Beeftink and Marcel Zwietering are gratefully acknowledged for their thorough reading of the manuscript.

7. Nomenclature

a	production constant formed by the number of virions produced per cell, multiplied by the number of cells flowing into the reactor per unit of time	(h^{-1})
B	number of entry sites on a cell	
C	number of non-infected insect cells	
D	dilution rate	(h^{-1})
f	fraction of virions	
F	fraction of cells with a certain infection type	
G	number of cells that are infected	
I	rate of virion use for infection purposes	(h^{-1})
k_g	first-order cell growth constant	(h^{-1})
P	propability that an entry site is infected	
p_{max}	maximum number of passages	
R	rate of virion release by the cells	(h^{-1})
$r_{x,\,in}$	ingoing flow of x	(h^{-1})
$r_{x,\,out}$	outgoing flow of x	(h^{-1})
$r_{x,\,prod}$	production rate of x	(h^{-1})
$r_{x,\,use}$	consumption rate of x	(h^{-1})
t	time	(h)

$t_{1/2}$ time at which the infection level in a continuous culture is half the pseudo-steady-state level (h)
v amount of virions
x number of cells or virions
Δt time step (h)
α number of entry sites per cell that are not occupied by any virion
β number of entry sites per cell that is occupied by A-NOVs
λ number of entry sites per cell that is occupied by D-NOVs
κ number of entry sites per cell that is occupied by I-NOVs

Subscripts

d defective interfering particle (D-NOV)
i infectious non-occluded virus (I-NOV)
m reactor number in the series
a NOV leading to an abortive infection (A-NOV)
o not infected
p passage number
tot total

8. References

Faulkner, P. (1981) Baculoviruses. In: Pathogenesis of invertebrate microbial diseases. (Davidson, E.A. Ed.)., Osmun & Co., Allanheld, New Jersey, USA, 3-37.

De Gooijer, C.D., Van Lier, F.L.J., Van den End, E.J., Vlak, J.M. and Tramper, J. (1989) A model for baculovirus production with continuous insect cell cultures. Appl. Microb. Biotechnol. **30,** 497-501.

Hink, W.F. (1970) Established cell line from the cabbage looper, *Trichoplusia ni.* Nature **226,** 466-467.

Klöppinger, M., Fertig, G., Fraune, E. and Miltenburger, H.G. (1990) Multistage production of *Autographa californica* nuclear polyhedrosis virus in insect cell cultures. Cytotechnology **4,** 271-278.

Kompier, R., Tramper, J. and Vlak, J.M. (1988) A continuous process for the production of baculovirus using insect-cell cultures. Biotechnol. Lett. **10,** 849-854.

Kool, M., Voncken, J.W., Van Lier, F.L.J., Tramper, J. and Vlak, J.M. (1991) Detection and analysis of a mutant with defective interfering properties during the continuous production of *Autographa californica* nuclear polyhedrosis virus in bioreactors. Virology **183,** 739-746.

Kool, M., Van Lier, F.L.J., Vlak, J.M. and Tramper, J. (1990) Production of (recombinant) baculoviruses in a two stage bioreactor system using insect-cell cultures. In: Agricultural Biotechnology in focus in the Netherlands. (Dekkers, J.J, Van der Plas, H.C. and Vuijk, D.H. Eds.)., Pudoc, Wageningen, The Netherlands, 64-69.

Levenspiel, O. (1972) Chemical reaction engineering. J. Wiley & Sons, New York, USA, 253-296.

Van Lier, F.L.J., Van den End, E.J., De Gooijer, C.D., Vlak, J.M. and Tramper, J. (1990) Continuous production of baculovirus in a cascade of insect-cell reactors. Appl. Microb. Biotechnol. **33,** 43-47.

Van Lier, F.L.J., Van der Meijs, W.C.J., Grobben, N.G., Olie, R.A., Vlak, J.M. and Tramper, J. (1992) Continuous ß-galactosidase production with a recombinant baculovirus insect-cell system in bioreactors. J. of Biotechnol. **22,** 291-298.

Luckow, V.A. and Summers, M.D. (1988) Trends in the development of baculovirus expression vectors. Bio/Technol. **6,** 47-55.

Von Magnus, P. (1959) Incomplete forms of influenza virus. Adv. Virus Res. **2,** 59-79.

Seth, P., Fitzgerald, D., Willingham, M. and Pastan, I. (1986) Pathway of adenovirus entry into cells. In: Virus attachment and entry into cells. Proceedings of an ASM conference held in Philadelphia, Pennsylvania, USA, April 1985. (Crowell, R.L. and Lonberg-Holm, K. Eds.)., 191-195.

Smith, G.E. and Summers, M.D. (1978) Analysis of baculovirus genomes with restriction endonucleases. Virology **89,** 517-527.

Tramper, J. and Vlak, J.M. (1988) Some engineering and economic aspects of continuous cultivation of insect cells for the production of baculoviruses. Ann. N.Y. Acad. Sci. **469,** 279-288

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J. and McCawley, P. (1977) The establishment of two cell lines of the insect *Spodoptera frugiperda* (Lepidoptera:Noctuidae). In Vitro **13,** 213-217.

Vlak, J.M. (1979) The proteins of nonoccluded *Autographa californica* nuclear polyhedrosis virus produced in an established cell line of Spodoptera frugiperda. J. Invertebr. Pathol. **34,** 110-118.

Vlak, J.M. and Keus, R.J.A. (1990) The baculovirus expression vector system for production of viral vaccines. In: Advances in Biotechnological Processes. (Mizrahi, A. Ed.)., Alan R. Liss Inc., New York, USA, **14,** 91-128.

Volkman, L.E., Summers, M.D. and Hsieh, C-H. (1976) Occluded and nonoccluded nuclear polyhedrosis virus grown in *Trichoplusia ni*: comparative neutralization, comparative infectivity, and in vitro growth studies. J. Virol. **19,** 820-832.

Wickham, T.J., Granados, R.R., Wood, H.A., Hammer, D.A. and Shuler, M.L. (1990) General analysis of receptor-mediated viral attachment to cell surfaces. Biophys. J. **58,** 1501-1516.

Wickham, T.J., Davis, T., Granados, R.R., Hammer, D.A., Shuler, M.L. and Wood, H.A. (1991) Baculovirus defective interfering particles are responsible for variations in recombinant protein production as a function of multiplicity of infection. Biotechnol. Lett. **7**, 483-488.

Chapter 6 has been accepted for publication as: van Lier, F.L.J., van den Hombergh, J.P.T.W., de Gooijer, C.D., den Boer, M.M., Vlak, J.M. and Tramper, J. (1995) Long-term semi-continuous production of recombinant baculovirus protein in a repeated (fed-)batch two-stage reactor system. Enzyme Microb. Technol. In press.

6

Long-term semi-continuous production of recombinant baculovirus protein in a repeated (fed-)batch two-stage reactor system

Summary

The baculovirus expression system is commonly used in the research and development area and in the production of diagnostics and vaccines. Because the infection of insect-cell cultures with a (recombinant) baculovirus is a lytic process the running time of an infected batch insect-cell reactor is limited. Another disadvantage of the system is the instability of the virus. In this study a two-stage reactor system was tested for its suitability for long-term semi-continuous operation. Three experimental set-ups were tested involving repeated infections in a reactor fed with cell suspension from a separate cell-growth reactor. As virus inoculum part of a previous infection was used. Best performance with respect to long-term operation was obtained with a repeated batch system. Twelve consecutive productive runs, consisting of infections during five days, could be performed. The titers of infectious extra-cellular virus could be described well with an infection model previously developed in our laboratory.

1. Introduction

The baculovirus expression vector system has established itself as a powerful tool in the expression of foreign genes. The system is mainly used in research and development and for production of diagnostics and vaccines (Vlak 1990). The first steps towards clinical application have already been taken (Glaser 1993). A major disadvantage of the system, however, is the lytic character of the infection process which limits the production time of a batch process.

A continuous production process in a two-stage reactor system is possible by exploiting the typical bi-phasic infection cycle of the virus. During the first phase infected cells produce non-occluded viruses (NOVs) which are released into the medium and which are capable of infecting other insect cells. When infected with wild-type virus, the infected cells will produce virus particles packed in polyhedra during the second phase of the infection process. In a baculovirus expression vector usually the gene coding for the major late protein (polyhedrin) is replaced by a gene of choice and hence the infected cell will produce the desired protein during the second phase of virus infection (O' Reilly et al. 1992, King and Possee 1992). An alternative site for insertion is the locus of the p10 gene which has, like the polyhedrin gene, a strong promoter and which is also highly expressed during the second phase of infection (Vlak et al. 1990).

For a continuous production process insect-cell culturing and infection of cells have to be physically separated. This can be achieved by using a reactor system consisting of two continuously operated reactors in series. In the first reactor the insect cells are cultured and the effluent is used as influent for the second reactor where the cells are infected. In such a reactor system wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) (Kompier et al. 1988) and recombinant ß-galactosidase (van Lier et al. 1992) have been successfully produced. In both studies, however, after four weeks of more or less stable production the productivity rapidly decreased. This decrease in production has been ascribed to interference of

defective virus particles with intact virus (Kool et al. 1991).

To make the continuous system suitable for large-scale production the level of defective NOVs (D-NOVs) should be held low. This implies that a low multiplicity of infection (m.o.i.; number of infectious viruses available per cell) should be maintained in the infection vessel. This can not be achieved in a continuous reactor system since NOV concentration gradually builds up (about 200 NOVs are produced per cell). A batch infection has the disadvantage of long down times as a result of cleaning and sterilization of the reactor after each run. With a semi-continuous system based on repeated (fed-)batch infections the m.o.i. can be better controlled. By using a two-reactor setup the down time can be minimized (van Lier et al. 1991, de Gooijer et al. 1992, Zhang et al. 1993).

Hink and Strauss (Hink and Strauss 1980, Hink 1982) described a production system where *Trichoplusia ni* Tn368 cells were cultured in a stirred reactor. AcNPV was propagated in a series of four spinner flasks. Every 24 hours the content from each infection vessel was pumped to the next infection vessel and the first infection vessel was filled from the cell-growth reactor and inoculated with virus. Production of polyhedra stayed constant during four runs and then declined. This decline was probably due to lack of nutrients or accumulation of waste metabolites since medium was re-used. Klöppinger et al. (1990) reported on semi-continuous production of polyhedra in a reactor system consisting of two bioreactors. Part of the cultured cells from the first reactor was pumped into a second reactor for infection, the remaining cells were used as inoculum for a new fed-batch culture of cells. When the virus propagation reactor was harvested, part of the contents stayed in the reactor to infect the next cell batch. Klöppinger et al. (1990) observed "a constant production for several weeks". In a third study on repeated batch production, *Bombyx mori* Bm5 cells were infected with a recombinant BmNPV expressing bacterial chloramphenicol acetyltransferase (Zhang et al. 1993). In that study, four consecutive runs were performed and production of the two-stage bioreactor system was constant for 27 days.

Especially for clinical applications it is important to get insight in the stability of the baculovirus expression vector. In our laboratory a model was developed describing the infection process in insect-cell reactor configurations (de Gooijer et al. 1992). It is assumed that each insect cell has a limited number of so-called "entry sites" where virus can enter the cell. Furthermore, three types of viruses are taken into account: infectious virus (I-NOV), defective virus (D-NOVS) and abortive virus (A-NOV). Most virus belongs to this last group that uses an entry-site but is incapable of a permissive infection. With this model continuous reactor runs could be well described. This model can also be used to describe a series of repeated batch runs. De Gooijer et al. (1992) did a model simulation for a system similar to the one described by Klöppinger et al. (1990) and Zhang et al. (1993). The model predicted that after about 12 consecutive batch runs the I-NOV concentration would decrease as a result of defective interference of D-NOVs.

In the present study it was investigated how many consecutive batch infections could be done before the production declined as a result of defective virus build up. Beside the repeated batch infection experiment, two other experiments were performed where infection was conducted in a fed-batch mode. The experimental data were used to evaluate the model of de Gooijer et al. (1992) in predicting the productivity decline.

2. Materials and methods

Cells, virus, medium

The IPLB-Sf-21 cell line isolated from *Spodoptera frugiperda* by Vaughn et al. (1977) was used. The cells were grown in TNM-FH medium (Hink 1970) supplemented with 10% fetal calf serum. Cells were maintained in 25 cm^2 tissue culture flasks (Greiner). For growth in suspension methyl cellulose with a final concentration of 0.1% (w/v) was added to the medium. The recombinant baculovirus was an *Autographa*

californica multiple-capsid nuclear polyhedrosis virus (AcMNPV) containing the lacZ gene of *Escherichia coli* instead of the polyhedrin gene (Summers and Smith 1987).

Bioreactor configuration

The bioreactor system consisted of two 1 dm^3 round-bottomed fermentors or two 2 dm^3 flat-bottomed fermentors (Applikon) in series. The reactors were equipped with marine impellers and maintained at 28°C with an internal heating system connected to a waterbath with forced cooling. Air was introduced with a flow of 10 dm^3/h in the head space of the reactors through a 0.2 μm filter. Liquid was transported via silicone tubing by peristaltic pumps (Watson-Marlow). Infection of the cell-growth reactor by virus from the infection reactor was prevented with a drop former.

The first experiment consisted of a series of batch infections. The reactors were operated as shown in figure 1a. The batch time was five days. To inoculate a batch of cells, 20 cm^3 suspension of the previous run was left in the reactor.

In the second experiment (figure 1b) the infection reactor was filled with 0.3 dm^3 cell suspension. During two days cell suspension was added continuously to the infection reactor. Additionally, upon reaching the final working volume the reactor was operated batch-wise during 1 day. To inoculate a new infection cycle 20 cm^3 of suspension was left in the reactor. The one day batch period served two purposes: it prolonged the residence time of cells in the infection reactor and it allowed for a cell suspension build-up in the cell-growth reactor needed for starting the next infection.

The third experiment was performed analogous to the second experiment except that here a three day fed-batch period was used and that the reactor volumes differed (figure 1c).

During all operation schemes the cell-growth reactor was fed continuously with medium. During the first experiment the effluent of the cell-growth reactor was bleeded when the working volume was reached. The operation of the bioreactor systems was such that the residence time of

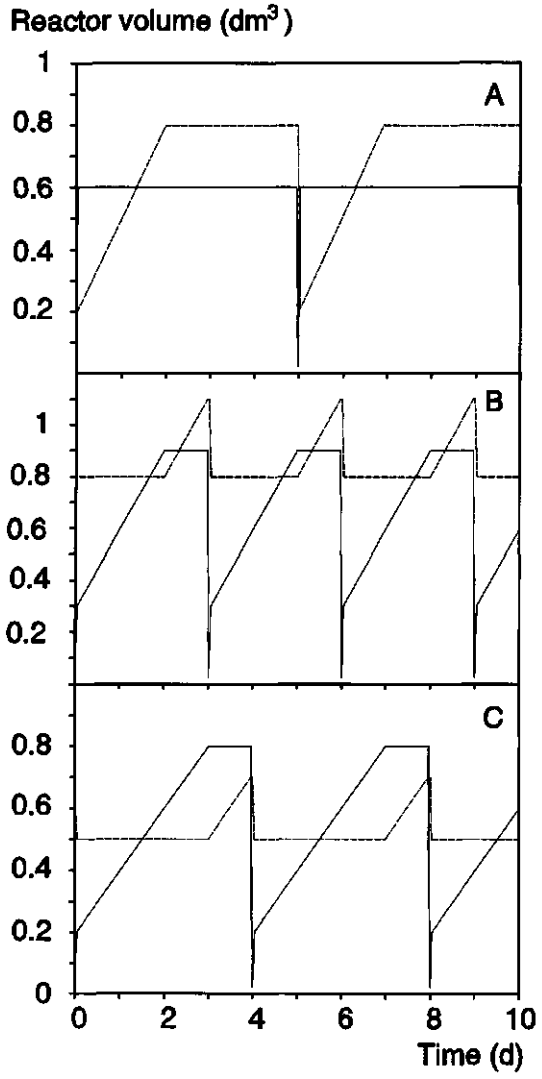

Figure 1.
Reactor volumes during the infection processes. Solid lines: infection reactor, broken lines: cell-growth reactor. A: 5-day batch infections, B: 2 day fed-batch / 1 day batch infections, C: 3 day fed-batch / 1 day batch infections.

the cells in the cell-growth reactors were similar. Hence physiological differences of the cells before infection between the three experiments were eliminated as much as possible.

Bioreactor samples

Samples from the bioreactor were forced into the sample flasks by pressurizing the reactor. The sample system was cleaned by blowing sterile air through the system. Samples were taken daily. The sample volumes were about 1 cm^3 for the cell-growth reactor and about 5 cm^3 for the infection reactor. In both samples the cell number was determined.

Four cm^3 of the samples from the infection reactor were centrifuged (1500 x g for 5 min). The cell pellet was resuspended in 4 cm^3 phosphate buffered saline (PBS) and centrifuged again. The pellet was finally resuspended in 1 cm^3 PBS. Both the cell suspension and the supernatant from the washing step were stored at -20°C. The supernatant from the first centrifuge step was filtered through a 0.45 μm filter and split into two aliquots of which one was stored at 4°C for virus titer determination and the other at -20°C for a ß-galactosidase assay.

Cell counts

Cells were counted in a bright-line Neubauer haemocytometer. To distinguish between viable and non-viable cells the trypan blue exclusion method was used.

ß-galactosidase assay

The cell pellet stored at -20°C was lysed by sonification (three cycles of 30 s with 30 s intervals). ß-galactosidase activity was determined in the lysed pellet and both supernatant fractions stored at -20°C.

The activity of ß-galactosidase was measured by following the hydrolysis of 2-nitrophenyl-galactopyranoside (ONPG) spectrophotometrically at 420 nm. The assay was executed at 28°C. The activity of ß-galactosidase was expressed as μmol ONPG cleaved per minute. An

extinction coefficient of $4.5x10^3$ $M^{-1}.m^{-1}$ was used.

Virus titer

The titer of infectious virus was determined in the supernatant fraction stored at 4°C using an end point dilution method as described by Vlak (1979). To screen for wells containing infected cells 10 mm^3 of a 0.4 $g.dm^{-3}$ solution of 5-bromo-4-chloro-3-indolyl-ß-galactopyranoside (X-gal) was added to each well.

3. Results and discussion

Viable-cell concentration in both the cell-growth and infection reactor at the start of each infection run was relatively constant during the repeated batch infection experiment (figure 2a). Since the residence time of the cells was similar in all three reactor configurations, similar cell densities could be expected at the beginning of each infection. However, cell densities differed from about $0.8x10^6$ cells/cm^3 (batch system) to about $1.6x10^6$ cells/cm^3 (3 day fed-batch).

During each infection the viable-cell concentration in the infection reactor decreased. Viable-cell concentrations during the two-day repeated fed-batch experiment showed a similar behaviour (figure 2b). During the three-day repeated fed-batch experiment (figure 1c), however, viable-cell density at the start of each infection run was not constant and the viable-cell density in the infection reactor did not decrease as distinctly as in the other two experiments (figure 2c).

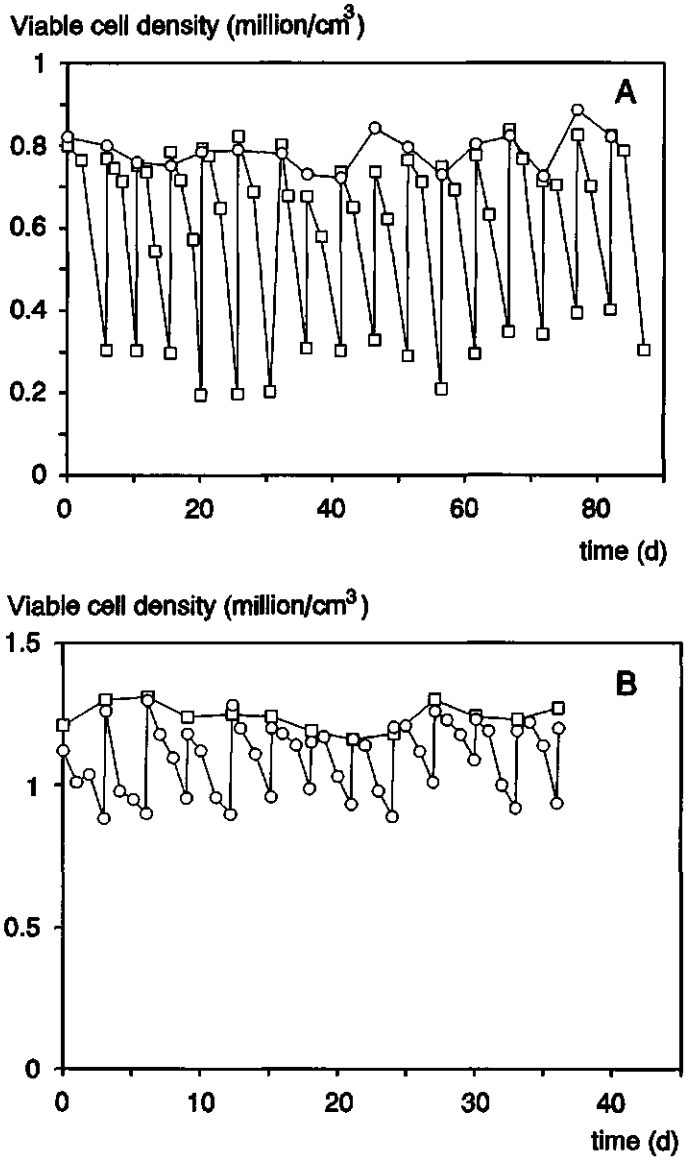

Figure 2 A, B.
Viable cell density. ○: cell-growth reactor, □: infection reactor. A: 5-day batch infections, B: 2 day fed-batch / 1 day batch infections.

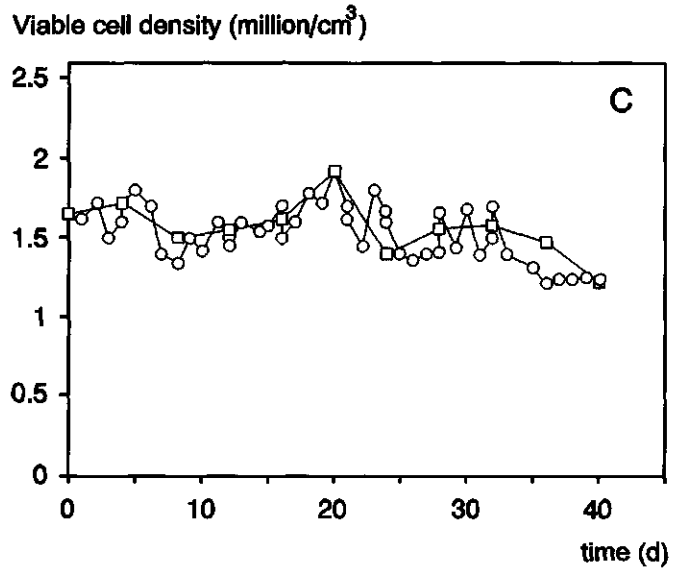

Figure 2 C.
Viable cell density 3 day fed-batch / 1 day batch infections. ○: cell-growth reactor, □: infection reactor.

During twelve consecutive infections in the infection reactor of the batch operated system (figure 1a) ß-galactosidase production was reproducible and averaged 22 U/cm^3 (figure 3a). After the twelfth run production of ß-galactosidase decreased and stayed at about 3 U/cm^3. A total of 21 consecutive runs was performed (not all data shown). Production of ß-galactosidase during the two-day fed-batch operated infection experiment (figure 1b) declined after the fifth consecutive infection (figure 3b). During the first five infections the ß-galactosidase production was about 14 U/cm^3, comparable to the ß-galactosidase activity in the batch operated system three days after start of infection. ß-galactosidase activity in the infection reactor operated with the three-day fed-batch regime (figure 1c) was considerably higher than in both other experiments. Here,

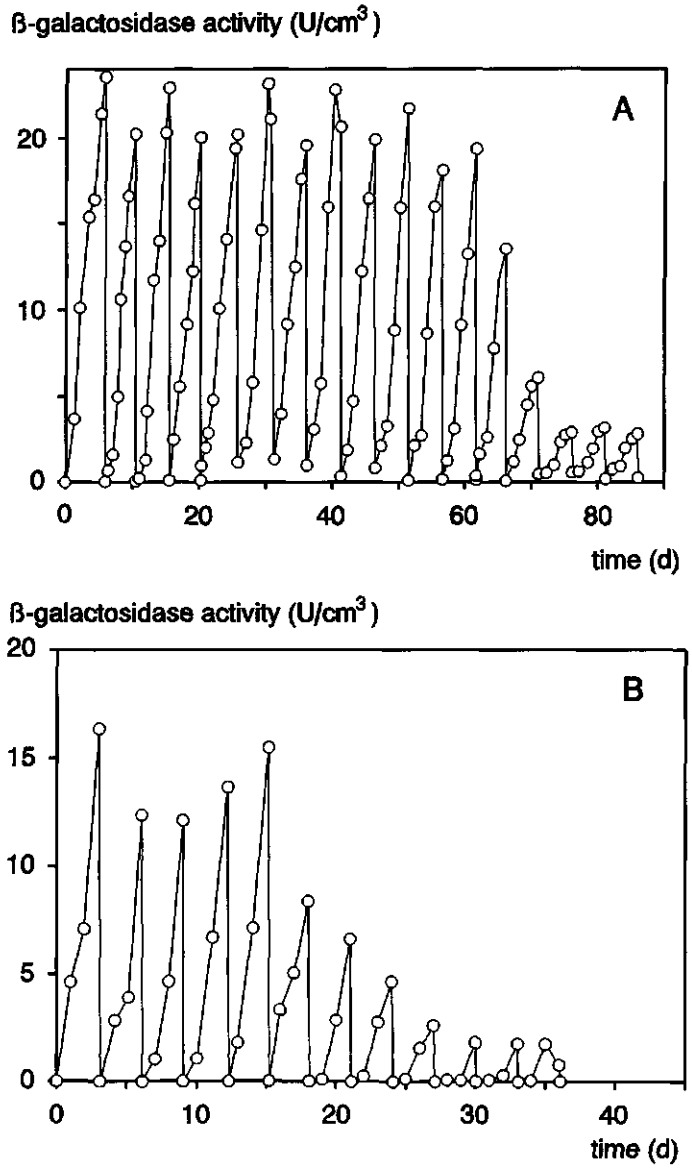

Figure 3 A, B.
ß-galactosidase activity. A: 5-day batch infections, B: 2 day fed-batch / 1 day batch infections.

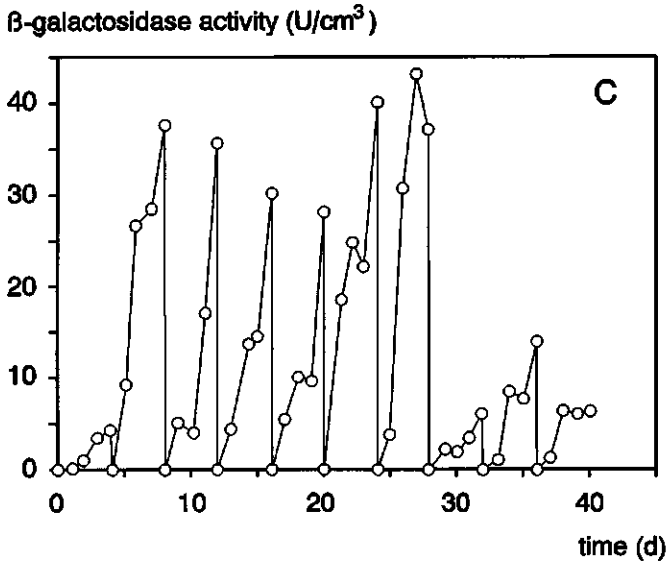

Figure 3 C.
ß-galactosidase activity 3 day fed-batch / 1 day batch infections.

production was on average 35 U/cm^3 during the first seven runs with exception of the first infection (figure 3c).

The production time of the repeated batch configuration is by far the longest. This can be expected because batch operation diminishes the build-up of D-NOVs compared to fed-batch operated systems. In a fed-batch mode of infection more virus passages can occur due to the continuous supply of non-infected cells. The fed-batch infections, however, offer a tool for model evaluation. It should be noted that long-term operation is not per se an optimization criterion. Practical reasons (maintenance, working hours) or regulatory affairs may limit the running time of a cultivation.

The lacZ gene of *E. coli* is often used as a model gene for measuring baculovirus-mediated recombinant-protein expression. Caution must be

taken, however, when data obtained with lacZ-AcMNPV vectors are compared. Differences in levels of ß-galactosidase expression can occur as a result of differences in cell density (resulting in different metabolic state of cell suspensions), differences in baculovirus vectors and differences in cell lines used (Wickham et al. 1992). Also differences in m.o.i. can influence ß-galactosidase production (Licari and Bailey 1991, Neutra et al. 1992). Furthermore, the experimental determination of ß-galactosidase activity (differences in temperature, extinction coefficient or pH) may introduce (seeming) discrepancies when different studies are compared.

Nevertheless, in table 1 production data obtained with the reactor configurations described in this paper are compared with data from the literature. These literature data are comparable with respect to the ß-galactosidase activity assay conditions. In all cases the same extinction coefficient ($4.5x10^3$ $M^{-1}.m^{-1}$) was used. The productivity of the repeated batch system is low as compared to the literature data on single batches (data are all from day 5 post infection). However, using the same virus, cells, medium and culture conditions values of about 20 $U/10^6$ cells were found in our laboratory in batch cultures (van Lier et al. 1992). Furthermore, since the bioreactors were only aerated through the head space, oxygen limitation could also be responsible for lower yield. The productivities of the continuous system and the reactor configurations described in this study are in the same order of magnitude. The three-day fed-batch reactor system shows a somewhat higher productivity. This suggests that a fed-batch operated system can give rise to a higher productivity. This is, however, unlikely since the average residence time of the cells in the infection reactor is less than in the batch operated infection reactor. The higher productivity can be attributed to the higher cell density.

Table 1.
Comparison of several reactor systems used to produce baculovirus-expressed ß-galactosidase. STR: stirred tank reactor, AR: airlift reactor. Literature: [1] van Lier et al. 1992, [2] Neutra et al. 1992, [3] Murhammer and Goochee 1988, [4] King et al. 1992.

System	ß-gal. activity in harvest (U/cm^3)	Volume of harvest per day (cm^3/d)	Productivity ($U/cm^3.d$)	Total running time (d)
Continuous (STR)[1]	10	320	4	25
Batch (shake flask)[2]	200	8	40	5
Batch (AR)[3]	50	114	10	5
Batch (AR)[4]	64	1560	13	5
Repeated batch	22	120	4.4	60
Repeated fed-batch/batch (2/1 d)	14	300	4.7	15
Repeated fed-batch/batch (3/1 d)	35	200	8.8	28

In all three experiments performed, the decrease in ß-galactosidase production in the infection reactor coincided with a decrease in titer of infectious virus (figure 4). This decrease is due to interference of defective rec-AcMNPV mutants which lack the ability to produce ß-galactosidase (Kool et al. 1991) or compete for replication factors (Kool et al. 1993).

The kinetics of the mechanism of interference by defective AcMNPV was modeled by de Gooijer et al. (1992) for continuous systems. This

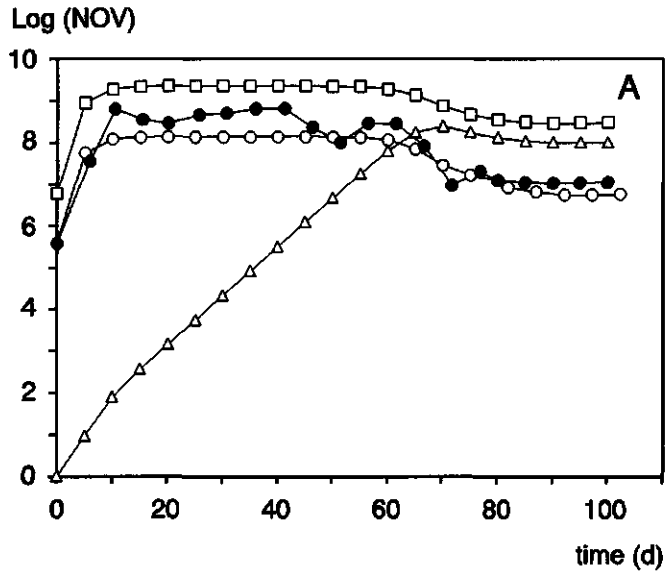

Figure 4 A.
NOV titers 5-day batch infections. ●: measured I-NOV titer, ○: computed I-NOV titer, □: computed A-NOV titer, △: computed D-NOV titer.

model is capable of predicting the titer of NOVs and distinguishes between infectious NOVs (I-NOVs), defective NOVs (D-NOVs) and abortive NOVs (A-NOVs). For the repeated batch experiment as described in this study de Gooijer et al. (1992) calculated the virus titer profiles as shown in figure 4a. The virus titer profiles of both other experimental systems used in this study were calculated with the same system-independent parameters as used for the batch operated system (table 2).

Table 2.
Input parameters for the baculovirus infection model (de Gooijer et al. 1992).

Variable	Value	Unit
Number of D-NOVs at startup	0	-
Number of cell entry sites	33	$cell^{-1}$
Number of virions produced at a correct infection	3340	$cell^{-1}$
of which A-NOVs	94	%
of which D-NOVs	10^{-6}	%
Number of virions produced at a defective infection	1600	$cell^{-1}$
of which A-NOVs	71	%
of which D-NOVs	25.5	%

The calculated NOV titers are given in figure 4b and c. In case of the two-day fed-batch infection the calculated I-NOV declined somewhat later than the measured titer. The model predicts the measured data well with respect to the repeated batch infection and the three-day fed-batch infection experiment. Especially the time when the I-NOV titer dropped is well predicted.

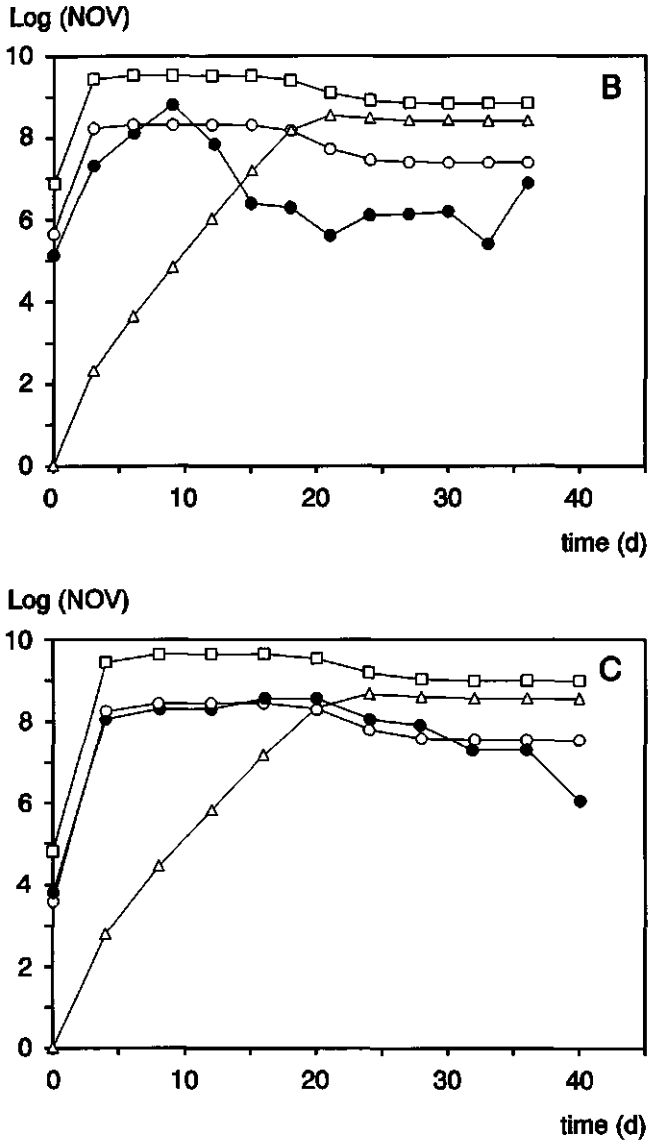

Figure 4 B, C.
NOV titers. ●: measured I-NOV titer, ○: computed I-NOV titer, □: computed A-NOV titer, △: computed D-NOV titer. B: 2 day fed-batch / 1 day batch infections, C: 3 day fed-batch / 1 day batch infections.

4. Conclusions

The use of repeated infections, each starting with an inoculum from the previous infection, increased the running time of a two-stage reactor system as compared to a continuously operated two-stage reactor system. There is, however, a limit to the number of consecutive infections that can be performed without significant loss in productivity. This number depends on the mode of operation of the reactor system. From the three modes of operation tested, a scheme of consecutive 5 day batch infections performed best in terms of long-term operation.

With the model developed to describe the infection process (de Gooijer et al. 1992) the number of consecutive infections that can be performed before defective baculoviruses become predominant in the infection reactor can be predicted well.

5. Acknowledgements

Arie van Oorschot and Rick Koken are greatfully acknowledged for adaptation of the computer model to repeated batch operations. We thank Fred van den End and Magda Usmany for technical assistance and Marcel Kool for helpful discussions. This work was financed in part by the Dutch Programme Committee for Agricultural Biotechnology.

6. References

de Gooijer, C.D., Koken, R.H.M., van Lier, F.L.J., Kool, M., Vlak, J.M. and Tramper, J (1992) A structured dynamic model for the baculovirus infection process in insect cell reactor configurations. Biotechnol. Bioeng. **40**, 537-548.

Glaser, V. Biotechnology industry's interest grows in baculovirus expression systems (1993) Gen. Engin. News **13**, 10-11.

Hink, W.F. (1970) Established cell line from the cabbage looper, *Trichoplusia ni*. Nature **226**, 466-467.

Hink, W.F. (1982) Production of *Autographa californica* nuclear polyhedrosis virus in cells from large-scale suspension cultures. In: Kurstak, E. (ed.) Microbial and viral pesticides. Marcel Dekker Inc., New York, 493-506.

Hink, W.F. and Strauss, E.M. (1980) Semi-continuous culture of the TN-368 cell line in fermentors with virus production in harvested cells. In: Kurstak, E., Maramorosch, K. and Dübendorfer, A. (eds.) Invertebrate systems in vitro. Elsevier/North-Holland Biomedical Press, Amsterdam, 27-33.

King, G.A., Daugulis, A.J., Faulkner, P. and Goosen, M.F.A. (1992) Recombinant ß-galactosidase production in serum-free medium by insect cells in a 14-L airlift bioreactor. Biotechnol. Progr. **8**, 567-571.

King, L.A. and Possee, R.D. (1992) The baculovirus expression system: A laboratory guide. Chapman & Hall. London 229 pp.

Klöppinger, M., Fertig, G., Fraune, E. and Miltenburger, H.G. (1990) Multistage production of Autographa californica nuclear polyhedrosis virus in insect cell cultures. Cytotechnol. **4**, 271-278.

Kompier, R., Tramper, J. and Vlak, J.M. (1988) A continuous process for production of baculovirus using insect-cell cultures. Biotechnol. Lett. **10**, 849-854.

Kool, M., Voncken, J.W., van Lier, F.L.J., Tramper, J. and Vlak, J.M. (1991) Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with defective interfering properties. Virology **183**, 739-746.

Kool, M., van den Berg, P.M.M.M., Tramper, J., Goldbach, R.W. and Vlak, J.M. (1993) Location of two putative origins of DNA replication of *Autographa californica* nuclear polyhedrosis virus. Virology **192**, 94-101.

Licari, P and Bailey, J.E. (1991) Factors influencing recombinant protein yields in an insect cell-baculovirus expression system: multiplicity of infection and intracellular protein degradation. Biotechnol. Bioeng. **37**, 238-246.

Murhammer, D.W. and Goochee, C.F. (1988) Scaleup of insect cell cultures: protective effects of pluronic F-68. Bio/Technol. **6**, 1411-1418.

Neutra, R., Levi, B.-Z. and Shoham, Y. (1992) Optimization of protein-production by the baculovirus expression vector system in shake flasks. Appl. Microbiol. Biotechnol. **37**, 74-78.

O' Reilly, D.R., Miller, L.K. and Luckow, V.E. (1992) Baculovirus expression vectors: A laboratory manual. W.H. Freeman & Co. New York, 347 pp.

Summers, M.D. and Smith, G.E. (1987) A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricult. Exp. Stn. Bull. No. 1555.

van Lier, F.L.J., van der Meijs, W.C.J., Grobben, N.G., Olie, R.A., Vlak, J.M. and Tramper, J. (1992) Continuous ß-galactosidase production with a recombinant baculovirus insect-cell system in bioreactors. J. Biotechnol. **22**, 291-298.

van Lier, F.L.J., de Gooijer C.D., Kool, M., Vlak, J.M. and Tramper, J. (1991) Production of (recombinant) baculovirus in two-stage bioreactor systems. In: Proceedings of the eighth international conference on invertebrate and fish tissue culture held in conjunction with the 1991 world congress on cell and tissue culture, June 16-20 1991, Anaheim, California Fraser, M.J. (ed.) Tissue Culture Association, Columbia, USA, 147-152.

Vlak, J.M. (1979) The proteins of nonoccluded nuclear polyhedrosis virus produced in an established cell line of *Spodoptera frugiperda*. J. Invertebr. Pathol. **34**, 110-118.

Vlak, J.M. (1990) The baculovirus expression vector system for production of viral vaccines. Adv. Biotechnol. Processes **14**, 91-128.

Vlak, J.M., Schouten, A, Usmany, M., Belsham, G.J., Klinge-Roode, E.C., Maule, A.J. van Lent, J.W. and Zuidema, D. (1990) Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. Virology **179**, 312-320.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J. and McCawley, P.W. (1977) The establishment of two cell lines of the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro **13**, 213-217.

Wickham, T.J., Davis, T., Granados, R.R., Shuler, M.L. and Wood, H.A. (1992) Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. Biotechnol. Progr. **8**, 391-396.

Zhang, J., Kalogerakis, N., Behie, L.A. and Iatrou, K. (1993) A two-stage bioreactor system for the production of recombinant protein using a genetically engineered baculovirus/insect cell system. Biotechnol. Bioeng. **42**, 357-366.

7

General discussion

Summary

Among the recombinant-protein expression systems available the baculovirus expression (BEV) system has established itself as a powerful research tool. Large quantities of recombinant protein can be produced in a relatively short time. In this chapter the most commonly used host cells (including insect cells) were compared with respect to several applications. It was assessed in which areas expression of proteins with insect cells can be successfully applied.

1. Introduction

In carpentry it is obvious which tool to use for a job: although it is possible to drive a nail into wood with a spanner, a hammer is here the appropriate tool. For production of recombinant proteins several tools (i.e. expression vector systems) are available, but it is not that obvious when to use which expression vector. This chapter focuses on the baculovirus expression vector (BEV) system. Through comparison of the BEV system with other expression vectors it will be assessed in which applications the BEV system is the appropriate tool.

When an ideal expression vector would exist, it would have the following characteristics:

- The recombinant protein is identical to its natural counterpart.
- The genetically modified cells are easy to culture in low-cost and well defined media.
- The foreign gene is highly expressed.
- The foreign gene is stably integrated in the cell.
- The foreign gene is easily inserted in the vector and the host cell is transformed effectively with the vector.
- The vector and host cell have no toxic or pathogenic properties.
- The genetically modified cell does not survive outside its culture environment.

In reality, however, expression vectors have only a few of the listed characteristics and it is the nature of the recombinant protein and its application which determine what are the most important characteristics.

2. Biological activity of expressed proteins

The first demand in all applications is that the produced protein is biologically active. The nature of the protein sets demands for the capacity of the host cell to process the protein in the proper way. Often the mere translation of DNA into an amino acid sequence is not enough to produce an active protein. Proteolytic cleavage, phosphorylation, amidation and glycosylation are examples of post-translational processes which may have to occur within the host cell. The complexity of post-translational processes a host cell can handle is correlated to its place in evolution. In table 1 four host cells are compared regarding several post-translational modifications. Hot topic regarding post-translational modification is glycosylation. Many proteins from mammalian origin depend on glycosylation to function properly. This glycosylation is often complex and although yeast cells are capable of glycosylation their capability is limited. It is particulary on the subject of glycosylation where there is a discussion about the use of insect cells as opposed to mammalian cells. Insect cells seem to be more limited in their ability to glycosilate proteins. The oligosaccharide side chains of proteins produced by insect cells are often smaller compared to the same protein produced in mammalian cells and are usually less complex (Kuroda et al. 1990). In most cases this does not affect biological activity. However, when the protein is intended for use as a drug, complete similarity to a natural occurring protein makes registration easier.

Table 1.
Comparison of capability of post-translational modifications for four host cells.

Processing	- Host cell -			
	E. coli	Yeast	Insect cells	Mammalian cells
proteolytic cleavage	±	±	+	+
glycosylation	-	±	+	+
folding	±	+	+	+
phosphorylation	-	+	+	+
acylation	-	+	+	+
amidation	-	-	+	+

3. Culture of host cells

Although bacteria are inferior with respect to post-translational modification, bacteria are easy to culture, product is often obtained in high yields and media are relatively simple. As a result of this, bacteria are the obvious host cells for production of relative simple proteins (i.e. proteins requiring non or limited post-translational processing). Since production with bacteria is relatively cheap it can even be economically to use bacteria for more complicated proteins and perform post-translational modifications after fermentation. In *Escherichia coli* fermentation for example proteins are often produced in denatured form in so-called inclusion bodies. After fermentation these inclusion bodies are recovered and solubilized. When diluted in a suitable buffer the protein refolds to its natural form.

The production of proteins with prokaryotic host cells is a still evolving area. The boundaries are still moving.

When the protein is too complex to be produced in *E. coli*,

mammalian cells (and in particular chinese hamster ovary (CHO) cells) are usually the alternative. Many of the initial draw-backs in mammalian cell cultivation have been solved during the last decade. Media optimalisation has resulted in better defined and less costly media. Especially the omission of serum from media has been a great step forward. The sensitivity of animal cells to the hydrodynamical forces occurring in bioreactors has been characterized and the effects on the cells can be limited by adequate design of the bioreactor and by adding protective agents like Pluronic® F68.

Compared to mammalian cells insect cells are easier to culture. Insect cells are less sensitive to physical and nutritional factors. The cells can grow on surfaces as well as in suspension. Detachment from a surface can be achieved by simple mechanical methods. Media development is somewhat behind the development of mammalian cell culture media. Therefore insect cell media are still relative expensive. Insect cells can easily be grown without addition of serum. Based on culture characteristics, insect cells offer an attractive alternative to mammalian cells.

4. Expression

Initially expression levels in bacteria were far superior to expression in animal cells. Within the animal-cell group, insect cells were said to have high expression levels, approaching those for bacteria. Expression levels up to 500 mg/dm^3 were claimed (Luckow and Summer 1988). However it turned out to be difficult to obtain those high levels. Expression levels vary widely. Moreover the expression levels of mammalian cells have been increased, due to improvement of expression systems. The initial advantage of high expression levels insect-cell culture possessed as opposed to mammalian-cell culture thus seems to be lost.

The major advantage of insect-cell expression compared to mammalian-cell culture is the ease with which the cells can be genetically modified. If high expression is obtained it is present from the beginning

whereas mammalian cells have to be treated (e.g. with methotrexate) for some time before the maximal expression level is obtained. The ease of use of the baculovirus expression vector has been increased by the introduction of several commercial expression kits.

5. Safety

Two major risks have to be considered when genetically modified organisms (GMO's) are used. The GMO can be hazardous to man thus being a potential risk for workers or, when it escapes from the production facility, to people in the environment. This risk is of course not restricted to GMO's but is also present when culturing natural pathogenic organisms. Another concern is the escape into the environment of a GMO that can compete with its non-modified counterpart. In the worst case the ecological system is unbalanced.

By its nature the baculovirus vector is a safe vector. Baculoviruses are very specific and only infect their hosts and sometimes a few related insects. When the virus is used for recombination a gene essential for *in vivo* replication is replaced by the inserted gene. Therefore the baculovirus will not be capable of surviving in the environment for long.

Another safety aspect of cell culture in general is the possible presence of adventitious agents in the culture. Since insect cells require culture conditions differing from mammalian cell culture (see table 2) propagation of micro-organisms hazardous for man is generally not supported. Furthermore most vertebrate viruses are incapable of replication in insect cells and the other way round insect viruses are specific for insects. As always there are exceptions: viruses that replicate both in insect and mammalian cells such as the arboviruses. Most of the viruses in this group, however, replicate only in dipteran cells. Since insect cells supporting baculovirus replication stem from lepidopteran insects the problem is diminished to the vesicular stomatitus virus (VSV) which has a

very wide host range.

Table 2.
Comparison of culture conditions

	insect cells	mammalian cells
pH	6.3	6.9-7.2
temperature	27°C	37°C
medium osmolarity	320 mOsm	220 mOsm

6. Application of the baculovirus expression vector

Generally speaking the field of protein production is divided between bacterial and mammalian cells. In the following the production of bulk products such as enzymes and antibiotics is omitted since this is the territory of the microbial expression systems. As already mentioned bacterial fermentation plays also a significant role in the production of specialized proteins used for diagnostic and therapeutic applications. The higher downstream and handling costs of these systems are compensated by the lower upstream costs (Bisbee 1994). The two major players on the stage of therapeutic protein production are *E. coli* and CHO cells. A considerable background of information is available on these systems, which makes it easier when a process has to be validated for acceptance by health authorities.

The baculovirus expression vector system is a tool that can produce fast and in a considerable amount complicated proteins. Therefore it is an excellent tool to use in research. The system can also be used in production processes, which is an obvious route to take when a high producing BEV

is available. The BEV system has however two disadvantages which were addressed in our laboratory. The BEV causes lysis of the insect cells thus making a continuous process difficult. Furthermore the baculovirus is not stable upon serial passage.

In 1988 results of a continuous insect-cell process on a lab scale were presented (Kompier et al. 1988). The authors used a two-stage production process where cell cultivation and virus propagation were separated in two reactors. The virus propagation reactor was continuously fed with cells coming from the cell-culture reactor. The incoming cells were infected by extracelluar virus produced by the already infected cells. The efficiency of cell usage was low in this system, but production could be maintained for one month.

In chapter 2 of the thesis results are presented of a similar system where the efficiency of cell usage is improved by changing the residence-time distribution of the virus propagation reactor. This reactor configuration produced encapsulated virus (polyhedra) for only two weeks.

To gain more insight in the phenomenon of declining polyhedra production, a recombinant baculovirus was used in the reactor system as described by Kompier et al. (1988). The recombinant baculovirus, in which the polyhedrin gene was replaced by the lacZ gene from *E. coli*, allowed the study of polyhedrin promoter-driven expression alone, independent of other factors affecting polyhedra formation. This is described in chapter 3. This study showed that the decrease in production was a result of reduced polyhedrin promoter-driven synthesis. Further work of Kool et al. (1991) on samples of the bioreactor revealed that reduction of production is due to the occurrence of defective mutants of the baculovirus. These defective viruses lack the polyhedrin promoter-driven gene and the ability to replicate without the presence of intact helper virus. When a helper virus is present in an infected cell, the defective virus replicates faster than the intact virus thus interfering with infectious virus replication.

There is another gene on the baculovirus genome which is suitable as site for insertion of a foreign gene. This gene, denoted p10, is, like the

polyhedrin gene, not necessary for *in vitro* replication and is highly expressed. In contrast to the polyhedrin gene it is preserved in the defective virus. To investigate if the p10 promoter is still active on the defective virus a recombinant baculovirus with the lacZ gene from *E. coli* under control of the p10 promoter was used in the continuous two-stage reactor system. This is described in chapter 4. Both polyhedron production and ß-galactosidase production decreased simultaneously after about four weeks of running the bioreactor process, indicating that the defective virus particles miss one or more genes essential for p10 promoter-driven synthesis.

The kinetics of baculovirus infection was subject of the development of a model described in chapter 5. This mathematical model is capable of describing the preceding experiments well. It was also used to model an experiment where repeated batch infections were performed. The simulated repeated batch process consisted of a reactor which was filled with cells from a second reactor, the cell-culture reactor. To inoculate the production reactor with virus only during the first run fresh virus was used. In the subsequent runs part of the infected-cell suspension was left behind to serve as inoculum. During infection the cell-culture reactor was fed with medium to generate cells for a next infection run.

With this set-up it was calculated that the decrease in productivity was postponed as a result of defective virus build up. By keeping the virus concentration low the chance that a cell is infected with both a defective and an intact virus becomes low, thus preventing rapid defective virus build up.

In chapter 6 the experimental results from a repeated batch reactor configuration as was modelled are given. The occurrence of defective virus is indeed postponed and production declines at the predicted time post infection. Two additional experimental set-ups involving repeated fed-batch infections also behaved according to model prediction.

7. Conclusion

The baculovirus expression system is especially suitable when a large amount of complex protein has to produced in a limited time. For production processes the lytic character of the infection is a disadvantage. It is however, possible to use extra-cellular virus obtained from infected-cell culture to infect new batches. This reuse of virus is limited, but since the nature of this limitation is known and predictable it should not be an obstacle for process design. Furthermore the decline in production due to the increasing passage number of virus is reproducible and therefore validation of a process should be possible. For a validated process, however, two banks will be necessary: a cell and a virus bank which both have to be tested.

8. References

Bisbee, C.C. Opportunities in and alternative strategies for biomanufacturing success. Gen. Eng. News **14** (14), 8.

Kool, M., Voncken, J.W., van Lier, F.L.J., Tramper, J. and Vlak, J.M. (1991) Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with defective interfering properties. Virology **183**, 739-746.

Kompier, R., Tramper, J. and Vlak, J.M. (1988) A continuous process for production of baculovirus using insect-cell cultures. Biotechnol. Lett. **10**, 849-854.

Kuroda, K., Geyer, H., Geyer, R., Doerfler, W. and Klenk, H.-D. (1990) The oligosaccharides of influenza virus hemagglutinin expressed in insect cells by a baculovirus vector. Virology **174**, 418-429.

Luckow, V.E. and Summers, M.D. (1988) Trends in the development of baculovirus expression vectors. Bio/Technol. **6**, 47-55.

Summary

Baculoviruses are insect-pathogenic viruses with a narrow host range. The viruses can be an alternative to chemical insecticides. From research aimed at improving the efficacy of the viruses in insect control another application evolved: the use of the baculovirus to express foreign proteins in insect cells. To produce large amounts of baculovirus or baculovirus-expressed proteins, large-scale culture and subsequent infection of insect cells is necessary. Chapter 1 of this thesis reviews the research on factors influencing insect-cell culture and infection and the status of production with insect-cell cultures.

One of the production strategies reviewed in chapter 1 is a continuous two-stage bioreactor configuration. In the first reactor cells are cultured and the effluent is led to second reactor where the cells are infected with the baculovirus. The first results reported with this system showed two disadvantages. The number of cells infected was relatively small and production in the infection reactor was limited to about one month.

To increase the number of infected cells the residence time distribution in the infection reactor was altered (chapter 2). The infection reactor was replaced by two infection reactors in series, each containing half the volume of the original infection reactor. Therefore, the mixing characteristics of the infection part of the production system became more plug-flow like. This resulted in a higher number of cells which produced polyhedra (encapsulated virus). However, the time during which the cells in the infection reactors produced polyhedra was diminished to about two weeks.

To gain more insight in the decrease of production a recombinant virus was used in a study described in chapter 3. In this recombinant virus the polyhedrin gene (coding for the major protein in the virus matrix) was replaced by the lacZ gene of *Escherichia coli*. Production of ß-galactosidase in a continuous two-reactor configuration gave analogous

results to production with the wild-type virus. Production was maintained for about four weeks then it rapidly decreased. Upon restriction enzyme analysis it was shown that the decrease of ß-galactosidase resulted from the disappearance of the gene from the virus population rather than inactivation of the gene.

Research at the department of Virology of the Agricultural University of Wageningen revealed that the reduction of production resulted from the occurrence of defective mutants of the baculovirus. These defective virus lack the polyhedrin-promoter driven gene and became predominant in the bioreactor due to interference with the replication of non-mutated virus.

On the defective viruses another highly expressed gene, the p10 gene, was found to be still present. A recombinant virus containing the lacZ from *E. coli* under control of the p10 promoter was used in the study described in chapter 4. The recombinant virus produced both polyhedra and ß-galactosidase in a continuous bioreactor system. Again production lasted for about four weeks both for polyhedra and ß-galactosidase. This indicates that the presence of the p10-promoter driven gene per se is not enough for recombinant protein production.

In chapter 5 a model is presented which describes the kinetics of virus infection. Besides the infectious virus particles and the defective mutants, a third virus type is taken into account. This so-called abortive virus is capable of blocking an entry site of a cell but is not leading to production of new virus particles. By assuming a limited amount of entry sites on an insect cell it was possible to calculate when production of infectious virus decreased. The model indicates that the build-up of defective viruses can be postponed by keeping the number of infectious viruses per cell low. A way of accomplishing this is the use of repeated batch infections.

In chapter 6 experiments with repeated (fed-)batch infections are presented. These experiments confirmed calculations with the model. A production involving a series of batch infections resulted in prolongation of production time to 60 days.

The application of the baculovirus expression vector in the light of the findings described in the thesis is discussed in chapter 7.

Samenvatting

Baculovirussen zijn insekt pathogene virussen met een beperkt gastheer bereik. Door de specificiteit van de virussen zijn ze een mogelijk alternatief voor het gebruik van chemische gewasbeschermingsmiddelen. Uit onderzoek gericht op het verbeteren van de effectiviteit van de virussen kwam een andere toepassing naar voren: het gebruik van het baculovirus als expressievector van eiwitten in insektecellen. Om grote hoeveelheden virus, of in insektecellen tot expressie gebracht eiwit, te produceren is het nodig om insektecellen op grote schaal te kweken en vervolgens te infecteren. Hoofdstuk 1 van het proefschrift geeft een overzicht van de factoren die groei en infectie van insektecellen beïnvloeden en van de stand van zaken met betrekking tot de produktie met behulp van insektecel cultures.

Eén van de produktiewijzen beschreven in hoofdstuk 1 is de continue produktie in een tweetraps bioreactor configuratie. In de eerste reactor worden de cellen gekweekt en het effluent wordt naar de tweede reactor gepompt waar de cellen worden geïnfecteerd met het virus. De eerste resultaten met het genoemde systeem leverden twee tegenvallende aspecten op. Het aantal cellen dat geïnfecteerd werd was laag en de produktie van virus in de infectie reactor stopte na ongeveer één maand.

Om het aantal cellen dat polyeders (ingepakte virusdeeltjes) bevatte te verhogen werd de verblijftijdspreiding in de infectiereactor veranderd (hoofdstuk 2). De infectiereactor werd vervangen door twee reactoren in serie die elk de helft van het volume van de oorspronkelijke reactor bevatten. Hierdoor benaderde het menggedrag van het infectiedeel van het produktiesysteem een propstroom. Het gevolg hiervan was een verhoogd aantal cellen dat polyeders bevatte. Daar stond echter tegenover dat de tijd gedurende welke de cellen polyeders produceerden afnam tot ongeveer twee weken.

Om meer inzicht te krijgen in het waarom van het stoppen van de produktie werd een recombinant virus gebruikt in de studie beschreven in

hoofdstuk 3. In deze recombinant was het polyhedrine gen (dat codeert voor het belangrijkste eiwit in de eiwitmantel van het virus) vervangen door het lacZ gen van *Escherichia coli*. De produktie van ß-galactosidase in een continu tweetraps reactor systeem gaf analoge resultaten als de produktie met het wild-type virus. De produktie duurde ongeveer vier weken en liep toen snel terug.

Uit onderzoek door de vakgroep Virologie van de Landbouw-universiteit Wageningen bleek dat het teruglopen van de produktie het gevolg was van het ontstaan van zogenaamde defecte mutanten van het baculovirus. Deze defecte mutanten missen het door de polyhedrine promoter gecontroleerde gen en door te interfereren met de replicatie van niet gemuteerd virus krijgt de mutant de overhand in de reactor.

In het genoom van het defecte virus bevindt zich nog wel een ander gen dat een hoge expressie kent, het p10 gen. Een recombinant virus dat het lacZ gen onder controle van de p10 promoter bevatte was onderwerp van studie in hoofdstuk 4. Het recombinante virus produceerde zowel polyeders als ß-galactosidase in een continu bioreactor systeem. Ook hier duurde de produktie slechts vier weken voor zowel de polyeders als het ß-galactosidase. Dit geeft aan dat het aanwezig zijn van het door de p10 promoter gecontroleerde gen op zichzelf niet voldoende is voor de produktie van het recombinant eiwit.

In hoofdstuk 5 wordt een model gepresenteerd dat de kinetiek van de virus infectie beschrijft. Naast de infectieuze virussen en de defecte virussen wordt een derde klasse van virussen ten tonele gevoerd. Deze zogenoemde abortieve virussen zijn in staat om een zogenaamde "entry site"(plaats waar een virus de cel binnen kan dringen) te bezetten maar leiden niet tot de produktie van nieuwe virus deeltjes. Door er van uit te gaan dat er slechts een beperkt aantal "entry sites" per insektecel aanwezig is was het mogelijk te berekenen wanneer de produktie van infectieus virus afnam. Het model gaf verder aan dat de toename van defecte virussen kan worden uitgesteld door het aantal infectieuze virussen per cel laag te houden. Een manier om dit te bewerkstelligen is door gebruik te maken van opeenvolgende "batch" infecties.

In hoofdstuk 6 worden experimenten met opeenvolgende "(fed-)batch" infecties beschreven. Deze experimenten bevestigden de

berekeningen met het model. Een produktie bestaande uit een serie van batch infecties resulteerde in een verlenging van de produktietijd tot 60 dagen.

De toepassing van het baculovirus expressie-systeem in het licht van het onderzoek beschreven in het proefschrift, is onderwerp van hoofdstuk 7.

Bibliography

De Gooijer, C.D., van Lier, F.L.J., van den End, E.J., Vlak, J.M. and Tramper, J. (1989) A model for baculovirus production with continuous insect cell cultures. Appl. Microbiol. Biotechnol. **30**, 497-501.

De Gooijer, C.D., Koken, R.H.M., Van Lier, F.L.J., Kool, M., Vlak, J.M. and Tramper, J. (1992). A structured dynamic model for the baculovirus infection process in insect cell reactor configurations. Biotechnol. Bioeng. **40**, 537-548.

Kool, M., van Lier, F.L.J., Vlak, J.M. and Tramper, J. (1990) Production of (recombinant) baculoviruses in a two stage bioreactor system using insect-cell cultures. In: Dekkers, J.J., van der Plas, H.C. and Vuijk, D.H. (eds) Agricultural biotechnology in focus in the Netherlands. Pudoc Wageningen 64-69.

Kool, M., Voncken, J.W., van Lier, F.L.J., Tramper, J. and Vlak, J.M. (1991) Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with defective interfering properties. Virology **183**, 739-746.

Tramper, J., van den End, E.J., de Gooijer, C.D., Kompier, R., van Lier, F.L.J., Usmany, M. and Vlak, J.M. (1990) Production of baculovirus in a continuous insect-cell culture. Bioreactor design, operation and modeling. Ann. N.Y. Acad. Sci. **589**, 423-430.

Tramper, J., Van Lier, F.L.J., Kool, M., De Gooijer, C.D. and Vlak, J.M. (1992). Production of (recombinant) baculoviruses in insect-cell bioreactors. In: Vardar-Sukan, F.F. and Sukan, S. (eds.) Recent advances in biotechnology. Kluwer Academic Publishers, The Netherlands, 263-284.

Van Lier, F.L.J., van den End, E.J., de Gooijer, C.D., Vlak, J.M. and Tramper, J. (1990) Continuous production of baculovirus in a cascade of insect-cell reactors. Appl. Microbiol. Biotechnol. **33**, 43-47.

Van Lier, F.L.J., Kool, M., van den End, E.J., de Gooijer, C.D., Usmany, M., Vlak, J.M. and Tramper, J. (1990) Production of baculovirus or recombinant derivatives in continuous insect-cell bioreactors. Ann. N.Y. Acad. Sci. **613**, 183-190.

Van Lier, F.L.J., de Gooijer, C.D., Kool, M., Vlak, J.M. and Tramper, J. (1991). Production of (recombinant) baculovirus in two-stage bioreactor systems. In: Fraser, M.J. (ed.) Proceedings of the eighth International Conference on Invertebrate and Fish Culture held in conjunction with the 1991 World Congress on Cell and Tissue Culture, June 16-20 1991, Anaheim, California. Tissue culture association, Columbia, USA, 147-152.

van Lier, F.L.J., van der Meijs, W.C.J., Grobben, N.G., Olie, R.A., Vlak, J.M. and Tramper, J. (1992) Continuous ß-galactosidase production with a recombinant baculovirus insect-cell system in bioreactors. J. Biotechnol. **22**, 291-298.

Van Lier, Vlak, J.M. en Tramper, J. (1991) Production of baculovirus-expressed proteins from suspension cultures of insect cells. In: Spier, R.E. en Griffiths, J.B. (eds.) Animal cell biotechnology - volume V. Academic Press, London, 169-188.

Van Lier, F.L.J., van Duijnhoven, G.C.F., de Vaan, M.M.J.A.C.M., Vlak, J.M. and Tramper, J. (1994) Continuous ß-galactosidase production in insect cells with a baculovirus vector based on the p10 gene in a two-stage bioreactor system. Biotechnol. Progr. **10**, 60-64.

Van Lier, F.L.J., van den Hombergh, J.P.T.W., de Gooijer, C.D., den Boer, M.M., Vlak, J.M. and Tramper, J. (1995) Long-term semi-continuous production of recombinant baculovirus protein in a repeated (fed-)batch two-stage reactor system. Enz. Microb. Technol. In press.

Van Lier, F.L.J. (1995) Regulatory issues in the use of insect-cell cultures. Submitted.

Vlak, J.M., van Lier, F.L.J., Voncken, J.W., Kool, M., van den End, E.J., Usmany, M., de Gooijer, C.D. and Tramper, J. (1990) Design and operation of insect cell bioreactors for continuous production of baculovirus recombinant proteins. In: White, M.D., Reuveny, S. en Shafferman, A. (eds.) Biologicals from recombinant microorganisms and animal cells. Production and recovery. VCH Weinheim/Deerfield Beach and Balaban, Rehovot/Philadelphia, 69-81.

Curriculum vitae

Geboren 10 juni 1964 te Arnhem kreeg ik de namen Franciscus Leonardus Johannes van Lier mee. Na de lagere school volgde ik een VWO opleiding aan het Neder-Rijn college te Arnhem, waar ik in 1982 het diploma voor behaalde.

Van 1982 tot 1988 studeerde ik moleculaire wetenschappen aan de Landbouwuniversiteit te Wageningen. De afstudeervakken waren Organische Chemie en Proceskunde en als bijvak het predoctorale deel van de lerarenopleiding Scheikunde.

Van 1988 tot en met 1992 was ik als assistent in opleiding bij de sectie Proceskunde van de Landbouwuniversiteit bezig met het onderzoek beschreven in dit proefschrift.

In 1992 en 1993 was ik werkzaam bij de stichting Bio-Cat en gestationeerd bij het bedrijf Bio-Intermediair alwaar ik betrokken was bij de opbouw van het kwaliteitssysteem en de initiële validatie.

In 1994 trad ik in dienst bij Bio-Intermediair, eerst als medewerker bij de toen zogeheten "Technical support" afdeling en later dat jaar als hoofd van de afdeling Fermentatie van zoogdiercellen.