# Cell culture derived AgMNPV bioinsecticide: biological constraints and bioprocess issues

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## Abstract

We have studied parameters for optimizing the *Spodoptera frugiperda* (Sf9) cell culture and viral infection for the production of *Anticarsia gemmatalis* multiple nucleopolyhedrosis virus (AgMNPV) polyhedra inclusion bodies (PIBs) in shaker-Schott or spinner bottles and bioreactors. We have assayed the $k_La$ of the systems, initial cell seeding, cell culture volume, dissolved oxygen (DO), multiplicity of infection (MOI), nutrients consumption, and metabolites production. The medium surface oxygen transfer was shown to be higher in shaker bottles than in spinner ones, which was in direct correlation to the higher cell density obtained. Best quantitative performances of PIBs production were obtained with a SF900II medium volume/shaker-bottle volume ratio of 15% and MOI of 0.5 to 1 performed at a cell concentration at infection (CCI) of 1 to $2.5 \times 10^6$ cells/ml in a medium containing enough glucose and glutamine. Upon infection, a decrease in the cell multiplication was observed to be dependent on the MOI used, and the $\mu X$ at the exponential growth phase in infected and non-infected cultures were, respectively, of 0.2832 and 0.3914 (day$^{-1}$). The glucose consumption and lactate production were higher in the infected cultures ($\mu$Glucose and $\mu$Lactate of, respectively, 0.0248 and $0.0089 \times 10^{-8}$ g/cell$\times$day in infected cultures and 0.0151 and $0.0046 \times 10^{-8}$ g/cell$\times$day in non infected ones). The glutamine consumption did not differ in both cultures ($\mu$Glutamine of 0.0034 and $0.0037 \times 10^{-8}$ g/cell$\times$day in, respectively, infected and non infected cultures). When a virus MOI of 0.1 to 1 was used for infection, a higher concentration of PIBs/ml was obtained. This was in direct correlation to a higher cell concentration present in these cultures, where a decrease in cell multiplication due to virus infection is minimized. When a MOI of 1 was used, a more effective decrease in cell multiplication was observed and a lower concentration of PIBs/ml was obtained, but with the best performance of PIBs/cell. Correlations between MOI and CCI indicate that a MOI 0.1 to 1.4 and a CCI of $10^6$ to $2 \times 10^6$ cells/ml led to the best PIBs production performances. The virulence of PIBs produced in cultures infected at low or high MOI showed comparable $DL_{50}$. Culture and infection in scaling-up conditions, performed in a bioreactor, were shown to provide the cells with a better environment and be capable of potentially improving the shaker-Schott findings. For an accurate qualitative control of PIB virulence, hemolymph from AgMNPV infected *Anticarsia gemmatalis* was used as starting material for passages in Sf9 cells. These led to a loss of virulence among the PIBs with an increase in the $DL_{50}$. The loss

of virulence was accompanied by a loss in budded virus titer, a decreased number of PIBs produced and an altered DNA restriction pattern, suggesting the generation of defective interference particles (DIPs). Transmission electron microscopy (TEM) studies revealed that after cell passages, PIBs lacking virions were progressively synthesized. The study described here point out the biological constraints and bioprocess issues for the preparation of AgMNPV PIBs for biological control.

*Abbreviations:* AgMNPV – *Anticarsia gemmatalis* multiple nucleopolyhedrosis virus; BVs – budded virus; DIPs – defective interference particles; DO – dissolved oxygen; MOI – multiplicity of infection; PIBs – polyhedral inclusion bodies; Sf9 – *Spodoptera frugiperda*; TEM – Transmission electronic microscopy

## Introduction

The potential of entomopathogenic viruses as bio-insecticides has been well described (Richards et al. 1998; Chakraborty et al. 1999; Moscardi 1999). The baculoviruses, naturally occurring arthropod-specific viruses, are effective alternatives to chemical insecticides. During virus replication virions are found inside occlusion bodies constituted mainly by polyhedrin (PIBs) which, in nature, protect them from inactivation. Attempts to use baculoviruses in biological control, mainly *Lepidoptera*, has been done in several countries in the world and the most successful one is related to the soybean caterpillar control by the *Anticarsia gemmatalis* multiple nucleopolyhedrosis virus (AgMNPV). For more than 20 years the biological control program of Embrapa (Brazilian Agricultural Research Corporation) has proved the AgMNPV bioinsecticide value. Annually, approximately 1.4 million hectares of soybean are treated with AgMNPV and an increasing demand takes place (Moscardi 1999; Embrapa 2004). Nevertheless, only 1.4 out of 21 million soybean hectares are today treated in Brazil with the commercially available AgMNPV bioinsecticide. Among the drawbacks to foster the AgMNPV production and application, one includes the technical and economical difficulties for PIBs production with a cell culture based technology (Bonning et al. 1995; Pereira et al. 2001; Lua et al. 2002). Currently the production is conducted in farmer's field and lead to a cost for the farmer lower than the cost of chemical insecticides. Despite the success and commercial viability of the program, the AgMNPV field production is limited by abiotic and biotic factors that affect host abundance (Moscardi 1999). Considering technical and economical issues a cell culture technology for AgMNPV production would be very welcome.

Cell culture bioprocesses for virus production are today quite developed allowing for instance several viral vaccines production in bioreactors of thousand of liters (Marks 2003). But for a bioinsecticide production, we face an uncommon difficulty which is not presented for viral vaccine production. The challenge is to produce large amounts of highly virulent virus samples. For a viral vaccine, the main property has to be the immunogenicity and its virulence is not taken care, the final product is anyway inactivated or has to be kept attenuated. But for a bioinsecticide, virulence is essential and several attempts are under investigation to genetically modify the viruses conferring them a high virulence (Bonning and Hammock 1995, 1996; Wood and Granados 1991; Rodrigues 2001). It is quite well known that virus passaging in cell cultures leads to attenuation and this is not different for baculoviruses. Extensive changes in the baculovirus genome take place during passages in cell cultures (Bonning et al. 1995; Lua et al. 2002). So, a cell culture based bioprocess for baculovirus bioinsecticide production has to fulfill at least two basic requirements, (a) the production of virulent virions inside PIBs and, (b) a low cost and commercially competitive final product.

Insect cell cultures are easier to handle than mammalian cells, being capable to multiply in monolayers or in suspension at temperatures ranging from 25 to 30 °C. They show, for instance, a good resistance to shear stress as well as to pH variations and as a consequence the culture procedures can be based on technologies currently

used for animal cells, such as tissue culture flasks and spinners bottles and, in addition, can also be performed in orbital shaker bottles and on simpler media (Weiss and Vaughn 1986; Mitsuhashi 1998; Pereira et al. 2001; Weber et al. 2002).

The study presented in this paper describes a quantitative and qualitative analysis of AgMNPV PIBs production in lepidopteran cells, and discusses biological constraints and bioprocess issues related to attempts to establish a cell culture based bioprocess production.

## Materials and methods

### Cell cultures

*Spodoptera frugiperda* (Sf9) cells (ATCC 1711) were grown and maintained in the serum free SF900II (Life Tecnologies) medium supplemented with 0.2% of Pluronic F68 (Gibco BRL). Media were always supplemented with 1% of gentamicin. Cells were cultivated, at 100 rpm and 28 °C, in suspension using shaker-Schott (Schott) under orbital agitation or spinner (Bellco) bottles or a 1L working volume Inceltech/SGI Discovery 100 (Inceltech, France) bioreactor provided with a MRU control system for temperature (Pt100 sensor), pH (Mettler Toledo probe), stirring (magnetic transmission), dissolved oxygen (0 to 50 and 0 to 200 ml/min inlet flow). The bioreactor is controlled and the data are collected by a computer (Bioac software (Inceltech, France)).

The medium-surface volumetric oxygen transfer rate ($k_La$) in both, the schott-shaker and spinner systems, were determined in the conditions under which the cultures were performed. Medium in nitrogen atmosphere was exposed to air and dissolved oxygen concentration ($C$) was monitored. $k_La$ was calculated as the slope of $\ln[1 - (C/C_s)]$ as function of time, where $C_s$ is $C$ at saturation with air (100%).

Different cell seeding and medium volume/bottle volume ratios were assayed. Cell viability was determined by trypan blue exclusion and viable cell counting was done in a hemacytometer. Glucose, glutamine and lactate concentrations in the culture supernatants were determined by enzymatic reaction using the YSI 2700 analyzer (Yellow Springs). Specific cell growth, nutrient consumption or metabolite production were calculated. Experiments were delineated in order to establish optimized conditions for Sf9 cell growth by studying parameters such as $k_La$ of the system, culture volume and cell seeding, growth and metabolism.

### Virus infection, PIBs production and virulence

Wild type AgMNPV, isolated from the hemolymph of experimentally infected *Anticarsia gemmatalis* larvae was used for serial passages in Sf9 cells. At least 60 larvae were fed at 4th instar with AgMNPV polyhedra inclusion bodies (PIBs) enriched diet and 4 days after the hemolymph was collected, immediately diluted in culture medium to prevent oxidation, passed through 0.22μm filter and stored at 4 °C. AgMNPV budded virus (BVs) titers in the hemolymph were determined in Sf9 cultures in 96-well microplates by end point dilution, calculated according to Reed and Muench 1938, and expressed as $TCID_{50}$/ml.

Serial passages of BVs were performed in Sf9 cell cultures in order to examine the ability of these cells to produce BVs and PIBs. After centrifugation at 3000 rpm, the supernatants of infected cultures were collected and stored at 4 °C for BV titration, as described above. The culture sediments, with the cells containing PIBs, were treated for 10 min with 1 ml of 0.5% sodium dodecyl sulfate (SDS) for $10^6$ cells and centrifuged at 20.000 rpm for 20 min. The sediments were then suspended in PBS and the PIBs counted in hemacytometer. For the extraction of PIBs from infected larvaea similar procedure was used (O'Reilly et al. 1992).

Quantitative studies were done by varying the multiplicity of BV infection (MOI) from 0.1 to 5 and the Sf9 cell concentrations at infection (CCI) from 0.5 to $4.5 \times 10^6$ cells/ml and evaluating the kinetics of cell growth after infection as well as the number of PIBs produced. Qualitative studies were done by evaluating the PIBs virulence by induction of larvae mortality.

The evaluation of PIBs virulence was assayed in bioassays as described elsewhere (Medugno et al. 1997). Briefly, PIBs were diluted in distilled water and 5μl of each dilution was applied to the surface of the larvae diet. One third-instar larva was placed in each 50 ml cup containing the inoculated diet where it remained for 48 h, and then transferred to PIBs-free diet. Controls included distilled water and known infectious PIBs suspension. Bioassays were performed with 60 larvae per

sample dilution. Mortality was assessed daily and recorded until pupation or death. The results are expressed in $DL_{50}$ with standard deviation.

## DNA restriction analysis (REN)

The genomic REN digestion pattern of viral DNA was evaluated as described elsewhere (O'Reilly et al. 1992). Briefly, DNA was extracted from SDS treated cell cultures infected with AgMNPV of low or high cell passages by using a phenol-chloroform procedure. The AgMNPV 2D isolate was used as a virus reference (Johnson and Maruniak 1989). The DNA samples were then digested separately with the endonucleases HindIII and PstI at 37 °C for 4 h. Each reaction was done in a final volume of 25 μl containing 1 μg DNA and 2 μl of enzymes with appropriated buffers. The products were then submitted to separation in 0.8% agarose gels and stained with ethidium bromide. Images were obtained using a photodigital system (Strategene Eagle Eye).

## Transmission electron microscopy

For transmission electron microscopy (TEM), PIBs were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences) and 2% paraformaldehyde (Sigma) in 0.1M sodium cacodilate buffer (pH 7.2)(Serva). After postfixation in 2% $OsO_4$ (Merck) in 0.1 M sodium cacodilate buffer (pH 7.2) and staining in 0.5% uranyl acetate solution (Baker) containing 0.5% sucrose (Merck), they were dehydrated in a series of crescent concentration of ethanol solutions (Synth), immersed in 100% ethanol and propylene oxide (Aldrich), and embedded in epoxy resin (Polybed). Thin sections (60 to 70 nm) cut with a Sorvall MT6000 ultramicrotome were stained with 2% uranyl acetate and lead citrate (Bozzola and Russel 1992). Observations and electron micrographs were performed in a LEO 906E TEM (80 kV) and images processed by computers.

## Results

### Sf9 cell cultures in shaker and spinner bottles

As shown in Figure 1, the orbital agitation provided by shakers allowed a better oxygen transfer through the medium surface than that observed when suspended magnetic stirrer, present in spinner bottles, are used. Depending on the volume used in the shaker-Schott bottles, from 5 to 30% of the total volume, the $k_La$ obtained varied from 67.3 to 11.4 $h^{-1}$, respectively. In contrast, in spinner bottles with 50 and 100% of the recommended working volumes, the $k_La$ were of 2.89 and 0.88 $h^{-1}$, respectively. In correlation to the $k_La$ values, we could observe that cultures performed with high (20% and 25%) medium volume/shaker-bottle volume ratio failed to express good kinetics of cell growth (Figure 2a). In spite of a high $k_La$, cultures performed with very low volume/shaker-bottle volume ratio (5%) also failed to express good kinetics of cell growth (Figure 2a), and this may be due to the higher hydrodynamic stress produced in such conditions.

Experiments conducted in order to examine the influence of cell seeding on the kinetics of Sf9 cell growth showed that, in both shaker-Schott and spinner bottles, initial cell densities lower than $5 \times 10^5$ cells/ml led to a kinetic of cell growth with an extended lag-phase (Figure 2b and 2c). The cultures performed in spinner bottles failed to express good performances of cell growth (Figure 2c), never attaining cell densities as high as those obtained in cultures performed in shaker-Schott bottles (Figure 2a and b).

The best results of cell growth were observed when Sf9 cells were cultivated in a shaker-Schott bottles with a medium volume/shaker-bottle volume ratio of 15% and using a cell seeding of 0.5 to $1 \times 10^6$ cells/ml. At these conditions cell concentrations of 8.5 to $9 \times 10^6$ cells/ml could be obtained after 6 days of culture. Experiments of AgMNPV infection were then conducted using these basic conditions.

### Sf9 cell metabolism and AgMNPV infection

As shown in Figure 3, during the Sf9 cell growth in shaker bottles, glucose and glutamine were consumed and lactate was produced. In spite of a higher glucose consumption, nor the glucose neither the glutamine did appear to be limiting factors to the cell growth. The cell growth reached a peak at 7 days of culture with $9.1 \times 10^6$ ells/ml, when the medium still contained reasonable amounts of glucose (5.8 g/l) and glutamine (1.7 g/l)
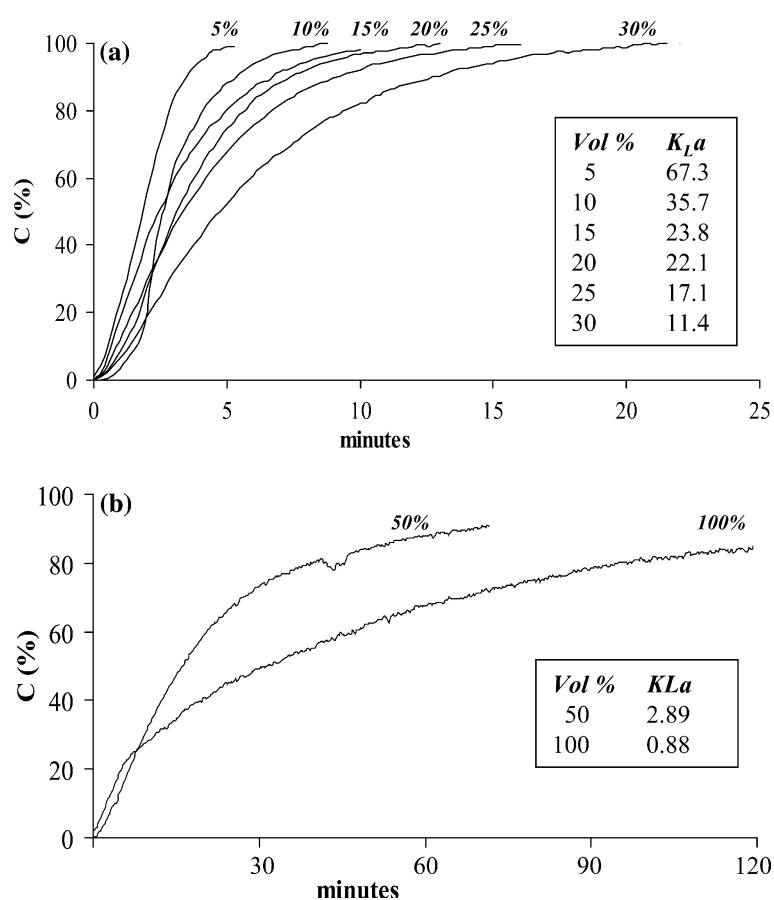
*Figure 1.* Kinetics of oxygen transfer in shaker-Schott (a) and spinner bottles (b). Bottles with different medium volumes (from 5% to 30% of total volume for shaker-Schott and 50% or 100% for spinners) at 28 °C and 100 rpm were used for measuring the oxygen transfer through the liquid surface. $k_L a$ was determined as indicated in material and methods. *C* dissolved oxygen concentration.

(Figure 3a). Upon AgMNPV infection, an important limitation of cell growth was observed. In the virus infected cultures Figure 3b) the $\mu X$, at the exponential growth phase, was of 0.28 day$^{-1}$ and the cultures attained $2.8 \times 10^6$ cells/ml. It contrasted to the values of, respectively, 0.39 day$^{-1}$ and $9.1 \times 10^6$ cells/ml obtained in non infected cultures. Nevertheless, the glucose consumption and lactate production rate, at the exponential phase, were higher in infected cultures ($\mu$glucose of 0.025 and $\mu$lactate of 0.0089), than in non-infected ones ($\mu$glucose of 0.015 and $\mu$lactate of 0.0046).

Depending on the multiplicity of infection (MOI) used for AgMNPV infection, a different degree of cell growth inhibition was observed Figure 4a). The infection with 0.1 MOI had little effect and the cell growth reached a density of

$5.3 \times 10^6$ cells/ml at day 5. The increase in the MOI led to a higher degree of cell growth inhibition.

In the Figure 4b we show data related to the production of AgMNPV PIBs in Sf9 cell cultures on shaker-Schott bottles. Both the number of cells/ml and PIBs/ml decreased with the increase of MOI used for infection, the number of cells/ml ranging from $5.3 \times 10^6$ cells/ml at MOI 0.1 to $3 \times 10^6$ cells/ml at MOI 1, and the number of PIBs/ml ranging from $8 \times 10^7$ at MOI 0.1 to $5.5 \times 10^7$ at MOI 1. On the other hand the number of PIBs/cell increased in cultures infected at MOI 0.1 to 1 (15 to 18.3 PIBs/cell, respectively). The PIBs produced in the cultures infected at different MOI were tested in bioassays and showed comparable virulence.

As shown in Figure 5a, regardless the CCI used, an increase in the MOI led to a progressive decrease in the maximum cell concentration reached
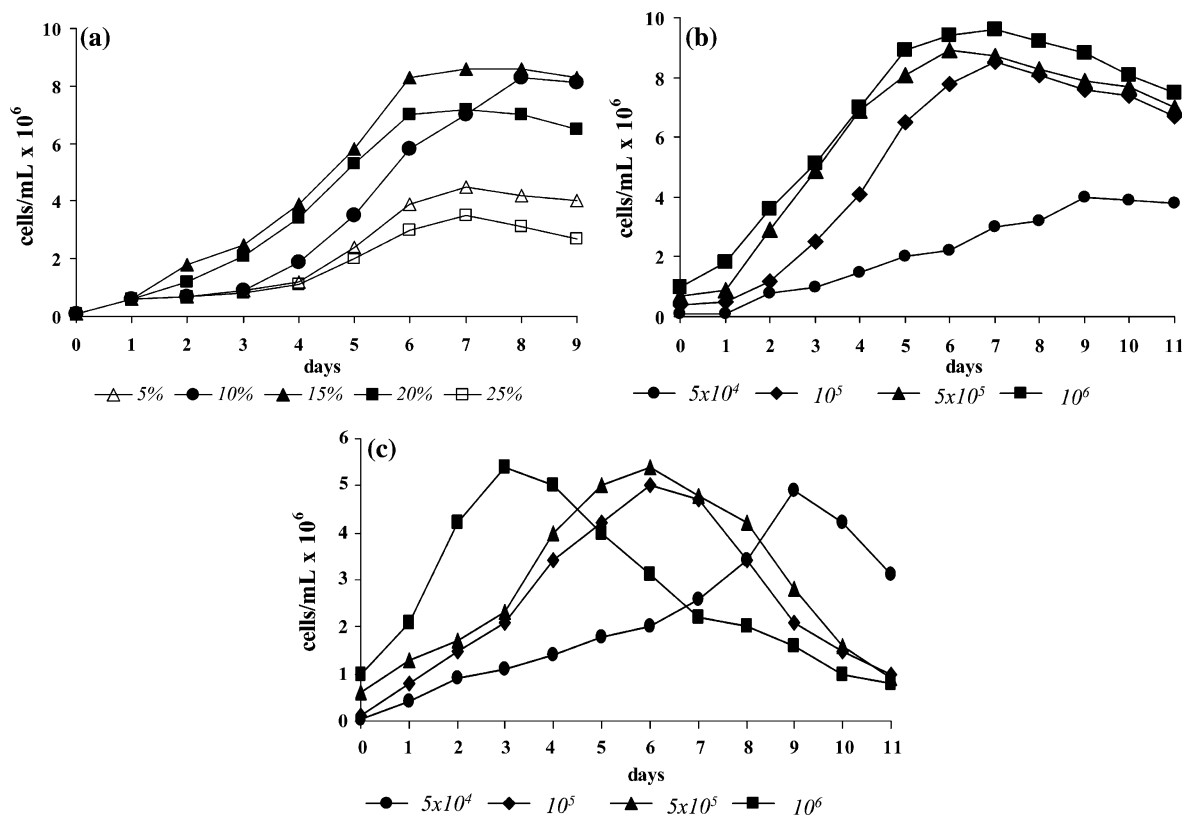
*Figure 2.* Kinetics of Sf9 cell growth in shaker-Schott (a and b) and spinner bottles (c). Cells were cultivated in 100 ml shaker-Schott or spinners bottles at 28 °C and 100 rpm. Different ratios medium/bottle volumes (a) were tested in cultures performed with $10^5$ cells/ml as initial cell seeding. Different cell seeding (b and c) was tested in cultures performed in shaker-Schott bottles (b) with a ratio medium/bottle volume of 15%, or in spinner bottles (c) with a ratio medium/bottle volume of 50%. Samples were daily collected and the viable cell concentrations determined. Results are the average of at least 3 independent experiments.

by the culture. In cell cultures infected with 0.1 to 0.7 MOI, the maximum cell concentration remained roughly unchanged but those infected with MOI > 0.7 showed a decrease in the cell concentration in function of the MOI. For instance, cultures at a CCI of $2 \pm 0.5 \times 10^6$ cells/ml upon infection with MOI 0.1, 3 or 5 reached a maximum cell concentration of respectively, $6.4 \times 10^6$, $5.5 \times 10^6$ and $4.3 \times 10^6$ cells/ml. The PIBs production in these cultures, as shown in Figure 5b, were relatively low ($< 5 \times 10^7$ PIBs/ml) in cultures infected at high or low CCI (respectively, $3.5 \pm 1 \times 10^6$ and $0.5 \pm 0.1 \times 10^6$ cells/ml) when compared to the PIBs production obtained in cell cultures infected at CCI of $10^6$ to $2.5 \times 10^6$ cells/ml ($> 10^8$ PIBs/ml) and not influenced by the MOI used (from 0.1 to 5).

To overcome possible oxygen limitation in shaker-Schott bottles, experiments in a bioreactor were performed under controlled dissolved oxygen (DO) concentration (Figure 6). Non-infected cells reached a maximum cell density at day 3 of culture ($9 \times 10^6$ cells/ml) when a plateau was reached and remained for 2 days. In paralleled cultures, at day 2, infection was done at MOI 1 leading to limitation in cell growth and to production of virus. The cell growth reached a peak at day 4 ($4.5 \times 10^6$ cell/ml), which remained for 72 h, with no apparent limitation of glucose and glutamine or inhibition by lactate. The values of PIBs production were of $6.3 \times 10^7$ PIBs/ml and 14.6 PIBs/cell. In both, control or infected cultures the nutrients consumption were higher than in shaker bottles.

As shown in Table 1, the *Anticarsia gemmatalis* hemolymph used as starting material for virus passages had a BV titer of $10^{7.8}$ TCID$_{50}$/ml. PIBs produced in larvae showed a DL$_{50}$ of $0.4 \times 10^3$. The
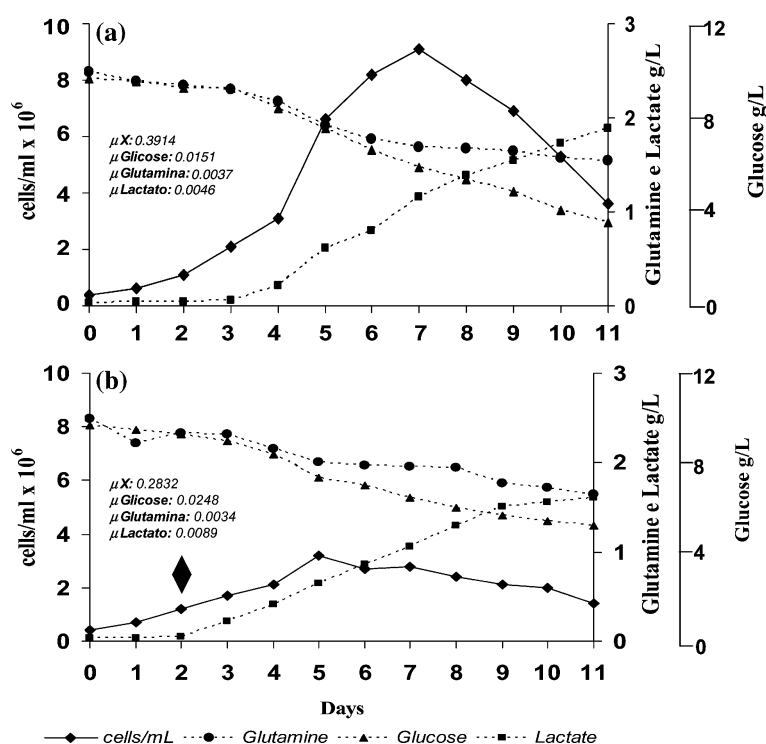
*Figure 3*. Kinetics of Sf9 cell growth and metabolism in control (a) or AgMNPV infected (b) cultures. $5 \times 10^5$ Sf9 cells/ml were inoculated in shaker-Schott bottles using a ratio medium/bottle volume of 15% and maintained at 28 °C and 100 rpm. Paralleled cultures were infected at day 2 (arrow) with 1 MOI of hemolymph derived-AgMNPV. Samples were daily collected for cell counting and glucose, glutamine and lactate quantification. Specific cell growth rate (day$^{-1}$) and glucose/glutamine consumption rate or lactate production rate ($10^{-8}$g/cell$\times$day) were calculated. Glucose, glutamine or lactate concentrations are expressed as g/l, the specific cell growth as $\mu X$ and the nutrients consumption or metabolite production as $\mu$glucose, $\mu$glutamine or $\mu$lactate. Results are the average of at least 3 independent experiments.

viral passages in Sf9 cell cultures led to a loss of virulence among the PIBs with an increase in the $DL_{50}$. At the 3rd passage in Sf9 cells the PIBs showed already a $DL_{50}$ of $1225 \times 10^3$, and from the 5th passage they were already not virulent enough to induce mortality. During cell passages, the loss of virulence was accompanied by a loss in BV titer (from $10^{7.32}$ $TCID_{50}$/ml at the 1st passage to $10^{5.11}$ $TCID_{50}$/ml at the 5th passage), and the number of PIBs produced (from 27.6 PIBs/cell at the 1st passage to 6.8 PIBs/cell at the 5th passage). After few passages in larvae, a highly virulent PIBs

*Table 1*. Productivity and virulence of AgMNPV samples after serial passages in Sf9 cell cultures.

| Virus Passage | BVs | PIBs | | |
|---|---|---|---|---|
| | titer *(TCID50/mL)* | total PIBs $\times 10^6$ | PIBs/cell | $DL_{50} \times 10^3$ |
| He | $10^{7.80 \pm 0.2}$ | | | $0.40 \pm 0.02$ |
| 1 | $10^{7.32 \pm 0.8}$ | $55.6 \pm 10.4$ | $27.6 \pm 5.6$ | $1.32 \pm 0.12$ |
| 2 | $10^{6.79 \pm 0.2}$ | $20.1 \pm 3.9$ | $14.1 \pm 1.9$ | $65.1 \pm 0.05$ |
| 3 | $10^{6.17 \pm 0.1}$ | $22.4 \pm 8.6$ | $14.5 \pm 0.5$ | $1225 \pm 90$ |
| 4 | $10^{5.10 \pm 0.1}$ | $19.2 \pm 6.8$ | $12.5 \pm 0.5$ | $< 50\%$ |
| 5 | $10^{5.11 \pm 0.1}$ | $6.8 \pm 2.2$ | $6.8 \pm 2.2$ | no death |

Sf9 cells ($10^6$ cells/mL) were cultivated in schott-shaker system (20 ml, 100 rpm) and infected with 1 MOI of hemolymph (He) obtained from *Anticarsia gemmatalis* larvae infected with AgMNPV for 3 days. 8 days after infection the BVs (supernatant) and PIBs (cell fraction) were collected and 5 serial repeated passages were performed. Supernatants and cell fractions were collected for, respectively, BVs titration and a quantitative (total PIBs or PIBs/cell) or qualitative ($DL_{50}$) analysis of PIBs.
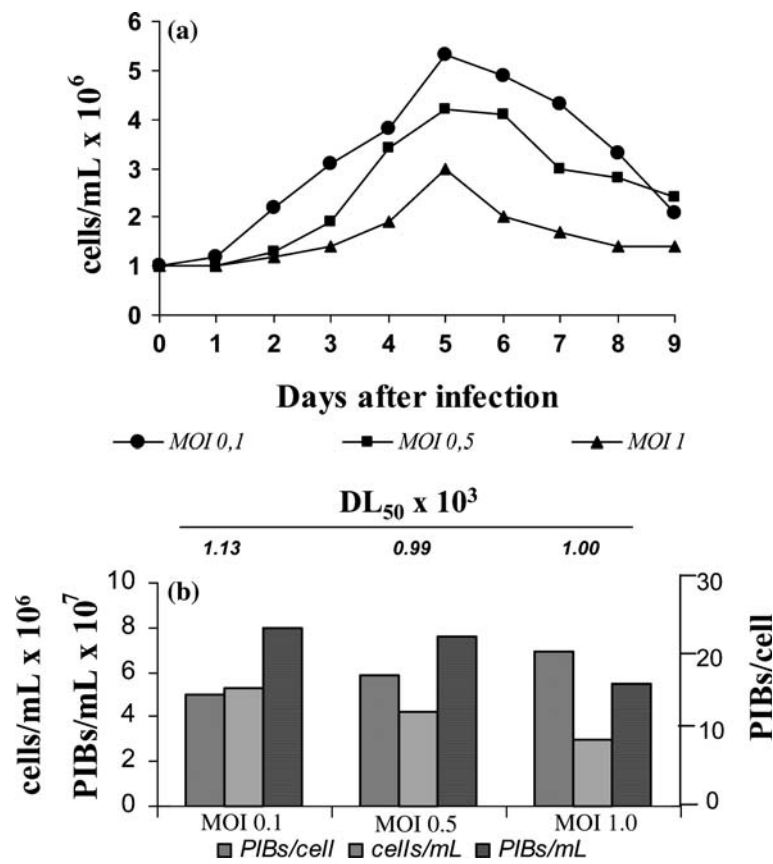
*Figure 4*. Kinetics of Sf9 cell growth upon AgMNPV infection (a) and AgMNPV-PIBs production and virulence (b). $5 \times 10^5$ Sf9 cells/ml were cultivated in shaker-Schott bottles using a ratio medium/bottle volume of 15% and maintained at 28 °C and 100 rpm. At a concentration of $10^6$ cells/ml they were infected with different MOI of hemolymph derived-AgMNPV. Samples were daily collected for the evaluation of viable cell concentration and at day 9 of infection, the cells were collected and the PIBs extracted, counted and tested in bioassays. Data are the average of at least 3 independent experiments. Cell counting is expressed as cells/ml $\times 10^6$, AgMNPV-PIBs production as PIBs/ml $\times 10^7$ or PIBs/cell, and AgMNPV-PIBs virulence as $DL_{50} \times 10^3$.

population could be obtained again (data not shown).

The *Hin*dIII and *Pst*I endonuclease digestion pattern of DNA synthesized in Sf9 cells upon infection with different AgMNPV isolates is shown in Figure 7. The data indicate that genomic changes occurred in AgMNPV during the serial passaging in Sf9 cell culture. At the 6th passage in Sf9 cells the *Hin*dIII and *Pst*I REN digestion patterns showed already clear differences, suggesting the generation of defective interference particles (DIPs). On the other hand only slight differences were shown between low passages Ag-MNPV and AgMNPV 2D REN digestion pattern.

Samples from serial passages were subject of ultrastructural studies in view of identifying changes during the passages in cultures. TEM

studies, showed in Figure 8, revealed that PIBs lacking virions were progressively synthesized after cell passages. At 4th passage most of the PIBs were found to contain few virions.

### Discussion

Baculoviruses and lepidopteran cells have a great interest for biotechnology due to the possibility of gene expression and the use as biopesticides. These can replace the chemical ones because of their specificity and safeness for the environment and public health. A drawback for the use of AgMNPV as biopesticide is the scaling-up of production and the loss of polyhedra virulence after passages in cell culture. Due to the costs involved in its production,
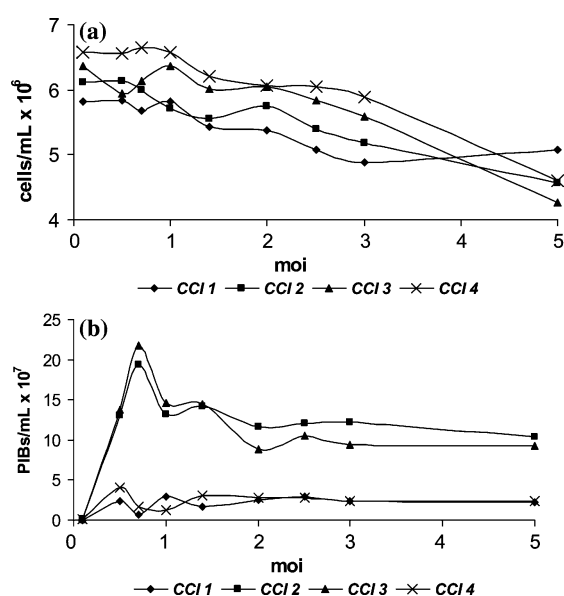
*Figure 5.* Maximal cell (a) and PIBs concentration (b) in Sf9 cell cultures infected with AgMNPV at different multiplication of infection (moi) and different cell concentrations at infection (CCI). $5 \times 10^5$ Sf9 cells/ml were inoculated in shaker-Schott bottles using a ratio medium/bottle volume of 15% and maintained at 28 °C and 100 rpm. Paralleled cultures were infected with AgMNPV at the indicated moi (0.1, 0.5, 0.7, 1, 1.4, 2, 2.5, 3 and 5) and CCI (CCI 1: $5 \pm 1 \times 10^5$ cells/ml; CCI 2: $1 \pm 0.4 \times 10^6$ cells/ml; CCI 3: $2 \pm 0.5 \times 10^6$ cells/ml; CCI 4: $3.5 \pm 1 \times 10^6$ cells/ml). Samples were daily collected for cell and PIBs counting and are expressed as cells/ml and PIBs/ml.

as compared to traditional larvae process of production, the use of a shaker bottle system at industrial scale should be seriously considered. Insect cells are natural host cells for baculoviruses and upon infection, polyhedral inclusion bodies (PIBs) as well as budded virus (BV) are produced. The virion containing -PIBs are ingested by insect larvae as food contaminants and after digestion in the insect midgut, virions may replicate in midgut cells and attain other tissues, leading to larvae disintegration, which becomes a fluid sac containing large amounts of PIBs. The AgMNPV PIBs, commercially produced in farmer's field, are currently used as bioinsecticide against the velvetbean caterpillar *Anticarsia gemmatalis*, and studies of cell cultures derived PIBs production in industrial scale are envisaged (Moscardi 1999; Pereira et al. 2001).

Facing economical reasons and in view of the hydrodynamic stress resistance of *Spodoptera*

*frugiperda* cells (Sf9) (Weiss and Vaughn 1986; Mitsuhashi 1998; Palomares and Ramirez 1997), we have examined the suitableness of shaker-Schott bottles for Sf9 cell multiplication and AgMNPV infection. Beside the nutrients disposable in the culture medium, the availability of dissolved oxygen represents a central parameter for the cultures to attain high cell densities. When compared to the spinner suspension system, the shaker-Schott system not only open the possibility to work with a large range of medium volume/bottle volume ratio but also allow a much higher transfer of oxygen through the liquid surface, as evidenced by the $k_{L}a$ (Figure 1). The higher Sf9 cell density ($\sim 9 \times 10^6$ cells/ml) was obtained in shaker-Schott bottles with a medium volume/shaker bottle volume of 15% (Figure 2a). In cultures performed at higher ratios a deficient oxygen transfer possibly inhibited the cell growth ($\sim 3.5 \times 10^6$ cells/ml) and in those at lower ratios, although at higher oxygen transfer, shear stress may have been responsible for growth inhibition ($\sim 4 \times 10^6$ cells/ml) Figure 2a). The initial cell seeding for cultures performed in shaker-Schott bottles (medium volume/bottle volume ratio of 15%) and spinner bottles (medium volume/bottle volume of 50%) was shown to be stricter for spinner bottle cultures than for the shaker bottles (Figure 2b and c). In shaker bottle cultures an initial cell seeding ranging from $10^5$ to $10^6$ cells/ml allowed satisfactory cell culture kinetics with high final cell density ($\sim 8 \times 10^6$ cells/ml), but for spinner cell cultures to show good kinetics a cell seeding of $10^6$ cells/ml was necessary and led to lower final cell densities ($\sim 5.5 \times 10^6$ cells/ml Figure 2b and c). Although good performances can usually also be obtained in spinner cultures after optimization, the shaker bottle system was shown to be very convenient for insect cell cultures and the higher medium surface oxygen transfer observed in this system (Figure 1) seems to play a central role.

Upon AgMNPV infection, a decrease in the cell multiplication was observed to be dependent on the multiplicity of infection (MOI) used (Figure 3 to 5). Nevertheless, the glucose consumption and lactate production were higher in the infected cultures, the glutamine consumption being not altered by the virus infection. These observations pointed to a major role of glucose as a nutrient for Sf9 cell cultures, confirming previous observations
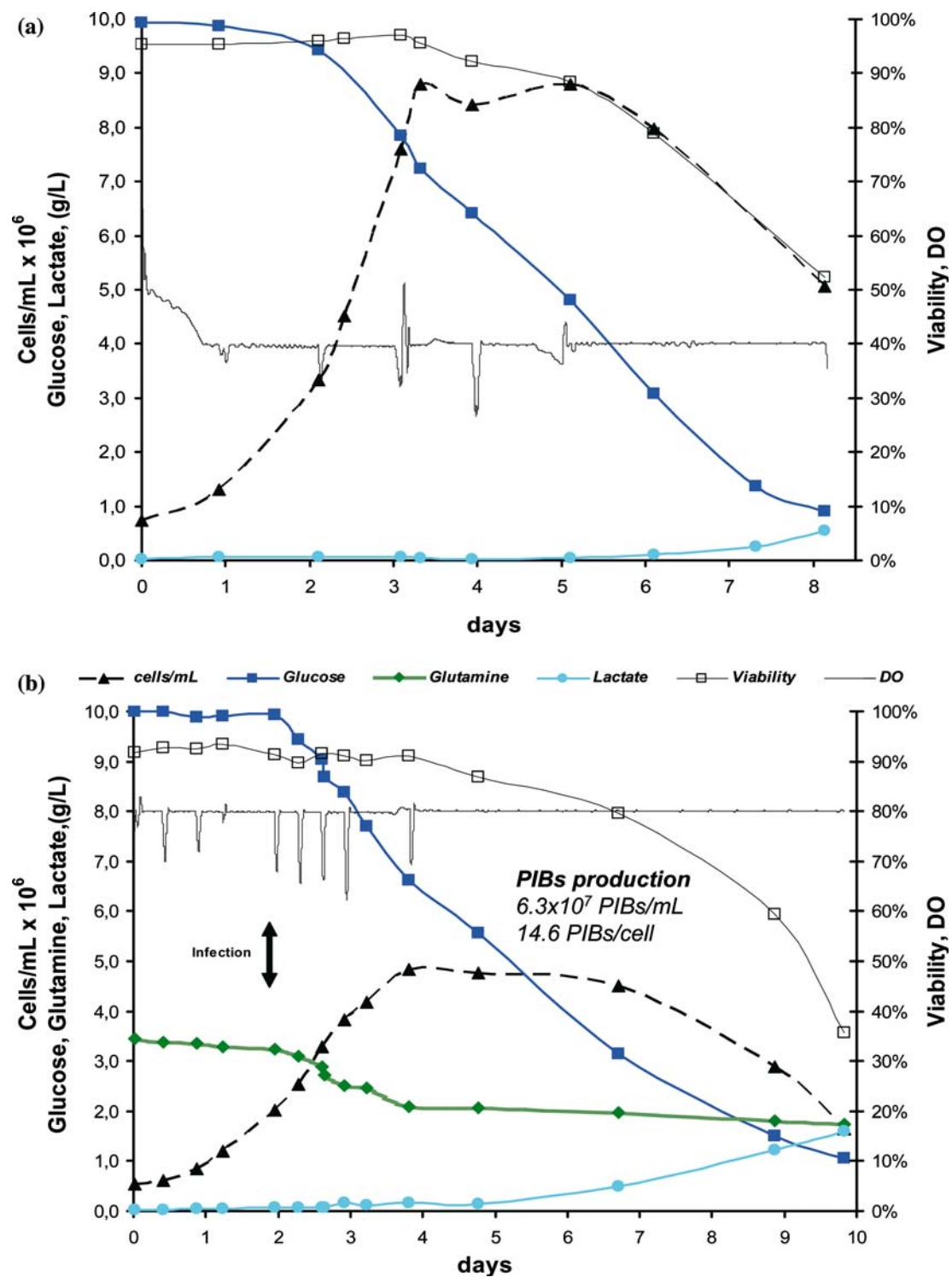
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*Figure 6.* Kinetics of Sf9 cell growth and metabolism in bioreactor under controlled dissolved oxygen concentration. Control (a) and infected (b) Sf9 cell cultures were performed in 1L medium at 28 °C and 100 rpm. AgMNPV infection was done at day 2 with MOI 1. Samples were daily collected for cell counting, expressed as viable cells×10⁶. Glucose, glutamine and lactate were quantified and expressed as g/l. PIBs were counted and expressed as PIBs/ml and PIBs/cell.

on similar systems (Ikonomou et al. 2003). The best quantitative performances of PIBs/cell production were obtained when cultures were infected with a MOI of 0.5 to 1 (Figures 4 and 5). When lower MOI was used, a higher concentration of PIBs/ml was obtained, which is in direct correlation to a higher cell concentration present in these cultures, where a decrease in cell multiplication due to virus infection is minimized (Figures 4 and 5). The virulence of PIBs produced in cultures infected at low or high MOI showed comparable $DL_{50}$, in spite of possible secondary viral infections taking place in the cultures infected with low MOI (Figure 4).

Studies performed to investigate the influence of MOI and CCI on the cell growth and PIBs production (Figure 5) have shown that, independent of the CCI used, higher MOI induced higher decrease in the maximum cell concentration in the cultures. MOIs ranging from 0.1 to 0.7 did not influence the maximum cell culture concentration when compared to non-infected cultures. The maximum PIBs concentration produced were quite low ($< 5 \times 10^7$ PIBs/ml) in cultures infected at high or low CCI (respectively, $3.5 \pm 1 \times 10^6$ and $0.5 \pm 0.1 \times 10^6$ cells/ml) and best results ($> 10^8$ PIBs/ml) were obtained when a CCI ranging from $10^6$ to $2.5 \times 10^6$ cells/ml was used

for infection, regardless the MOI used (from 0.1 to 5). Possibly, these poor performances of PIBs production with high or low CCI are linked to, respectively, the low specific cell growth and the low cell density present in the cultures at the infection moment. Based on these data we predict the following parameters as optimal for production: MOI 0.1 to 1.4 and CCI of $10^6$ to $2 \times 10^6$ cells/ml.

Assays of cell growth in a bioreactor showed that controlled DO allowed the cultures to reach the same final cell density ($9 \times 10^6$ cells/ml) but much faster (already at day 3). Upon infection, the culture attained a higher final cell density peak ($4.5 \times 10^6$ cells/ml) which remained for 72h (Figure 6). The PIBs production in the infected cultures performed in a bioreactor ($6.3 \times 10^7$ PIBs/ml, 14.6 PIBs/cell), as compared to that observed in shaker bottles, was higher in terms of PIBs/ml, but lower in terms of PIBs/cell. Nutrients consumption was shown to be higher than that observed in shaker bottles, possibly due to the better conditions provided by the DO availability. So, the Sf9 cell culture and AgMNPV infection in scaling-up conditions were shown to provide the cells with a suitable environment and potentially be capable of improving the shaker-Schott findings of cell growth and PIBs production. Nevertheless,
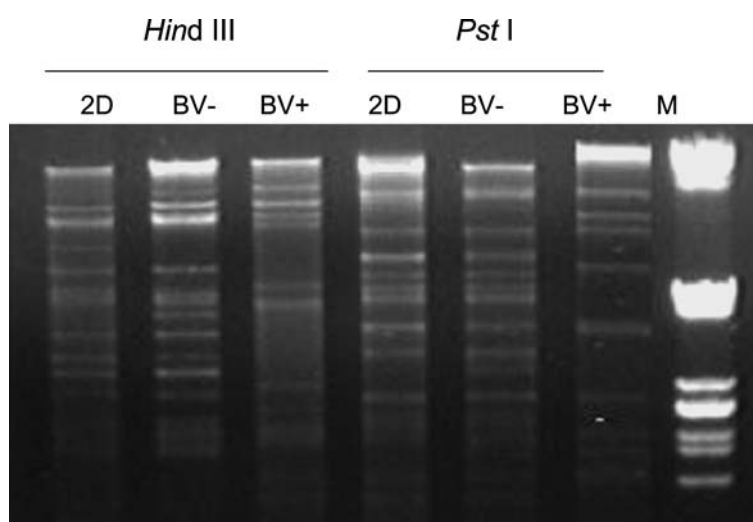


*Figure 7.* Restriction analysis of DNA from PIBs produced in cells infected with AgMNPV of low (BV-) or high passages (BV+). $10^6$ *Anticarsia gemmatalis* derived cells (UFL-AG-286) were infected with 1 MOI of budded viruses (BV) of AgMNPV isolates with low (1st passage) or high passages (6th passage) in Sf9 cell cultures (respectively, BV- or BV+), or with 1 MOI of the reference low passage AgMNPV-2D. At day 5 of infection the viral DNA was extracted from cell lysates, submitted to cleavage by the restriction enzymes (*Hin*dIII and *Pst*I) and the products were analyzed in agarose gel. M represents molecular weight standards.
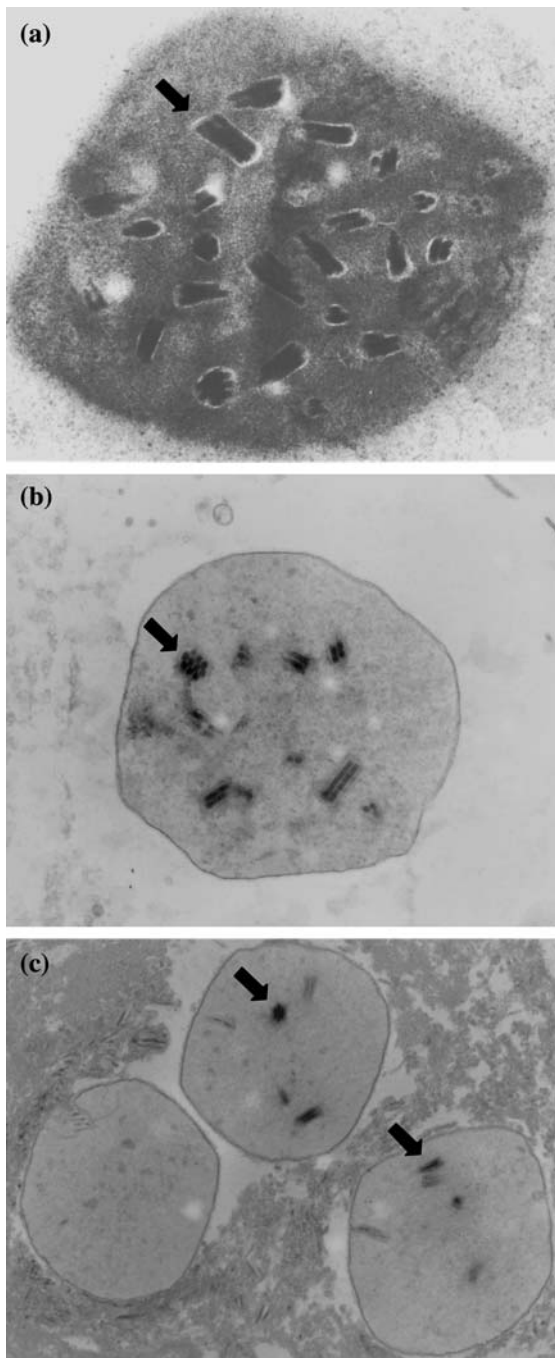
*Figure 8.* Transmission electron microscopy (TEM) of PIBs originated from Sf9 cells infected with AgMNPV of 1st (a), 2nd (b) and 4th (c) passages on Sf9 cell cultures. Arrows indicate the virions.

metabolic, shear stress are under investigation in order to improve and optimize the bioprocess performance.

For an accurate qualitative control of PIBs virulence, hemolymph from AgMNPV infected *Anticarsia gemmatalis* was used as starting material for passages in Sf9 cells and the PIBs production was tested for virulence in bioassays. Viral passages in Sf9 cell cultures led to a loss of virulence among the PIBs with an increase in the $DL_{50}$. During cell passages the loss of virulence was accompanied by a loss in BV titer, in the number of PIBs produced and also by an alteration of the DNA restriction pattern, indicating the synthesis of defective PIBs. At the 5th passage in Sf9 cells the PIBs were already not virulent enough to induce mortality (Table 1 and Figure 7). Ultrastructural studies, performed by TEM, revealed that after cell passages PIBs lacking virions were progressively synthesized (Figure 8).

Since one of the major characteristics of a Ag-MNPV bioinsecticide as a biological product is its ability to kill the larvae, the loss of virulence taking place among the PIBs produced in cell cultures, represents a great challenge to establish a cell culture bioprocess. It confers to this product a differential goal when compared to other viral cell culture products, such as vaccines, where a major characteristic is not virulence but immunogenicity. By not fully understood mechanisms, most of the viruses undergo a loss of virulence during cell culture passages and it is often welcome since attenuation is a desirable issue. A better comprehension of viral attenuation will be of utmost importance in virology and studies of baculovirus virulence control during cell passaging, would be a great contribution in view of establishing a bioinsecticide.

In conclusion, the study described here points out a biological constraint for the production of AgMNPV bioinsecticide, related to the loss of PIBs virulence, and a possible bioprocess issue, related to properties of insect cell growing under orbital shaker agitation, in view of dealing with biological and economical requirements for bioinsecticide production.

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