Special Issue on

Industrial Biotechnology-Made in Germany: The path from policies to sustainable energy, commodity and specialty products

Edited by:

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Establishment and Characterization of three New Embryonic Spodoptera littoralis Cell Lines and Testing their Susceptibility to SpliMNPV

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Abstract

Baculoviruses have a significant potential as biological pesticides. Spodoptera littoralis multicapsid nucleopolyhedrovirus (SpliMNPV) could thus find an application to protect plants against the African Cotton Leafworm. For the in vitro production of SpliMNPV a cellular system has to be established. For this purpose three new continuous cell lines were established from the embryonic tissue of the cotton leaf worm S. littoralis. The three cell lines were designated Spli-C, Spli-S and Spli-B. They consisted mostly of spherical cells, but also contained spindle and giant cells. The population doubling time for the three cell lines Spli-C, Spli-S and Spli-B were 30.5, 31 and 44.5 hrs, respectively, at passage 19, while at passage 120 it decreased to 26, 27 and 32 hrs, respectively. RAPD and DAF DNA fingerprint confirmed that the cell lines originated from S. littoralis tissues. The lactate dehydrogenase (LDH) isozyme analysis demonstrated a distinguishable difference between the three new S. littoralis cell lines and the other insect cell lines which we use in our laboratory. All three new cell lines were susceptible to SpliMNPV and thus are suitable for virus multiplication. Cells were immobilized using sodium cellulose sulfate (NaCS) and poly diallyl dimethyl ammoniumchloride (PDADMAC) capsules to protect cells from shear stress. This is caused during cultivation by agitation and gas sparging during supply of sufficient oxygen in order to reach high cell densities. The cell densities increased from 4-5x10^6 cells/ml in suspension culture to 1.3x10^7 cells/ml in capsules. Our results suggest that large-scale production of SpliMNPV as a biopesticide is possible with these cell lines.

INTRODUCTION

Insect cell culture is becoming an important tool in different fields of study, such as cell physiology, genetics, immunology, developmental biology and microbial pathology [1-3]. But also for biotechnological applications. Recently, insect cell cultures have been widely used in the production of viral insecticides [4, 5], recombinant proteins and vaccines [6-8]. The first continuous insect cell line was established by Grace in 1962 [9]. Since then many cell lines have been developed, but only very few of them are used in the industrial field.

S. littoralis is considered to be one of the most destructive agricultural insect pests, attacking more than 40 plant families, including about 87 economically important species such as apples, corn, cotton, tomatoes and potatoes. It undergoes many generations per year, and a significant part of the pest population has become resistant to chemical pesticides [10,11]. S. littoralis has been classified by the European and Mediterranean Plant Protection Organization (EPPO) as an A2 quarantine pest. In the US, it is considered an exotic organism with a high risk of becoming invasive [12,13].

The insect cell lines used for viral pesticide production have to be susceptible to the virus, support viral replication and produce high yield of budded virus and occlusion bodies. The cell lines also should be able to grow in suspension culture and in low cost serum free medium [14]. For commercial application, high cell densities offer advantages, e.g. in the production of viral pesticides in compact bioreactors with high volumetric production rates, but there are factors that may prevent the cells from reaching sufficient high densities. The most important factors are oxygen limitation and cell damage as a result of agitation and gas sparging [15,16]. Cell culturing in capsules can overcome these problems since a high shaking rate can be applied to supply a sufficient amount of oxygen without damaging the cells. The cell encapsulation technique provides many advantages beyond, increasing oxygen supply as, protecting cells from shaking stress, making the medium exchange process easy without losing cells, and facilitating product purification [17,15]. Euroferm (Erlangen, Germany) is one of the companies that use the cell encapsulation technique for the production of insect-pathogenic viruses as biopesticides. The aim of this study was to establish new cell lines from S. littoralis embryonic tissue and to test their ability to support SpliMNPV replication for in vitro production of a viral insecticide using the cell encapsulation technique. Three cell
lines were successfully established, which are to our knowledge the first cell lines established from *S. littoralis* embryonic tissue.

**MATERIALS AND METHODS**

**Insect cell lines and virus**

The following insect cell lines Sf21 (*Spodoptera frugiperda*), Tni (*Trichoplusia ni*) and Cp (*Cydia pomonella*) were used in this study. Additionally, SpilMNPV and fertilized *Spodoptera littoralis* eggs were used to establish the new cell lines.

**Primary cultures**

Primary cultures of cells were prepared from *S. littoralis* eggs (Syngenta, Switzerland). The eggs were placed in a mesh strainer and washed with 1% Triton X-100 in order to disinfect the surface of the eggs; eggs were immersed three times in 5% sodium hypochlorite solution for 5 min each. Then, they were placed in 70% ethanol for 20 min. Afterwards, eggs were washed with PBS buffer (pH 6.3). Disinfected eggs were crushed in the Hink’s TNM-FH medium (Sigma), which was supplemented with 20% FBS, by using a spatula. This forced the cells to go through the mesh strainer. Finally, the cell suspension was dispensed in 24-multiporous plates and incubated at 27°C. No antibiotics were used in this experiment.

**Cell maintenance and subculture**

Cells were maintained by replacing half of the old medium with the same volume of fresh medium every 7-10 days. The cells were subcultured when they had covered 90% of the growth area. The subculturing was achieved by flushing the medium over the cell monolayer, and then the cell suspension was split at a ratio of 1:2 with fresh medium. The cells were adapted for growth in suspension culture after passage ten and grown in EX-cell 420 medium instead of Hink’s TNM-FH medium, which was used in the initiation of the primary culture. The concentration of FBS was decreased gradually from 20% to 15%, 10% and 5% at passages 5, 11 and 31 until passage 65, respectively. From then on, the cells were subcultured every 2-3 days.

**Cell morphology**

The morphology of the obtained cells was observed and photographed by using a Nikon inverted microscope with phase contrast. The cell size histogram of the three *S. littoralis* cell lines, in addition to the Sf21 cell line, were analyzed using a Moxi automated cell counter.

**Cell line growth curve, population doubling time and MTT calibration curve**

The growth curves of the three cell lines (designated as Spli-B, Spli-C and Spli-S) were determined at passages 19 and 120. The cells were seeded at a density of 4-6x10^4 cells/ml in an Erlenmeyer flask containing EX-Cell 420 medium (Sigma) supplemented with 10% FBS at passage 19 and 5% at passage 120. The growth culture was incubated at 27°C, 50 rpm. The cell density was determined each day by using a hemocytometer. The population doubling time was calculated by plotting the cell density against the growth time. An exponential regression was used to estimate cell doubling time during the logarithmic phase of growth [18]. An MTT calibration test was done with 2-3x10^5 cells for each cell line harvested 24 and 120 hrs post-seeding. Five different measurements of cell densities were carried out for each cell line. The activity of the mitochondrial dehydrogenase enzymes was assessed by monitoring the conversion of MTT [19].

**Isozyme analysis**

In this experiment, lactate dehydrogenase (LDH) was used for isozyme analysis. The three new *S. littoralis* cell lines were compared to the three insect cell lines Sf21, Tni and Cp. The cells were harvested at the logarithmic phase and washed twice with phosphate buffer (pH 6.3) by centrifugation at 180g for 8 min. The cell pellet (5-6x10^6 cells) was suspended in lysis buffer (20 mM phosphate buffer pH 7.4, 500 mM NaCl and 2% Nonidet P40) and sonicated for 15 min at 4°C. Cell extracts were centrifuged at 13,000 g for 30 min at 4°C, and the supernatant was transferred to a new tube. Extracted proteins were separated on 8% polyacrylamide gel electrophoresis at constant voltage, 70 V, for 15 min, then 120 V for 80-120 min, and finally the gels were stained to detect LDH enzymes [20]. The migration distance of each isoenzyme band was recorded. The relative mobility (Rm) was estimated by measuring the ratio of the migration distance of isoenzyme bands to the migration distance of the standard cell line [21,22,23], i.e. the Cp cell line with a LDH is 23,058 Da (UniProtKB databases).

**DNA fingerprinting**

The three *S. littoralis* cell lines were genetically characterized by DNA amplification fingerprinting (DAF) using three pairs of DAF primers which were originally designed for PCR amplification of mamalian aldolase 5’ CCGGACGGAAAGGAG-3’, 5’-CACAT-ACTGGGACCGTCTCA-3’, interleukin-1β 5’-ATGAGGATGACTTGACTGGCTGATGTACCAGTT-3’, and prolactin receptor 5’-CTG GGA CAG ATG GAG GAC TT-3’, 5’-GAGGTGCTGATGTACCAGTT-3’ and mammalian aldolase 5’ CCGGACGGAAAGGAG-3’, 5’-CACAT-ACTGGGACCGTCTCA-3’, and mammalian aldolase 5’ CCGGACGGAAAGGAG-3’, 5’-CACAT-ACTGGGACCGTCTCA-3’.

**Cryopreservation**

The cells with high viability (~98%) were stored by harvesting the cells at 180 g for 8 min. The cell pellet was resuspended in freezing medium and cell numbers were adjusted to 5x10^6 cells/ml. Two freezing media were used to store the cells. The first comprised 90% EX-Cell 420 medium plus 20% FBS and 10% DMSO. The second comprised 90% EX-Cell 420 medium plus 20% FBS and 10% glycerol. The cell suspension was dispensed into sterile cryotubes. The cryotubes were frozen at -20°C for 0.5-2 hours and transferred to -80°C for permanent storage.
Testing the susceptibility of the S. littoralis cell lines to SpliMNPV

The virus stock was prepared from SpliMNPV occlusion bodies, according to Reid and Lua, 2005 [25] with some modification. The occlusion bodies were solubilized by incubation in alkaline solution (pH 11) at 27°C until they lysed and then the solution was neutralized by insect medium to a final pH 6.0-6.4. Finally, the virus solution was sterilized by filtration (0.22 µm) and stored at 4°C. The newly established S. littoralis cell lines were seeded at a density of 4x10^5 cells/ml in a 6-well plate and inoculated with the virus before incubation at 27°C. The cells were checked each day under a microscope to follow the infection progress. The cells were seeded in a shaker flask, infected with the virus (culture supernatant), incubated at 27°C, 50 rpm, and the cell number and viability were checked each day. The virus was harvested by centrifugation at 180 g for 8 min before the cell viability dropped to 80%. Virus stock was stored at 4°C for experiments.

Viral occlusion bodies purification and quantification

SpliMNPV was quantified by counting the viral occlusion bodies (OBs) under a microscope. Infected cells were harvested by centrifugation at 13,000 rpm for 30 min, the pellet was resuspended in polyhedralysis buffer (10Mm Tris, 1mM EDTA, 0.072% SDS), and sonicated for 15 min. Then, the samples were centrifuged again at 13,000 rpm for 30 min. The supernatant that contains cell debris was removed and the pellet was resuspended again in the polyhedralysis buffer and sonicated again. This process was repeated several times to remove all the cell debris and obtained only the OBs in the samples. After that, the purified OBs were washed and resuspended in ultrapure water and stored at -20°C. The OBs were counted under phase contrast microscope using a haemocytometer and OB amount was estimated using the following formula:

\[ \text{Occlusion bodies (OBs)} = \text{counted OBs} \times \text{dilution factor}. \]

S. littoralis cell encapsulation

The cell encapsulation was done by mixing sodium cellulose sulfate (NaCS; Euroferm/Germany) with poly dialyl dimethyl ammonium chloride (low molecular weight pDADMAC; Sigma) to produce hollow spheres. The NaCS solution was prepared at a concentration of 1.9 % (W/W) in PBS (pH 6.3) in a small beaker. The beaker was covered with paraffin and aluminum foil, and left at RT overnight. The solution was strongly agitated by stirring for at least 2 hrs until it was homogenized completely. Then it was sterilized in an autoclave at 121°C for 5 min. The solution was taken out immediately at the end of sterilization and cooled down with agitation at RT for at least 2 hrs.

The pDADMAC solution was prepared at a concentration of 1.2% (W/W) in PBS (pH 6.3) and one drop of Tween-20 was added to each beaker. The solution was then agitated with a magnetic stirrer until it was homogenized. The beaker was covered with aluminum foil and the solution was sterilized, including the magnetic stirring bar, by autoclave using the same conditions used to sterilize the NaCS solution.

A sterile syringe (5 ml) was fixed on a stand, a small sterile tube was joined to one end and passed through a peristaltic pump, and a sterile needle was fixed to the other end of the tube. Cells with a viability of 95-98% were harvested, the cell pellet (5x10^6 cells) resuspended gently in 0.25 ml FBS, and 4.75 ml NaCS solution was added and mixed gently. The cell suspension was transferred to the syringe and dripped into the pDADMAC solution beaker placed on a magnetic stirrer so that each drop formed a capsule. Afterwards, capsules were washed three times with PBS (pH 6.3). Finally, EX-Cell 420 medium was added to the capsules, which then were transferred into a baffled flask, and placed in an incubator at 27°C, 70 rpm. The medium was changed 2 hrs after encapsulation to remove remaining traces of pDADMAC. Culture medium was changed each 3 days during the first 10 days, and every day thereafter. The cell density within the capsules was estimated by weighting 3-4 capsules. To dissolve the capsules 5-10 fold cellulase were added, and incubated at 27°C for about 2 hrs. Finally, the cell number was determined using a hemocytometer.

RESULTS AND DISCUSSION

Primary cultures

The primary cell cultures were prepared by crushing disinfected eggs in insect medium. The suspensions of embryonic tissue fragments were dispensed in 24-multiwell plates and incubated at 27°C. The tissue fragments started to attach to the bottom of the multiwell plates after 1-2 days. Thirty days later, a network of nerve-like structures was observed. Many vesicles appeared in the early stages of primary culture initiation. Thereafter, cells started to migrate from the embryonic tissue segments. The culture included adherent and suspended cells.
Various tissues of lepidopteran insects were used to establish many continuous cell lines [26]. The embryonic, fat and ovarian tissues are the most commonly used source [27]. The immature embryonic tissue is the most successful source for development of cell lines [28]. The opportunity to obtain a continuous cell line from immature tissue is higher than from mature tissues, because the embryonic tissues consist of many undifferentiated actively proliferating cells [26]. Therefore, in this study, we used embryonic tissues to get the first cell lines established from *S. littoralis*.

**Cell subculture**

The first successful subculture was achieved two months after establishing the primary culture. At the beginning, the cells were attached strongly to the bottom of the well. However, cells started to lose adherence and became easy to remove from the well bottom after six passages. Two weeks after establishing the first subculture, the second subculture was obtained, while the third subculture was established ten days later. Furthermore, after a few passages the cells started to grow quickly, which further shortened the period between the subcultures. At present, cells are subcultured every 2-3 days. Since the cells became well-adapted to the growth conditions, in EX-Cell 420 medium supplemented with 5% FBS, the split ratio was gradually increased from 1:2 at the beginning to 1:10. To prolong cell life, medium supplemented with 5% FBS, the split ratio was gradually increased. After a few passages the cells started to grow quickly, which further shortened the period between the subcultures. At present, cells are subcultured every 2-3 days. Since the cells became well-adapted to the growth conditions, in EX-Cell 420 medium supplemented with 5% FBS, the split ratio was gradually increased from 1:2 at the beginning to 1:10. To prolong cell life, medium supplemented with 5% FBS, the split ratio was gradually increased from 1:2 at the beginning to 1:10. To prolong cell life, medium supplemented with 5% FBS, the split ratio was gradually increased from 1:2 at the beginning. At present, cells are subcultured every 2-3 days. Since the cells become well-adapted to the growth conditions, in EX-Cell 420 medium supplemented with 5% FBS, the split ratio was gradually increased from 1:2 at the beginning to 1:10. To prolong cell life, medium supplemented with 5% FBS, the split ratio was gradually increased from 1:2 at the beginning to 1:10. To prolong cell life, medium supplemented with 5% FBS, the split ratio was gradually increased from 1:2 at the beginning to 1:10.

**Cell morphology**

The results showed that the populations of the Spli-B, Spli-C and Spli-S cell lines are heterogeneous. Moreover, at the beginning they were composed of different shapes and sizes, such as fibroblast-like cells, spherical cells, spindle-like cells, and trapezoid-like cells. After several subcultures, the dominant shapes were spherical but some spindle and giant cells were still detectable. The mean diameter of the Spli-C, Spli-S, and Spli-B cells are 16.65, 14.2 and 18.46 µm, respectively. The existence of various forms of cells during establishing cell lines was reported by other authors [29]. This was foreseeable since embryos differentiate into different tissues and thus will contain different types of cells [26]. Embryonic fragments will therefore lead to heterogeneous cell populations as has been demonstrated in this project. The cell size of the Spli-C, Spli-S, and Spli-B cell lines was within the assumed range size (5-20.11 µm) for insect cells [30].

**Cell line growth curve, population doubling time and MTT calibration curve**

The growth curves of the three cell lines were determined in suspension culture. The maximum population density of the Spli-C and Spli-S cells were 4-5x10^6 cells/ml, while for Spli-B it was 6x10^6 cells/ml. The growth rate of the Spli-C cell line was 0.582 (d^−1), which was higher than the other two cell lines, while the Spli-B cell line showed the slowest growth rate about 0.373 (d^−1). The population doubling time for Spli-B, Spli-C and Spli-S at passage 19 was 44.5, 30.5 and 31 hrs, respectively; while at passage 120 it changed to 32, 26 and 27 hrs, respectively. The three cell lines did not undergo a lag phase and the saturated phase occurred five days post cell seeding for Spli-C and seven days for Spli-S and Spli-B. The MTT calibration curve show stable slopes for all three cell lines when compared both at 24 and at 120 hrs. The cell growth curves are shown in Figure 2.

The maximum population density of the Spli-C, Spli-S and Spli-B cell lines was higher than other cell lines established from *S. littoralis* by other researchers. According to Knudson et al. 1980 [29], the maximum cell density of the *S. littoralis* UIV-SL573 cell line, which was established from the pupal ovary tissue, was 3x10^6 cells/ml 6-7 days post-seeding. This increment in cell density in this project could be because different tissues were used in establishing the corresponding cell lines.

**Isozyme analysis**

In order to distinguish between different insect cell lines, isozyme analysis was used as a protein fingerprint using...
polyacrylamide gel electrophoresis. It is well known that isoenzyme analysis using polyacrylamide gel is sufficiently sensitive to obtain clear isoenzyme patterns and distinguish between different cell lines [20]. The lactate dehydrogenase (LDH) isozyme analysis revealed one clear and sharp band for each cell line. The particularly fast \textit{S. littoralis} enzyme bands indicate that the \textit{S. littoralis} LDH has a molecular weight less than the other Lepidoptera cell lines tested. The LDH enzyme bands of the three \textit{S. littoralis} cell lines are identical. The relative mobility (Rm) of the Spli-C, Spli-S and Spli-B cell lines was 1.6, confirming that these three cell lines belong to the same species. The SF21 enzyme band is very close to the \textit{S. littoralis} bands, with an Rm of 1.47, which could be because they belong to the same genus \textit{Spodoptera}. On the other hand, the isozyme profile of the Tni cell line, with an Rm 0.67, differed significantly from the \textit{S. littoralis} cell lines, proving that there was no cross-contamination with the other insect cell lines used in our laboratory (Figure 3).

**DNA fingerprinting**

DNA fingerprinting was also used to confirm the identity of the new cell lines. Because cross-contamination is quite common in laboratories, it is necessary to use the DNA fingerprint to confirm the identity of cell lines and to detect any contamination. The DAF and RAPD are important DNA fingerprinting techniques that are widely used to identify cell lines and both can successfully distinguish between different cell lines [24,31]. This is because each species is genetically unique and a specific DNA fingerprint exists for each one [32].

The results of RAPD and DAF were different from one species to the other and from one primer to the other. Different DNA band numbers and sizes, ranging between 0.15 and 3 Kb, were observed. The three \textit{S. littoralis} cell lines showed similar DAF profile patterns with the aldolase primers. Six bands at approximately 340, 400, 500, 650, 700 and 920bp were seen in both the Spli-S and Spli-C. In the host five bands were recorded, which were equal in size to the bands obtained from Spli-S and Spli-C DNA preparations. Spli-B gave six bands and five of these bands were also similar to the host. Spli-B had one band at approximately 450 bp not found in the host, Spli-C or Spli-S. On the other hand, the SF21 showed five bands while seven and six bands were produced from Cp and Tn cell lines, respectively (Figure 4).

With the interleukin-1β primers, Spli-C and Spli-S showed six bands at approximately 200, 500, 600, 765, 1500 and 2400 bp and five of these bands were shared with the host, which also had five bands. Five bands were generated from Spli-B cells, with four bands being similar to the host and band five a different size at approximately 600 bp. The other insect cell lines SF21, Cp, and Tn clearly showed different patterns, where six bands were observed in SF21 and three and four bands were recorded in Cp and Tn respectively (Figure 4). The prolactin receptor primers failed to amplify in any case and thus there were no bands observed on the agarose gel. This could be due to the lack of complementary sequences for this set of primers in the insect cells’ genome [33].

The RAPD results with primer 2 showed five bands (310, 400, 600, 700 and 1250 bp) that were identical in the Spli-C, Spli-S and Spli-B as well as in the host. While with primer 1, the Spli-B showed a slightly different pattern from the other two. Three bands (2000, 2250 and 3000 bp) identical to those in the host were observed in the Spli-C and Spli-S cell lines, while the Spli-B cell line showed only two bands identical to the host. The third band, at about 2000 bp, was missing from the profile (Figure 5).

The DNA profile of the three \textit{S. littoralis} cell lines were identical to the host (eggs) DNA profile confirming that the new cell lines originated from the \textit{S. littoralis} species. The new cell lines clearly showed profile patterns different from the SF21, Tn and Cp cell lines, which are used in our laboratory as well.

The two DNA fingerprint techniques, DAF and RAPD,
successfully confirmed again that the three *S. littoralis* cell lines are nearly identical and belong to the same species and that they differ from the other insect cell lines that are used in our laboratory, even though they are closely related. The Spli-B cell line showed a slightly different pattern than Spli-C and Spli-S with the interleukin-1β primers and RAPD primer 1. This could be due to DNA methylation or mutation that may have prevented primers from annealing to this site in Spli-B genomic DNA. A comparable observation was recorded by McIntosh and his group in 1996 [24].

**Testing the susceptibility of the *S. littoralis* cell lines to SpliMNPV**

After establishing and characterizing the new cell lines, their susceptibility to SpliMNPV was tested to observe, if these cell lines are susceptible to the virus infection and also to determine the highest virus producer cell line. SpliMNPV successfully infected and replicated in all three cell lines. Cytopathic effects, such as hypertrophy of nuclei slow cell growth and large cell size, were observed after 1-2 days post-infection. Viral occlusion bodies (OBs) were observed inside the cell nuclei after 2-3 days and after 4-5 days many OBs were produced in the cells. A few days post-infection, some of the infected cells were lysed and OBs were released into the culture medium (Figure 6).

These observations indicate that the new *S. littoralis* cell lines were susceptible to SpliMNPV and they supported viral replication. The same observation was noticed by Shih [34]. The Viral occlusion bodies are highly detectable during the late stage of the viral infection, accumulated inside the infected cells, and were later released into the culture medium after cell death. The NPV OBs can be easily identified under a microscope due to their large size range from 0.15 to 15 µm [35,36].

In suspension culture, the cell density of infected cells

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**Figure 7** The growth curve of Spli-C cells for both uninfected and infected with SpliMNPV. Cells were cultured in EX-Cell 420 medium supplemented with 5% FBS at 27°C, 50 rpm.

**Figure 8** (A) The growth curve of the Spli-C cells in capsules and in suspension culture. (B) Spli-C cell growth in capsules after 1, 9, and 14 days (for both, uninfected and infected cells with SpliMNPV).
dramatically decreased compared to uninfected cells, and the growth rate of the infected cells was lower than noninfected cells. The cell viability also decreased after two days post infection as shown in Figure 7.

The Spli-C cells showed high susceptibility to the virus and high production of OBs (data not shown). The slow growth rate of the infected cells was expected since cell division of permissive insect cells undergoes arrestment when the cells are infected with baculovirus [37].

**S. littoralis** cell encapsulation

The Spli-C cell line that showed higher susceptible to Spli MNPV and higher viral occlusion bodies production (1x10⁷ OBs/ml) than the other two cell lines (data not shown) was immobilized using NaCS and PDADMAC. The resulting capsules were 3 mm in diameter. The Spli-C cells grew well aggregated inside the capsules, the cell growth rate in capsules was 0.553 (d⁻¹) and the cells filled the capsules after about two weeks. The maximum cell density of the Spli-C cells in capsules reached 1.3x10⁷ cells/ml while it was only 4-5x10⁶ cells/ml in suspension culture (Figure 8). The Spli-C cell encapsulation led to a roughly 3-fold increase in cell density. The Spli-C cells in the capsule were infected with SpliMNPV at MOI 1 to analyse the infection process in capsules, where the infected cells showed, as expected, lower cell densities compared to uninfected cells (Figure 8).

The cell encapsulation technique provides many advantages, such as increasing oxygen supply and protecting cells from shaking stress, which is an important requirement for increasing the cell densities and the product yield [15]. Therefore, increasing in Spli-C cell density was obtained due to cell culture in capsules. This is in good accordance with the literature [38,15]. As a result of increasing cell densities per ml by using this cell culture system, an increase viral particle production could be achieved. Son et al. 2006 [38] recorded that the production of polyhedral inclusion bodies (PIBs) in encapsulated cells increased 58 fold compared to cultivation in suspension culture. Insect cells need more oxygen compared to mammalian cells, and virally infected insect cells consume even more oxygen than uninfected cells [39]. Therefore, cell encapsulation is a good way to provide enough quantities of oxygen to the cells. Cell encapsulation is attractive for continuous large-scale production of baculovirus [40].

**CONCLUSION**

Due to the harmful effects of chemical pesticides on the environment and human health, in addition to pest resistance against these agents, alternatives in general and ecological alternatives in particular are urgently needed. Biopesticides are considered as such viable alternatives since they specifically target the pest and lack harmful effects to humans and the environment. SpliMNPV, as a biopesticide, is used actively to control the cotton leaf worm *S. littoralis* spest, which is considered the major pest on cotton and other economically important plants. Currently, the *in vivo* production, i.e. the controlled multiplication of the virus in the living pest organism, is the only method used for production of SpliMNPV as a commercial biopesticide product, despite the fact that it has several disadvantages such as the requirement for intensive labor and the poor quality of its product.

In this study, we developed biotechnological system for Spli MNPV production applying cell culture, where the viral pesticide produced using *in vitro* methods is of good quality and free of microbial contamination. But the main advantages of it are easy, cheap and space saving production conditions. Three new cell lines were developed and characterized and found to be able to support SpliMNPV replication. Immobilization led to particularly good growth in a baffled shaker flasks under suboptimal culture conditions. However, optimization is still required to obtain high cell densities and high viral OBs yield in a bioreactor system as necessary for an industrial product. Furthermore, the isolation of pure clones from the heterogeneous cell population of the best producer cell line, i.e. is needed, in order to develop a high virus producing cell colony. But our results already clearly demonstrate the feasibility of large scale production of SpliMNPV to be used as biopesticide using the cell encapsulation system.

The biopesticide market is rapidly growing by around 16% each year and the demand for the biopesticides is expected to rise steadily [41]. Since the market is predictable to reach $3.2 billion by 2017 and $4.5 billion by 2023 according to Global Industry Analysis, Inc. and Lux Research, the SpliMNPV pesticide market is expected to increase as well. with the biotechnological production system described above this need can be served easily and with good quality to secure important sectors of agricultural production. With the SpliMNPV agent a potent and urgently needed biopesticide with particularly friendly ecological properties will become available for such important agricultural products like corn, cotton, tomatoes and potatoes.

**ACKNOWLEDGEMENT**

The authors would like to thank Roland Reist and Oliver Kindler (Syngenta Crop Protection, Switzerland) for providing *S. littoralis* eggs and David Gryzwacz (University of Greenwich, UK) for providing *S. littoralis* NPV isolates. The authors would also like to thank Anette Amtmann for technical assistance. We would also like to thank the German Academic Exchange Service (DAAD) and the Ministry of Higher Education in Iraq for the scholarship to Ibrahim Ahmed that helped make this project possible. Special thanks are attributed to Cynthia L. Goodman (USDA/ARS/BCIRL, USA). Our thanks to Andreas Perlick (Institute of Bioprocess Engineering, Friedrich Alexander Universität, Erlangen-Nürnberg, Germany) for reviewing and correction of the manuscript.

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