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BIOREACTOR PRODUCTION PROCESS OF *Spodoptera frugiperda multiple nucleopolyhedrovirus* BIOPESTICIDE

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ABSTRACT

Spodoptera frugiperda (fall armyworm) is one of the most important maize pests in the world and the baculovirus *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV), a natural pathogen of this pest, has been used as a biopesticide for its control. At present, *in vivo* strategies at the commercial scale are employed by multiplying the virus in the host insect in biofactory facilities; however, *in vitro* large-scale production is an interesting alternative to overcome the limitations of baculoviruses massal production. This study aimed to develop the process of the SfMNPV *in vitro* production by evaluating the effects of different multiplicities of infection (MOI) and nutritional supplements, morphological and molecular analysis of the infection on the growth of Sf9 cells and virus production. The Bioreactor Stirred Tank Reactor (STR) approach with glutamine-supplemented Sf-900 III serum free culture medium, combined with the MOI of 1.0, showed the best viral production performance, with a specific productivity above 300 occlusion bodies (OBs)/cell and volumetric productivity of 9.0 x 10^{11} OBs/L.

Keywords: *Spodoptera frugiperda*. *in vitro* production. baculovirus. biological control. bioreactor.

1 INTRODUCTION

The fall armyworm (FAW) *Spodoptera frugiperda* is native to Western Hemisphere tropical regions, from the United States to Argentina [1]. It's a polyphagous noctuid, considered the most important insect pest in maize crops [2], with recent reports in Africa and Asia [3,4]. This pest has been controlled with chemical pesticides and genetically modified maize, generating resistant biotypes [1,5]. The *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV - *Alphabaculovirus*, *Baculoviridae)* is pathogenic to FAW, therefore has been considered as an alternative. There already are reports of successful use of SfMNPV as biopesticides [6,7]. Its massal production is done by infecting healthy larvae in biofactories, but it has disadvantages such as intensive labor required, increased costs, cannibalistic larval behavior and contamination problems [7,8]. Bioreactor *in vitro*-produced virus offers greater process control, better quality in smaller space [8]. The Sf9 cell line is compatible with wild SfMNPV isolates' replication [9], however virus's serial passage in cell may generate Few-Polyhedra (FP) mutants. FP-mutants may produce low yields with low virulence and with advantageous rate of virus replication [10]. At laboratory scale, SfMNPV *in vitro* production is considered commercially viable when it reaches 300 OBs/cell [8]. Therefore, this work aimed to scale up SfMNPV *in vitro* production, optimizing cell growth and viral parameters and compare OBs production with different MOIs to increase cost-benefit ratio.

2 MATERIAL & METHODS

2.1. Cell Line, Virus, and Medium

Sf9 cell line (ATCC code 12659-017) stock was maintained at 28ºC under 130 rpm agitation speed in an orbital shaker (Innova 4000, New Brunswick Scientific, Edison, NJ, USA). The SfMNPV-19 virus isolate were used at a stock suspension of 4.65 x 10^6 pfu/mL and was previously obtained [9]. All cell cultures used Sf-900™ III Serum Free Medium (Gibco, Waltham, MA, USA) supplemented with glutamine (Merck) at a proportion of 1.0 g/L at the end of the exponential growth phase.

2.2. Cell Culture in Bioreactor and Cell Growth Analysis

The cells were grown in Bioreactor Stirred Tank Reactor (STR) BioFlo 110 (New Brunswick Scientific) or Biostat B (Sartorius, Göttingen, Germany) with a working volume of 1.0 L. The following parameters were adopted: temperature 28ºC, Dissolved Oxygen (DO) 30% of air saturation, agitation 80 rpm, aeration flowrate of 200 mL/min, and initial cell concentration of 5 x 10^5 cells/mL. The bioreactor samples were typically collected twice a day for cells and were counted in a Neubauer chamber under phase-contrast microscopy at 200x. The viable cells were quantified with 0.4% Trypan blue (Sigma-Aldrich, San Luis, MO, USA).

2.3. Effect of Multiplicity of Infection (MOI) on SfMNPV *In vitro* Production

The influence of two different MOIs in the SfMNPV production was investigated: one batch with an MOI of 0.1 (Run 1), and another batch with an MOI of 1.0 (Run 2). The viral inoculum was tittered by the serial dilution method [11] and the medium tissue culture infectious dose (TCID50) value was calculated according to [12]. The virus infection was carried out when the cells reached the concentration of 3.5 x 10^6 cells/mL. Cell growth was monitored for viral infection parameters for 11 days in the batch with MOI 0.1 (Run 1) and 17 days in the batch with MOI 1.0 (Run 2).

2.4. OBs Analysis – Quantification, Visualization, Molecular Evaluation and Viral DNA Quantification

SfMNPV OBs were obtained from the bioreactor samples centrifuged and counted in a Neubauer chamber under phase-contrast microscopy at a 400x magnification. Transmission Electron Microscopy of SfMNPV-infected cells was performed at 7 days post infection (dpi) to confirm the quality of produced OBs. The samples were prepared according to [13] and visualized at the Zeiss TEM 109 electron microscope. Bioreactor samples inoculated with MOI 0.1 (Run 1) were submitted to PCR analysis and Sanger sequencing (Macrogen, Korea) to verify possible modifications in genes related to OB formation and structural nucleocapsid and envelope proteins. Specific primers were designed for polh, protf, fp25k, pp34, and p10 genes [14, 15]. The sequences were compared to the SfMNPV-19 sequence from GenBank (10455) using ClustalW in the MEGA X program [16]. Samples from bioreactor inoculated with MOI 0.1 (Run 1) and MOI 1.0 (Run 2) were submitted to qPCR analysis to compare the viral DNA quantification to OBs quantification as described in [15]. *In vivo*-produced OBs were extracted for absolute quantification. The qPCR for SfMNPV with specific primers for the sf32 gene was carried out in a Rotor gene 5plex HRM platform (Qiagen), according to [17].

3 RESULTS & DISCUSSION

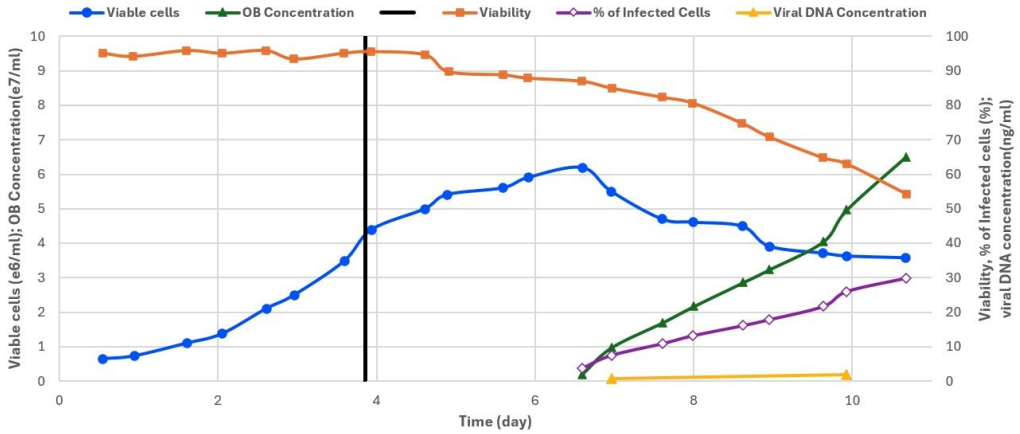

Figure 1 Run 1 - Sf9 cell growing in glutamine–supplemented bioreactor and infected with SfMNPV-19 at day 3.9 (MOI 0.1). Samples were collected for cell-counting, expressed as viable cells/mL, and viability (%). OBs were counted and expressed as OBs/mL. Cells with OBs were expressed as % of infected cells. The time axis was shifted to adjust the lag growth phase between runs. The vertical line represents infection time.

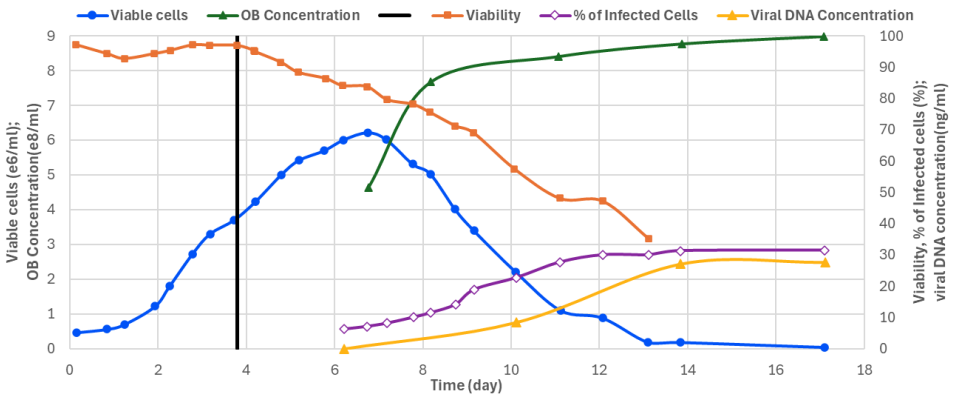

Figure 2 Run 2 - Sf9 cell growth in glutamine-supplemented bioreactor and infected with SfMNPV-19 at day 3.8 (MOI 1.0). Samples were collected for cell-counting, expressed as viable cells/mL, and viability (%). OBs were counted and expressed as OBs/mL. Cells with OBs were expressed as % of infected cells. Viral DNA concentrations were expressed in ng/mL. The vertical line represents infection time.

Table 1 Maximal viable cell and SfMNPV (OB) concentrations in Sf9 cell cultures using two multiplicities of infection (MOI). Percentage of viable cells that were infected, relation between OB and infected cells and concentration of viral DNA at 7dpi in these runs.

Run	MOI	Maximum Viable Cell Concentration (x10^6 cell/mL)	Maximum SfMNPV Concentrations (x10^7 OBs/mL)	Percentage of Infected Cells (%)	OBs/ Infected Cell	Viral DNA (ng/mL)
1	0.1	6.2	6.5	30.0	30.4	2.03
2	1.0	6.2	90.0	32.0	339	8.72

3.1. Effect of Viral Inoculum on SfMNPV Production

In Run 1 and 2, cell concentration peaked after about 2.5 dpi as the cell viability dropped and infection progressed (Figures 1 and 2). Run 2 was carried out for longer, but it is clear that, after about 10 dpi, cell infection was saturated even with the percentage

of cell infection reaching around 30% (Figure 2). although the maximum viable cell concentration and percentage of infected cells were similar in both experiments, the batch with a higher MOI presented better viral yields (Table 1). The increase in the MOI for 1.0 (Run 2) resulted in higher volumetric (9.0 x 10^8 OBs/mL) production of SfMNPV, as well as higher specific (340 OBs/cell) production since the percentage of infected cells were similar.

3.2. Molecular Analysis - Quantitative PCR Standardization and Sequencing Analysis of SfMNPV Produced in Bioreactor

To check the accuracy of qPCR quantification as a predictor for virus OBs production, the qPCR quantification of viral DNA was performed at 3, 7, 11 and 14 dpi in the bioreactor inoculated with MOI 1.0 (Run 2). The curve in viral DNA presented a fast-growing rate from 3 to 11 dpi, and then stabilized until 14 dpi. A similar pattern was observed for the curve of OBs/mL, as well as for the curve in the percentage of cells with OBs, indicating the accuracy of qPCR quantification as well as the best time for the viral quantification and showing a consistent correlation with other viral parameters (Figure 2). The analysis of agarose gel electrophoresis of PCR fragments revealed that all the samples had the expected gene size (754 bp—polh, 1217 bp—prot f, 625 bp—fp25k, 1063 bp—pp34, 495 bp—p10), indicating that bioreactor production with MOI 0.1 did not cause selection for defective viruses in these essential structural genes. The nucleotide sequences of these samples did not present any mutation or indel that induced amino acid changes compared to the sequence of the wild SfMNPV-19 isolate.

3.4. Morphological Analysis of SfMNPV Production

The optical microscopy from 0 to 7 dpi in all bioreactor batches with SfMNPV revealed the progress of infection over time, with an increasing number of cells presenting typical baculoviruses infection symptoms (cell hypertrophy, cell lysis, and dark nuclei). Ultrastructural analysis of the infected Sf9 cells showed production of many OBs compared to non-infected cells as well as virogenic stroma, nucleocapsids and virions formation. Another concern regarding SfMNPV *in vitro* production is the virulence loss due to the presence of few polyhedra mutants, aberrant OBs morphology, few virions inside OBs and defective interfering genotypes [10,18,19]. The ultrastructural analysis revealed OBs and virions without signals of critical modifications.

4 CONCLUSION

Based on the presented results, we conclude that the main factor of the infection process is to achieve a balance between an efficient MOI and the risk of generating mutants. Higher MOIs are more predictable, reproducible, and adjustable through time of infection [20], while lower MOIs tend to prevent mutant formation. The MOI of 1.0, despite presenting a similar percentage of cell infection as the MOI of 0.1, resulted in a specific productivity that was 10 times higher. This optimization enables high-scale production since it reached the minimum amount recommended by [8] for commercial production. A bioreactor with 300 L capacity, operating 20 times per year, with an average production of 10^{12} OBs/L, could produce enough virus to be applied to 60 thousand ha/year. Consequently, the *in vitro*-produced SfMNPV has the potential to protect the maize and other crops infested by FAW. It is essential, however, that the produced OBs have a similar virulence to those obtained from larvae. Further studies on the optimization process and evaluation of insect mortality (bioassays) by viruses produced in a bioreactor must be encouraged.

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