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Mass Production of HzSNPV Baculoviruses in Immobilized *Heliothis zea* (HzAM1) Insect Cell Culture

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Abstract *Heliothis zea* (HzAM1) insect cells were immobilized in microspheres by sodium-cellulosesulfate (NaCS) and polydiallyldimethylammoniumchloride (PDADMAC). The highest HzAMl cell density was 7.5×10^7 cells/mL in the microspheres. After infection of the immobilized cells by *Heliothis zea* single nuclear polyhedrosis virus (HzSNPV), the highest concentration of HzSNPV (polyhedral inclusion bodies: PIBs) produced was 2.87×10^{10} PIBs/mL in the microspheres.

Keywords: HzAM1 insect cell, HzSNPV, NaCS, PDADMAC, PIBs

INTRODUCTION

As an alternative to the use of chemical pesticides, the baculoviruses are valuable for use as biopesticides, due to their nonpathogenic properties in vertebrates, and being harmful to their insect host only [1,2]. Up until now, the production of baculoviruses has been performed in an insect cell culture system *in vitro*. However, the application of this system has been mostly limited to a bench scale application. One of the major problems with this system is that the cells are very shear-sensitive and are damaged by gas sparging and agitation. For insect cell cultures, this is a serious problem, since the cells are structurally more sensitive and demand more oxygen upon virus infection [3,4]. The lytic nature of the baculovirus-insect cell system also presents significant processing limitations on both production rate and product recovery trains [5,6]. Sf21 insect cells have been successfully immobilized and infected with AcMNPV by King *et al.* [7,8]. They were able to achieve about 8.0×10^7 cells/mL of Sf21 cell density in the microspheres using alginate/poly-L-lysine. When the cells were infected with an AcMNPV, a virus titer of 1.0×10^9 viruses/mL in the microspheres was reached. This was approximately 10 times higher than suspension culture at that time.

Therefore, the use of the use of an immobilization system has been shown as a solution for overcoming the above problems. The immobilized cells are protected from shear stresses due to gas sparging and agitation units and have an efficient oxygen supply, hence, achieving high cell density and product concentration [9-13].

In this study, *Heliothis zea* (HzAM1) insect cells were immobilized with sodium-cellulosesulfate (NaCS) and polydiallyldimethylammoniumchloride (PDADMAC) and cultured. After infection by HzSNPV baculoviruses, the production of polyhedral inclusion bodies of the baculovirus was investigated in the immobilized culture system.

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MATERIALS AND METHODS

Cell Culture and Virus Infection

Heliothis zea (HzAM1, Cynthia Goodman, USDA-ARS: USA) insect cells were cultured in SF900II medium (GIBCO BRL, USA) in shaker flasks. The suspended-cell viability and density were determined by the Trypan blue and the MTT-test methods. For the MTT-test, 1 mL of cell solution was taken from the cultured flasks and mixed with 0.25 mL of 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazoliumbromid (Sigma, USA 5% (w/v) MTT in PBS) and incubated at 27°C, in the dark, for 4 h Then, 1 mL of the incubated solution was added to 4 mL of lysis buffer (405 mL iso-propanol, 20 mL 1NHCl, and 75 mL SDS (20% (w/v)) and the mixture was supersonicated. The cell debris was removed by centrifugation at 3,000 rpm for 10 min, and the absorbance (λ=570 nm) of the supernatant was measured using a spectrophotometer.

When the cell density reached 1.0×10^6 cells/mL, with a viability above 90%, the cells were infected at a MOI (multiplicity of infection) of 1.0, by adding a virus-stock solution (MOI of 0.1) of HzSNPV (*Heliothis zea* single nuclear polyhedriosis virus, Cynthia Goodman, USDA-ARS: USA). The virus titer was determined by the end-point dilution assay [5]. The infected cells were then cultured in a culture flask.

Cell Immobilization

For encapsulation of HzAM1 cells, microspheres were produced in a one-step process. Sodium-cellulosesulfate

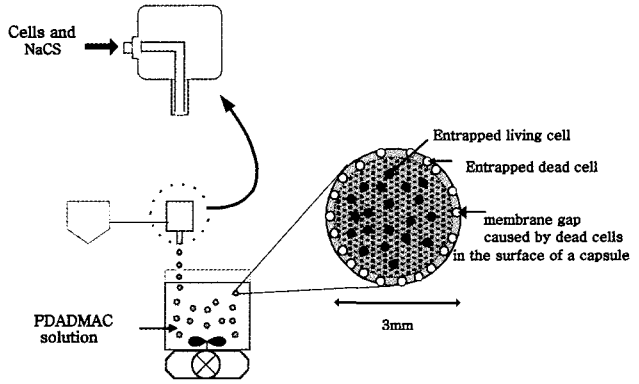

Fig. 1. Apparatus for encapsulation. The size of the produced microspheres is about 3 mm. The microspheres have membrane gaps, due to the death of cells protruding from the membrane of a sphere in the PDADMAC solution during stirring.

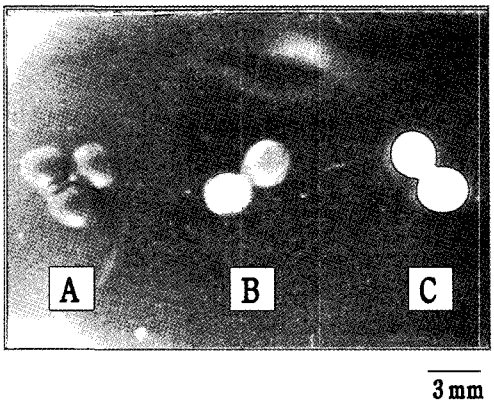

Fig. 2. Immobilized HzAM1 cells after an MTT-test. (A: at 72 h, B: 0 h and C: starving cells)

(NaCS, 3.5% (w/v), Technical University of Berlin, Germany) and poly-diallyldimethylammoniumchloride (PDA DMAC, 2.2% (w/v), Clariant GmbH, Gendorf, Germany) were prepared in 1% (w/v) NaCl solution and autoclaved (121°C, 10 min). The procedure of cell immobilization is shown in Fig. 1. Briefly, an encapsulation apparatus (Institute of Biotechnology, Technical University of Berlin, Germany) was connected by a silicone tube to the storage bin containing NaCS and the HzMA1 cells. The volume flow was adjusted to 200 mL/h. After dropping the mixed solution of NaCS and HzAM1 cells into the PDADMAC solution, the crosslinking reaction between the polymers was limited to the interface area of a drop, thereby, producing a stable membrane around a liquid core containing the cell suspension. The produced microspheres were stirred in the PDADMAC solution for 15 min, then washed extensively 3 times with phosphate buffered saline (0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8 g/L NaCl, and 1.15 g/L Na_2HPO_4), and finally transferred into culture flasks for cultivation.

Immobilized Cell Culture and Virus Infection

The microspheres (7.5 mL, wet volume) containing the cells were cultured in 50 mL of SF900II serum free medium in a 500-mL shaker flask with 4 baffles, at 27°C and 80 rpm. The medium was changed every two days. Ten microcapsules were taken daily from the culture flask for measurement of the immobilized cell density by the MTT-test.

When the immobilized cells reached the stationary growth phase in the microspheres, the cells were infected at a "theoretical" MOI of 1.0 with the previously prepared virus-stock solutions. The "theoretical" MOI of 1.0 was determined by the immobilized cell density, which was measured by the MTT-test before viral infection, as follows:

$$\text{"Theoretical" MOI} = \frac{\text{virus}\,[\text{mL}^{-1}]}{\text{cells in the hollow spheres}\,[\text{mL}^{-1}]} = 1.0$$

The MOI value is generally used to describe suspension culture experiments. Unlike suspension culture, most cells are hidden inside the microspheres and not all cells may be initially infected by the virus particles. Only the cells located next to pores or in the membrane of a sphere would be directly infected. Hence, the MOI value in these experiments is a theoretical value only and therefore referred to as the "theoretical" MOI. The medium was changed every day after viral infection.

Counting of HzSNPV (polyhedral inclusion bodies: PIBs)

One mL of the sample in the suspension culture or 5 microspheres of the immobilized culture which was homogenized in 20% (w/v) SDS solution, were centrifuged at 13,000 rpm for 15 min. The pellets were extensively washed in lysis buffer (1.21 g/L Tris, 0.37 g/L EDTA, and 0.72 g/L SDS) and supersonicated until cell membrane and debris were removed. Then, the samples were centrifuged again at 13,000 rpm for 15 min and the lysis buffer was added. This process was repeated until only PIBs remained. The PIBs in the clear solution were counted using a haemocytometer.

RESULTS

Fig. 2 shows the microspheres containing viable cells at 72 h (A), 0 h (B), and starving cells (C), after the MTT-test of the immobilized cell culture. The immobilized cell densities in the microspheres were 4.0×10^6 cells/mL at 0 h and 1.1×10^7 cells/mL at 72 h.

The highest cell density in the microspheres was 7.5×10^7 cells/mL. This was about 22 times higher than that in the suspension culture, which had 3.4×10^6 cells/mL (data not shown).

Production of Baculoviruses in the Suspended and Immobilized HzAM1 Cell Cultures

In the previous work, suspended HzAM1 cells were in-

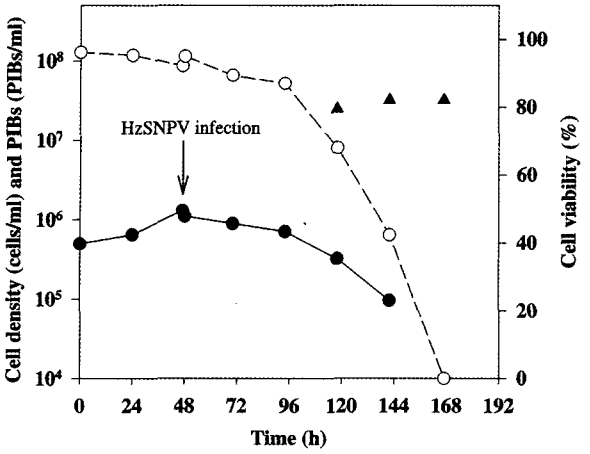

Fig. 3. Production of polyhedral inclusion bodies (PIBs) in suspended HzAM1 insect cell culture. The cells were infected by HzSNPV at a MOI of 1.0 (○: cell viability,●: cell density,▲: produced PIBs).

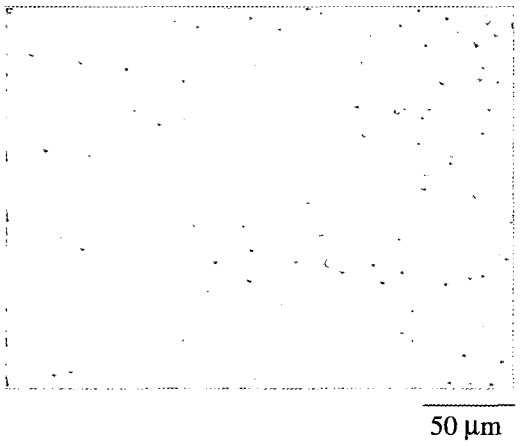

Fig. 4. HzSNPV polyhedral inclusion bodies (PIBs) produced by HzAM1 cells. The PIBs in clear solution could be detected under the microscope as being 2~5 μm in diameter.

fected with HzSNPV (MOI of 1.0) at the stationary phase for improving of the production of PIBs with the high cell density. However, the highest concentration of PIBs was not high (about 8.0×10^6 PIBs/mL) due to the limitations of oxygen and nutrients [2,5]. Thereby, in this study, suspended HzAM1 cells were infected with HzSNPV (MOI of 1.0) at the exponential phase as shown in Fig. 3. After the virus infection, the infected cell density significantly decreased within 96 hpi (hours post infection). The highest PIBs (polyhedral inclusion bodies) concentration of HzSNPV achieved was 3.2×10^7 PIBs/mL at 96 hpi (Fig. 3).

The PIBs produced were detected under the microscope at 48 hpi, as being about 2~5 μm in diameter, as shown in Fig. 4.

The immobilized HzAM1 cells were infected with the baculoviruses at a "theoretical" MOI of 1.0, in the stationary phase at 120 h, as shown in Fig. 5. After the virus infection, the medium was changed every day for maintenance of the optimal production environment. The PIBs attained a density of greater than 1.0×10^8 PIBs/mL, at 72 hpi in the microspheres. The PIB concentrations increased in the capsules even further, up to 1.0×10^{10} PIBs/mL. The highest PIBs concentration gained was 2.87×10^{10} PIBs/mL in the microspheres at 240 hpi. Additionally, the PIBs produced, up to 1.0×10^7 PIBs/mL, could be detected daily in the spent medium, after 72 hpi.

The production of PIBs, in the suspension and immobilized cultures, is shown in Table 1. The production rate of PIBs in the suspension culture (3.2×10^7 PIBs/mL for 4 days) was lower compared to that in the microspheres (6.0×10^8 PIBs/mL per day). The production term of the suspension was just 4 days, due to termination because of

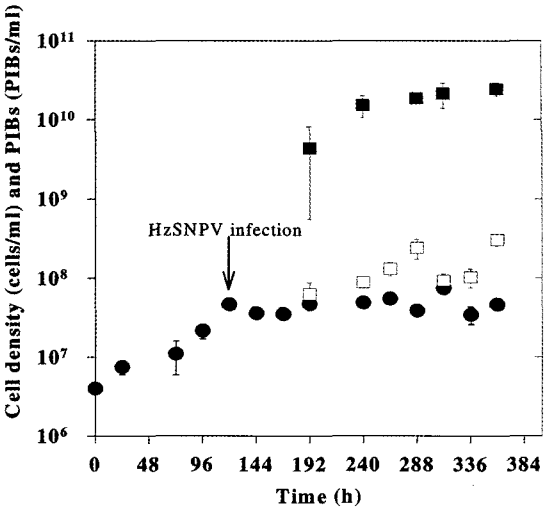

Fig. 5. Production of polyhedral inclusion bodies (PIBs) in immobilized HzAM1 insect cell culture. The cell density was measured by the MTT-test, and the cells were infected by HzSNPV at a "theoretical" MOI of 1.0 (●: immobilized cell density, ■: PIBs in the microspheres, □: PIBs in the spent medium).

Table 1. Production of PIBs in suspension and immobilized HzAM1 cell cultivation

Value	Suspension cultivation	Immobilized cultivation (in microspheres)
PIBs/mL & Production time (day)	3.2×10^7 (4)	6.0×10^8 (1)[c] 2.87×10^{10} (10)[d]
Relative productivities	1	75
TOI[a] (1.0×10^6 cells/mL)	1	50
Specific PIB productivity in 4 dpi[b] (PIBs/cell)	32	48

[a] TOI: time of infection: the cell density when the virus infection
[b] day post infection
[c] concentration of the produced PIBs/mL for one day
[d] concentration of the produced PIBs/mL for 10 days

the death of the infected cells. The relative PIBs productivity in immobilized HzAM1 cell culture was 75 times higher when compared to that in the suspension cultivation.

DISCUSSION

Unlike the cells in a suspension, immobilized insect cells could not be measured by trypan blue exclusion, since they form strong, dense cell clusters and are entrapped in the capsule membranes. Thus, it is difficult to separate the cells from each other, without destruction of a large portion of the cells to be counted. In fact, the immobilized cell densities counted by the trypan blue method, after rupturing the capsules, were always 10~30% lower than the values determined by the MTT-test, which does not rupture the spheres. However, this difference becomes 5% less when the MTT values of the cells, entrapped by the ruptured capsule membrane, were added to the values counted by the trypan blue method. This indicates that a fraction of the viable cells were excluded from the trypan blue method and destroyed, even after rupturing the capsules. Therefore, immobilized cell densities were determined by using MTT calibrated regressions by an MTT-test. In addition, noninfected and infected Cf-2C1 cell densities in the microspheres, measured by the MTT-test methods, were compared with the value determined by the trypan blue exclusion method. The result showed that the baculovirus infection did not cause any difference in the MTT conversion rate, which was less than 4% [14]. Therefore, the MTT-test method could be a simple test to use to determine the immobilized insect cell densities before and after virus infection.

In the immobilized state, a higher cell concentration and production rate, with the relative high TOI (50 times higher) could be achieved, as compared to that in the suspension culture. The specific PIBs productivity, of the immobilized cells for 4 days (48 PIBs/cell), was also higher than that in the suspension culture (32 PIBs/cell), as shown in Table 1. This indicates that a high TOI does not necessarily lead to a decrease in the productivity. It seems to be more important to maintain the optimal culture conditions with minimal biological and physical stress to the cells. The optimal culture conditions could be achieved in this immobilized culture process, thus, the baculovirus infection might occur in a secondary infection cycle, similar to *in vivo* conditions, by BVs (budded viruses) that are produced by prior infected cells in the capsules. Therefore, the supply of sufficient oxygen and nutrients were no different for both, the infected and noninfected cells in this immobilized culture system.

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