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An Immobilized Culture of *Choristoneura fumiferana* Cells for High Production of CfMNPV

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Abstract *Choristoneura fumiferana* (Cf-2C1) insect cells were cultured and immobilized by using cellulosesulfate (NaCS) and polydiallyldimethylammoniumchloride (PDADMAC). A concentration of CfMNPV (*Choristoneura fumiferana* multiple-nucleopolyhedrovirus) and a Cf-2C1 cell density in the microspheres have been achieved at the densities of 1.57×10^{10} PIBs/ml and 7.5×10^{7} cells/ml, respectively. Additionally, MTT-test was used to measure the viable cell density in the microspheres, and the confidence of MTT-test was investigated before and after baculovirus infection in the immobilized cell culture.

Key words: Cf-2C1 cells, CfMNPV, immobilization, MTT-test, polyhedral inclusion bodies

Baculoviruses are an alternative to the use of chemical pesticides for the control of insect pests in agriculture as well as an expression vector for the production of recombinant proteins of medical and pharmaceutical importance [9, 10, 12]. At present, various baculovirus-insect cell culture systems for scale-up have been investigated to achieve the commercial production of baculoviruses.

Cell immobilization has been shown to be one system for high production of baculoviruses, which protects cultured cells from shear stresses due to agitation and sparging that are common by used methods for supply of sufficient oxygen to cells [1-7, 15, 17], so that cell density and a high product concentration can be expected. Viable cell densities have been measured by trypan blue exclusion in animal cell cultures: Trypan blue is a stain recommended for use in dye exclusion procedures for viable cell counting. The principle involved is that live cells treated with trypan blue solution do not become dyed, whereas dead cells are dyed to an intensive blue color due to destruction and permeabilization of their cytoplasma membrane. Therefore, the staining facilitates the visualization of cell morphology. In immobilized culture systems, however, the trypan blue exclusion is not valid for the measurement of the viable cells, because the cells are entrapped by capsule polymer, thus making it impossible to distinguish between dead and living cells. In this study, Cf-2C1 cells were cultured and immobilized. The immobilized cell density was measured by MTT-test, and the effect of the baculovirus infection during the MTT-test in the microspheres was then studied to test the confidence of MTT-test in the immobilized culture.

Choristoneura fumiferana (Cf-2C1, Sardar S. Sohi: Canadian Forest Service, Canada) cells were cultured in SF900II (GIBCO BRL) medium, and their viability was determined by trypan blue method. Also, the viable cell density was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazoliumbromide: Sigma-Aldrich) test for comparing with trypan blue exclusion [8, 11, 14]. The medium was changed at 72 h. CfMNPV (*Choristoneura fumiferana* multiple nuclear polyhedrosis virus; Sardar S. Sohi: Canadian Forest Service, Canada) were used to produce virus stock solutions and polyhedral inclusion bodies (PIBs) of CfMNPV. When the insect cell densities reached 1×10^{6} cells/ml on the late exponential growth phase with the cell viability above 90%, the cells were infected with CfMNPV at a MOI (multiplicity of infection) of 0.1. The baculoviruses were harvested, when the viability of the host cells dropped below 80%. Host cells were removed by centrifugation (600 rpm, 10 min), and the supernatant containing the viruses was stored at 4°C in the dark for use as a virus stock solution. The viruses were titered by the end-point dilution method [13]. Polyhedral inclusion bodies (PIBs) of CfMNPV were produced in the suspended Cf-2C1 culture. When the cell densities reached 1×10^{6} cells/ml with the cell viability above 90%, the cells

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were centrifuged (900 rpm, 5 min) and the spent medium was removed. Then, the virus stock solution was added into the cells at a MOI of 1.0. Finally, the infected cells were cultured in 50 ml of fresh medium.

Cf-2C1 cells were immobilized by using 3.5% sodium-celluosesulfate (NaCS, Department of Bioprocess Engineering, TU-Berlin, Germany) and 2.2% poly-diallyldimethylammoniumchoride (PDADMAC, Clariant GmbH, Gendorf, Germany) in 1% NaCl solution. NaCS containing the cells was dropped into the PDADMAC solution. The drops were stirred in the PDADMAC solution for 15 min and transferred to a culture flask for incubation [16]. The immobilized cells (7.5 ml) were cultured in 50 ml of SF900II medium. The medium of the immobilized cells was changed every two days before CfMNPV infection and every day after the virus infection. Immobilized Cf-2C1 cell densities were measured by both MTT-test and trypan blue exclusion before and after virus infection in the stationary growth phase to evaluate whether MTT-reaction depends on the virus infection. The microspheres (noninfected or infected with viruses) were gently ruptured, and the cells released were carefully washed out with fresh medium. A portion of the suspended cells was counted by trypan blue and the other part was tested by MTT-test. When the immobilized cells reached the stationary growth phase in microspheres, the cells were infected by using CfMNPV at a "theoretical" MOI of 1.0 with the prepared virus-stock solution. The "theoretical" MOI of 1.0 was determined by the immobilized cell density that was measured by MTT-test as before the viral infection as follows:

$$\text{"Theoretical" MOI} = \frac{\text{virus }[1/\text{ml}]}{\text{cells in the hollow spheres }[1/\text{ml}]} = 1.0$$

Generally, MOI values are used to describe suspension culture experiments. Unlike in suspension culture, not all cells in a microsphere might be infected by the virus particles initially, since most cells are hidden inside of the microspheres. Only few cells located next to pores or in the membrane of a sphere would be directly infected. Hence, the MOI value in these experiments was a theoretical value, therefore, referred to as "theoretical" MOI. The PIBs (polyhedral inclusion bodies) produced from the ruptured capsules were counted by a microscope, as determined by Son [16]. One ml of the sample in the suspension culture or 5 microshperes in 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 polyhedra lysis buffer (1.21 g/l Tris, 0.37 g/l EDTA, 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 polyhedra lysis buffer was added. This process was repeated until the microscopic inspection showed that only PIBs remained in the sample. Polyhedral inclusion bodies in the lysis buffer were seen as 2- 5 μm large particles with 0.4% trypan blue exclusion under a light microscope (×200).

The very first polyhedral inclusion bodies could be detected inside of the insect cells at 24 h after the infection. The clear solution containing polyhedral inclusion bodies were counted with a hemacytometer and calculated by:

PIBs (PIBs/ml)=counted PIBs×dilution factor

Figure 1 shows polyhedral inclusion bodies of CfMNPV in Cf-2C1 cell culture at 48 hpi (hour post-infection).

Suspended Cf-2C1 cells were cultured for production of polyhedral inclusion bodies of CfMNPV. In the previous work [14], time of infection (TOI) was determined with 1.0×10^6 cells/ml at a MOI of 1.0 for high production of polyhedral inclusion bodies of CfMNPV. After infection of CfMNPV in the suspension culture, the infected cells did not grow for about 3 days, and the cell density decreased significantly. The infected cells could not be cultured after 6 days post-infection, because of the death of infected cells. The highest polyhedral inclusion bodies of CfMNPV obtained were 2.83×10^7 PIBs/ml at 120 (hour post-infection) (Fig. 2).

After MTT test of the immobilized cells, color from the cells in microspheres could be visuable. Figure 3 shows

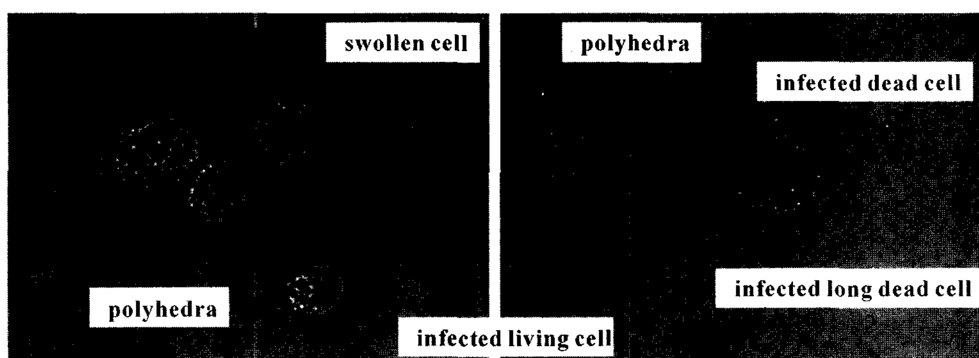

Fig. 1. Polyhedral inclusion bodies of Cf-2C1 cells at 48 hpi.

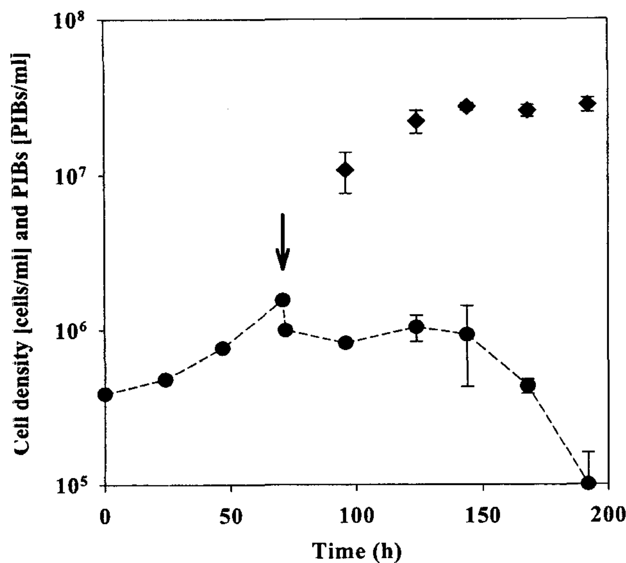

Fig. 2. Suspension culture of Cf-2C1 cells and CfMNPV infection (↓ : the time of CfMNPV infection at a MOI of 1.0).

the immobilized Cf-2C1 cells before (Fig. 3A) and after MTT-test (Fig. 3B, C, D, E). The colors produced by the enzymatic reaction between MTT and dehydrogenase in mitochondria of living cells were differentiated (Fig. 3C), however, the colors of microspheres containing dead cells were not changed by MTT-test (Fig. 3D). The sphere size was about 3 mm in diameter.

Cf-2C1 cell density was measured by MTT-test to compare with the trypan blue method to see whether the MTT reaction was stable under different culture conditions of suspended and immobilized cultures. Thus, the suspended cell densities were measured by trypan blue exclusion method, and the samples with the cells for MTT-test were taken at different incubation times: 24 h, 72 h and 144 h. Also, the relationship of cell densities between trypan blue method and MTT method was determined. As shown in Fig. 4, there was a good relationship between trypan blue and MTT-test, showing that the enzyme reaction of MTT is invariably constant regardless of cell growth phases.

Immobilized Cf-2C1 cell density was evaluated by both MTT-test and trypan blue exclusion methods. The immobilized cell density measured by MTT-test can be determined by the following equation:

$$CD\ (\text{cells/ml}) = K_{MTT} \times A$$

where CD is the viable cell density in the microspheres [cells/ml], K_{MTT} determined in Fig. 4 as 0.83×10^6 that is the average slope values of $1/1.2 \times 10^{-6}$ (1/ml), and A is a true absorbance including dilution factor in MTT-test. Figure 5 shows the noninfected (Fig. 5I) and infected (Fig. 5II) Cf-2C1 cells in the immobilized cultures. The cell densities were measured by MTT-test. In the Fig. 5I, the highest cell density reached 7.5×10^7 cells/ml in the microspheres, starting with the inoculation cell density of 4.5×10^6 cells/ml in the microspheres. The capsule samples were taken at 600 h, 768 h and 840 h for the measurement of cell density by using the trypan blue method. In infected immobilized Cf-2C1 cell culture (Fig. 5II), the highest cell density determined by MTT-test reached 7.0×10^7 cells/ml in the

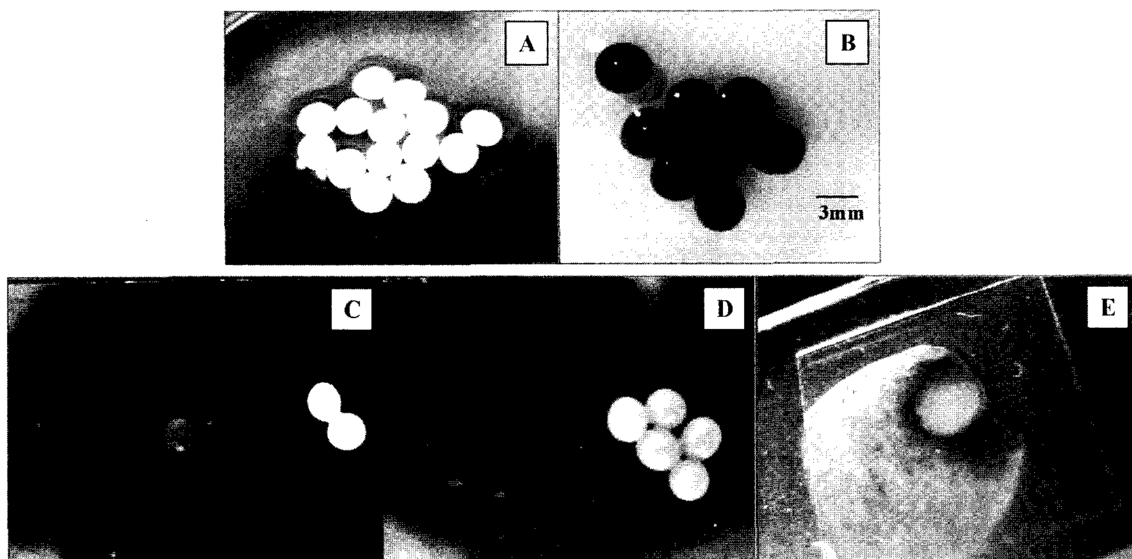

Fig. 3. Immobilized Cf-2C1 cells after the MTT-test (b/w-pictures).
A: capsules containing living cells before the MTT-test; B: capsules containing living cells after the MTT-test; C: capsules containing different concentrations of cells after the MTT-test; D: capsules containing living cells (left) and dead cells (right) after the MTT-test; E: capsule membrane and condensed cells after rupturing a capsule.

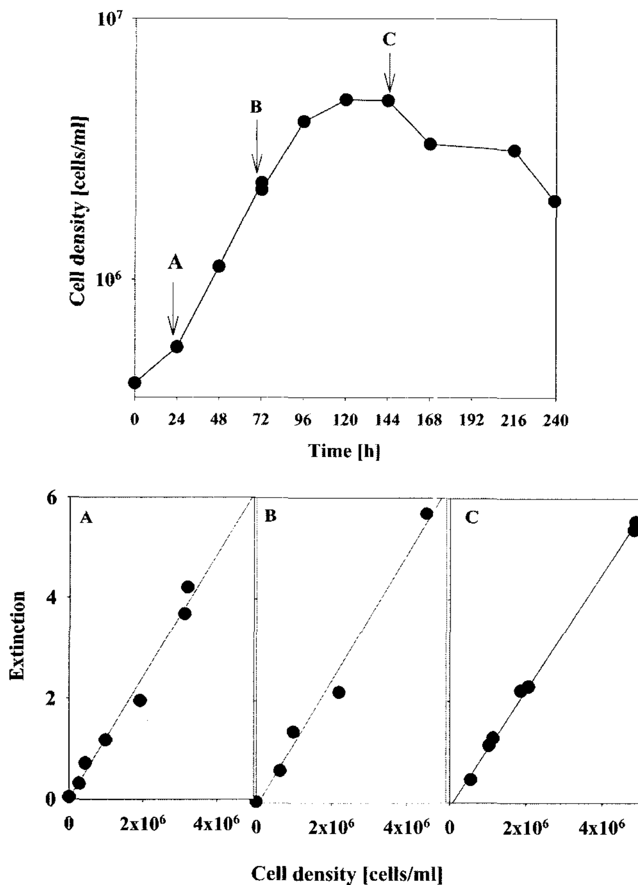

Fig. 4. Growth of Cf-2C1 cells determined by the trypan blue method.

The samples for MTT-test on different growth phases were taken at 24 h, 72 h, and 144 h as indicated by (↓). The cells were centrifuged at 72 h and fresh medium was added. The MTT calibration curves of the samples are shown in the graphs below (in the different growth phases at 24 h (A), 72 h (B) and 144 h (C)). The average value of the slopes is 1.2×10^{-6} (ml).

microspheres, starting with the inoculation cell density of 4.88×10^6 cells/ml in the microspheres. Polyhedral inclusion bodies produced by the immobilized cells increased significantly. Then, the highest concentration of polyhedral inclusion bodies obtained in the microspheres was 1.57×10^{10} PIBs/ml. The capsule samples for trypan blue exclusion were taken at 624 h and 840 h. The relationship between MTT-test and trypan blue method in the immobilized conditions before and after CfMNPV infection were evaluated, as shown in Fig. 5 (right). In particular, the regression differences throughout the cultivation period were not significant before and after virus infection. This indicates that there is no considerable difference between noninfected and infected insect cell metabolism regarding the dehydrogenase reaction with MTT.

In the case of the suspension culture, the MTT reaction was independent of cell growth phases. In the case of immobilized culture, the MTT reaction test could not be performed during the lag and the exponential growth phases, since it was difficult to wash out and maintain the living cells from the capsules during the test. Thus, the immobilized cells in the stationary phase were tested, and slopes of MTT-test in suspension and immobilized culture are shown in Table 1. The difference of K_{MTT} between noninfected and infected cells in immobilized culture was 0.04×10^6 (1/ml), indicating that the variation within the experimental results was less than 4%, therefore, not significant. The average K_{MTT} could be correlated to 0.83×10^6 (1/ml) in all the cell cultures of noninfected and infected conditions.

Therefore, MTT-test is applicable to determination of the viable insect cell densities in the immobilized system. The trypan blue exclusion is the common method used for measuring living cell density and cell viability. Unlike the cells in suspension condition, immobilized insect cells could not be measured by trypan blue exclusion, since they strongly form dense cell clusters and are entrapped in the polymer membranes. Thus, it is difficult to separate the cells from each other without destruction of a more or less 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 less by 5%, when the MTT values of the cells entrapped by the ruptured sphere membrane were added to the values by the trypan blue method. This shows that a fraction of viable cells was 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 the MTT-test. Noninfected and infected Cf-2C1 cell densities in the microspheres measured by the MTT-test 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. Regardless of infection or not, the MTT conversion and consequently the formazan formation were constant. The average MTT regression value of 1.2×10^{-6} (ml) was also constant. In the immobilized Cf-2C1 cell culture, the difference of MTT regression between infected and noninfected cells was less than 4%. The average value of the total slopes in the MTT calibration curves of suspension and immobilized cultures was about the same to 1.2×10^{-6} (ml). Finally, the enzyme reaction between MTT and dehydrogenase in mitochondria of viable cells was demonstrated to be not associated with the viral infection factor in insect cell culture. The highest immobilized cell density per ml volume reached about 15.6 times higher than the highest cell density in the suspension culture, and the highest polyhedral inclusion bodies of total culture volume in the immobilized culture were about 83 times

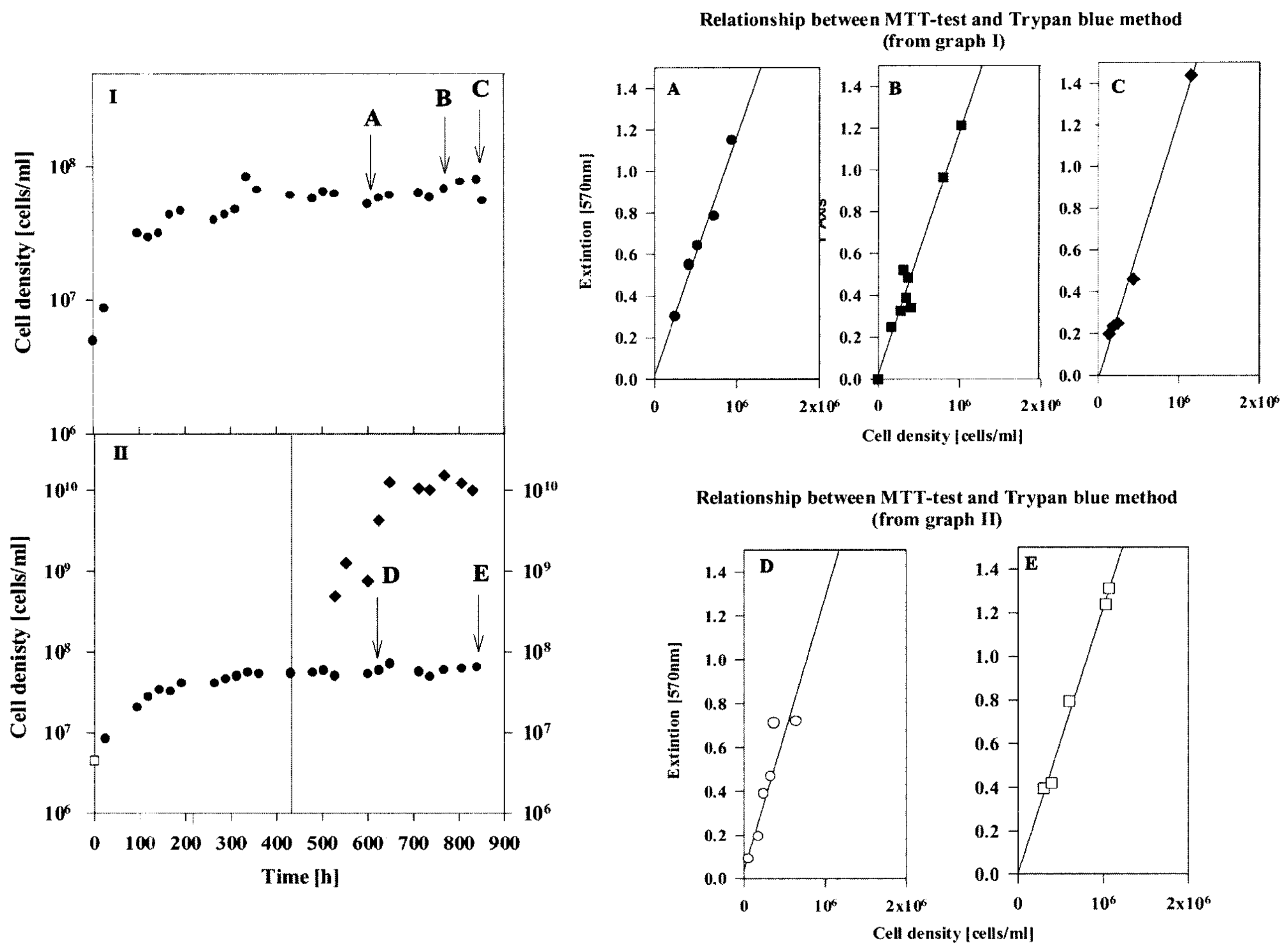

Fig. 5. Noninfected (I) and infected (II) Cf-2C1 cell cultures in immobilized condition.
The cell densities were measured by the MTT-test. In the graph I, the samples of noninfected cells for trypan blue exclusion were taken at 600 h, 768 h and 840 h as indicated by (↓). And in the graph II, the samples of infected cells for trypan blue exclusion were taken at 624 h and 840 h as indicated by (↓). (●: the cell density in the capsules, ◆: polyhedral inclusion bodies in the capsules, line: the time of CfMNPV infection). MTT-test calibration curves of the samples are shown in the right graphs (A: 600 h, slope: 1.15e-6; B: 768 h, slope: 1.16e-6; C: 840 h, slope: 1.25e-6; D: 624 h, slope: 1.24e-6; E: 840 h, slope: 1.22e-6).

higher than that in the suspension culture. This higher concentration of polyhedral inclusion bodies could have been obtained by the higher immobilized cell density, because of the optimum culture condition and sufficient supply of oxygen and nutrients to the cells without shear stresses. In conclusion, therefore, the high concentration of polyhedral inclusion bodies of CfMNPV could be obtained in the immobilized Cf-2C1 cell culture system by controlling the cell densities in the microspheres by simply using MTT-test.

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Table 1. K_{MTT} values of Cf-2C1 cells by the MTT-test.

	Suspension culture (Fig. 4)		Immobilized culture (Fig. 5)	
	Noninfected	Infected	Noninfected	Infected
Average of slopes	1.2×10^{-6}	-	1.18×10^{-6}	1.23×10^{-6}
Determined K_{MTT} (1/ml)	0.83×10^{6}	-	0.85×10^{6}	0.81×10^{6}
Average determined K_{MTT} (1/ml)		0.83×10^{6}		

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