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Evaluation of the serum-free medium MDSS2 for the production of poliovirus on Vero cells in bioreactors

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Abstract

The serum-free medium MDSS2 (Merten *et al.*, 1994), was used for cultivating Vero cells as well as for producing poliovirus (Sabin type 1) in static and in perfused microcarrier cultures. At slightly different growth rates of 0.0120/h and 0.0106/h, respectively, static cultures in serum-containing (SCM) and serum-free (SFM) medium produced titers of $10^{6.75}$ and $10^{6.67}$ TCID₅₀ per 50 μ l; signifying a specific productivity of 0.89 and 1.07 TCID₅₀/c.

Serum-free bioreactor cultures of Vero cells on DEAE-dextran microcarriers at 6.25 g/l produced cell densities of about 1.5×10^6 c/ml. After infection with virus (multiplicity of infection (MOI) 0.1–0.3) titers of about 6.3×10^8 TCID₅₀/ml were obtained, signifying an average specific productivity of 7.1 TCID₅₀/c.h. Although these values were 4 and 2 fold, respectively, higher than in classical resum-based production processes (Montagnon *et al.*, 1981), a reference culture, for which cell growth was done in SCM and only virus production was done in SFM, produced 2×10^9 TCID/ml with an average specific virus production rate of 18.9 TCID₅₀/c.h. The differences between the fully serum-free and our reference process were mainly due to physiological differences of cells grown in SCM and SFM and also due to strongly modified consumption kinetics after virus infection leading to limitations of one or several essential medium compounds, like glucose and amino acids. Avoiding these limitations by increasing the residual concentration of glucose, glutamine, histidine, and SH-amino acids, led to specific virus production rates (of about 17.9 TCID₅₀/c.h.) comparable to those found in the reference virus production process. The optimisation of the production of the poliovirus (Sabin 1) will be described with respect to the modification of the medium composition.

Introduction

The introduction of polio vaccines has largely contributed to human health in developed countries, where paralytic poliomyelitis could be practically eradicated. However, this infectious disease is still endemic in developing countries and nontemperate areas. Although the WHO projects a complete eradication of poliomyelitis at the year 2000 (WHO 1988, 1990) vaccination programmes have probably to be continued several more years in order to assure that no new break out might happen.

The use of cell culture for poliovirus production was introduced by Enders *et al.* (1949), greatly facil-

itating the preparation of a polio vaccine. Other milestones were the development of the microcarrier technology (van Wezel, 1967) and the introduction of Vero cells (Montagnon *et al.*, 1981, 1984) as production vehicle for facilitating and improving the industrial production process of an inactivated poliovirus vaccine.

The classical Vero based production process of poliovirus is based on a serum-containing medium (SCM), at least for the production of biomass (Montagnon *et al.*, 1981; Fabry *et al.*, 1989), whereas the media used for the virus production phase have generally not been published. However, the use of serum during a part of the production process bears sever-

al disadvantages: sera vary in quality and composition between batches and producers, and high quality sera are particularly expensive. They may also be contaminated by microorganisms (bacteria, mycoplasma, fungi, viruses, the BSE-agent, etc.).

Thus, the use of serum/protein-free media is a very important step towards a more controlled, better and less risky bioprocess. Therefore, we have developed the serum-free medium (SFM) MDSS2 which can be efficiently used for the culture of different cell lines and the production of various biologicals (Merten *et al.*, 1994). We could show that using MDSS2, rabies virus was very efficiently produced in perfused suspension cultures of BHK-21/BRS-Paris cells (Perrin *et al.*, 1995) and that various strains of influenza virus could be produced at high titers by using microcarrier cultures of MDCK cells and to somewhat lower titers by using microcarriers and suspension cultures of Vero and BHK-21/BRS-Paris cells (Merten *et al.*, 1996), respectively.

This paper describes the production of poliovirus (Sabin 1) on Vero cells, cultivated in serum-free conditions (MDSS2) as attached cells on microcarriers in stirred tank reactors. The optimisation of the serum-free virus production medium based on MDSS2, led to considerably better virus titers and virus production rates, than those reported previously (Montagnon *et al.*, 1981, 1984; Duchene *et al.*, 1990).

Materials and methods

Cell line and culture conditions: Vero-cells (WHO-1878) were obtained in the 137th passage from Dr. F. Horaud (Institut Pasteur, Unité de Virologie Médicale). The establishment of a working cell bank, the adaptation of the cells to growth in MDSS2 (Merten *et al.*, 1994), and the amplification of the cell mass took another 20 passages. The serum-free working cell bank had been established in MDSS2+DMSO+methylcellulose (Merten *et al.*, 1995), and was tested for the absence of mycoplasmas. The cells were thawed in SCM were adapted from cells grown in SFM to SCM-conditions during three passages. In general once a week, the cells were trypsinized and subcultivated in 50 ml medium (SFM or SCM) at 4×10^6 c/cm², as described previously (Merten *et al.*, 1995). Four days after the passage, 100% of the medium was changed.

Medium. The following media were used: MDSS2 (Axcell Biotechnologies, F-69610 Saint Genis l'Argentière ref. 34601), to which 1.2 g NaHCO₃/l was added, for the serum-free cultures; and DMEM (Axcell Biotechnologies, ref. 35213SP), to which FCS (fetal calf serum from Eurobio, F-91953 Les Ulis) and 1.2 g NaHCO₃/l were added, for the cultures in SCM.

Virus. Poliovirus type Sabin 1 with a titre of 10^9 TCID₅₀/ml. Cultures in six well plates (Corning 25810, 10 cm³-wells): The cultures were inoculated at 26.6×10^3 c/cm² in 3 ml of SFM (MDSS2) and SCM (DMEM+2%FCS) and incubated in a CO₂ incubator (5% CO₂, 95% air) at 37 °C. At 264 hours of culture time, the medium was eliminated, the cells were washed with PBS and infected with virus at a MOI of 12–18. During the virus production phase, the cells were incubated in the same medium as for cell growth, however, at 34 °C. The cultures were stopped six days after infection, the virus titer was determined.

Cultures in bioreactors

The following general conditions were employed: pH 7.20; regulation of the pH by surface injection of CO₂ or NaHCO₃ (75 g/l); pO₂: 20% air saturation, regulation by injection of air, O₂, and N₂ via solicon tubing (4 m/l); temperature: 36.8 °C for cell growth, 34 °C for virus production. The following reactors were used: Biolafitte ICC 1L and Inceltech LH series 210; both reactors were equipped with a spinfilter (pore size: 18 µm) for retaining microcarriers within the reactor during perfusion culture mode. In both cases a marine impeller was used as agitator, their working volume was 1.6 L.

Vero cells were cultivated on microcarriers: Superbead (Flow, cat n°: 60-085-12), concentration: 6.25 g/l. The microcarriers were twice washed in PBS, autoclaved at 121 °C for 30 minutes, and once washed and incubated in growth medium for at least 48 h before onset of the culture.

For starting the microcarrier cultures, the cells were trypsinized (Merten *et al.*, 1996), rigorously washed twice with growth medium in order to get rid of residual trypsin and incubated in the reactor in the presence of the microcarriers (starting volume: 1L). This suspension was discontinuously agitated (at 40 or 50 rpm) for 5 minutes with an interruption of 10 minutes. After 12 hours, the agitation was set continuously at 40 (cultures C₁ and C₂) or 50 rpm (cultures C₃ and C₄).

The perfusion mode was started when the cell density was about 4.5×10^5 c/ml. The dilution rate was chosen according to the cell density, generally it ranged between 0 and 0.038/h. For cell growth MDSS2 (cultures $C_1 - C_3$) and DMEM + 5% FCS (cultures were infected with virus at a MOI of 0.1–0.3 after having obtained about $1.4\text{--}1.5 \times 10^6$ c/ml for the cultures in SFM, or after a culture duration of about 175 h for the cultures in SCM. More details concerning virus inoculation and conditions during the virus production phase are presented in Table 1.

Sampling, analytics, calculations

Sampling: Samples were taken once or twice a day. 0.5 ml of the microcarrier suspension was treated with 0.5 ml crystal violet/citric acid (0.1% in 0.1 M citric and, + 1% Triton $\times 100$) at 37 °C for 1 h and the released cell nuclei were counted in a Mallassez chamber ($\rightarrow$ c/ml). During virus production phase and after detachment of all cells from the microcarriers, the cells were counted after treatment with trypane blue (0.2% in PBS) in a Mallassez chamber. For estimating the percentage of colonized microcarriers, and aliquote of the sample was put onto a slide and about 150 microcarriers were classified as confluent, with cells or without cells by using a microscope. The rest of the supernatant was centrifuged (800 rpm, 20 °C, 5 min) and frozen at -20 °C for further tests. In the case of virus containing samples, 1 ml of aliquotes of centrifuged supernatant were inactivated by β -propiolactone (final concentration: 1/4000, Morgeaux *et al.*, 1993) and stored at -20 °C; only those samples destined for virus titration were stored at -80 °C, in a non-inactivated form.

The determination of the glucose concentration was done by using the Trinder reagent (Sigma 315-100, method described by Sigma).

The amino acid analysis was done according to the method described by Smith and Panico (1985) (amino acid determination using reversed HPLC after precolumn-derivatation with o-phthalaldehyde). Briefly, 375 μ l of the sample were mixed with 25 μ l of internal standard (3-(2-thienyl)-DL-alanine at a final concentration of 15 mg/l) of 5% sulfosalicylic acid and filtered through 0.2 μ m. 150 μ l of the filtrate were mixed with 600 μ l H_2O and derivatized/further treated as described by Smith and Panica (1985). The calibration for each amino acid was done in the range of 1 to 25 mg/l. The HPLC was a Hewlett-Packard 1090 Liquid Chromatograph Series II equipped with automatic sample injection. The derivated samples were injected on a pre-

column/column Hewlett Packard ODS Hypersil 3 μ m, 100×4 mm/Hewlett Packard LiChrosper 100, RP-18, 5 μ m, 4×4 mm and chromatographed by using a gradient going from a solution of 50 mM sodium acetate, pH 5.5, 6% acetonitril to 50 mM sodium acetate, pH 5.5, 50% acetonitril. The detector was a Shimadzu Fluorescence HPLC monitor RF 535, using an excitation wave length of 340 nm and an emission wave length of 450 nm. The calculations were done by using the Nelson Analytical Model 2600 Chromatography Software Rev. 5.0.

The virus titration was done by using the dilution method according to the WHO (1990), whereby six parallel microcultures in 96 well were infected per virus (sample) dilution.

Calculations. The following calculations were performed:

$$\text{Specific growth rate: } \mu = \frac{\ln(\times_2/\times_1)}{(T_2 - T_1)} [1/h] \quad (1)$$

where $\times$ represents the viable cell density per ml, T represents the time points of sampling [h]; the subscripts 1 and 2 stand for two succeeding sampling points.

Overall specific virus production for dish cultures:

$$P = C/\times \quad (2)$$

where C is the virus titer in TCID₅₀/ml at 6 days postinfection, and $\times$ is the cell density per ml at virus inoculation.

Overall specific virus production rate for reactor processes:

$$P = \frac{\sum_{n=1}^n (2 \times (C_{n+1} - C_n))}{(T_n - T_1) [TCID_{50}/c \cdot h]} \quad (3)$$

where C represents the virus titer (TCID₅₀/ml), and $(T_n - T_1)$ represents the duration of the virus production up to obtention of the maximal titers. The phase after this point (constant virus titers or decreasing virus titers) was not taken into account.

Results

Cell growth of Vero cells and poliovirus production in static cultures

Static dish cultures of Vero cells in SCM (DNEM+2%FCS) and SFM (MDSS2) revealed a certain advantage of the SCM over the SFM with respect

Table 1. Detailed conditions used for performing bioreactor cultures of Vero for the production of polio virus

Cultures	Cell density for virus inoculation	Washing steps before virus inoculation	Medium change in %	Medium for virus production	Medium addition during virus production (mg/1 culture, d)
C ₁	1.4×10^6	–	72.2	MDSS2	–
C ₂	1.47×10^6	–	77.7	MDSS2	at 36.5 h PI: glucose: 1000 GLN: 300 at 0.0046/h
C ₃	1.48×10^6	+ (1)	85.2	MDSS2 (2)	at 14.75 h PI: glucose: 625 GLN: 300 HIS: 62.5 at 0.0048/h
C ₄	3.07×10^6	+ (3)	81.2	MDSS2	–

Notes: (1) one washing step using MDSS2. (2) this medium was enriched for methionine (+ 49 mg/l) and cystine (+ 62.57 mg/l). (3) two washing steps using PBS. PI: post-infection.

to cell growth. After a culture duration of 264 h $6.3 \pm 0.198 \times 10^5$ c/cm² and $4.38 \pm 0.311 \times 10^5$ c/cm², respectively, have been obtained, signifying a specific cell growth of 0.0120/h and 0.0106/h.

The virus titers obtained did not differ very much for both culture conditions, and 6 days postinfection $10^{6.75}$ and $10^{6.67}$ TCID_{50/50} µl were obtained for cultures done in SCM and SFM, respectively. Expressed as cell specific virus production, cells in SCM produced 0.89 TCID/c, whereas SFM cultures showed a higher specific production (1.07 TCID/c).

Stirred tank reactor cultures of Vero cells

As for the static batch cultures, Vero cells showed a somewhat reduced specific growth rate in SFM in comparison to cultures done in SCM (Figure 1A). About 175 h after onset of the cultures with about 2.3×10^5 c/mL 1.4 – 1.5×10^6 c/mL (cultures C₁–C₃, $\mu = 0.0109$ /h) and 3.1×10^6 c/mL (culture C₄, $\mu = 0.0146$ /h) were obtained in SFM and SCM, respectively. The perfusion was started at 77.75 h and 23.5 h for the SFM and SCM cultures, respectively; the dilution rate was normally chosen in accordance to the cell density and varied therefore from 0 to 0.038/h. The maximal dilution rate of about 0.038/h (Figure 1B) was largely sufficient for the alimentation of cell densities of about 1.5×10^6 c/ml (specific perfusion rate: 0.023–0.027 ml/10⁶c.h) under serum-free conditions. This rate seemed also to be sufficient (at the limit) for alimentering about 3×10^6

c/ml in the SCM conditions (specific perfusion rate: 0.011–0.016 ml/10⁶ c.h), although the residual glucose concentration was lower than 0.12 mM (not shown). However, first the cells grew at a specific growth rate equally or better than that observed in batch spinner cultures (Merten *et al.*, 1994) and, second, the cells were alimentered with fresh medium signifying that the daily glucose addition and consumption was about 12.5 mmol per liter culture.

The differences in the final cell density were mainly due to the relatively poor (inhomogeneous) distribution of the cells on the microcarriers cultivated in SFM in comparison to those in SCM (Figure 1C). Whereas for culture C₄, done in SCM, already 63% of the microcarriers were confluent and only 2% were cell-free about 80 h after onset of the culture, for culture C₃ done in SFM, only 2% of the microcarriers were completely covered and 35% of the carriers were without any cell. At the time of virus infection (at about 173 h after onset of the culture) for culture C₄, 90% of the carriers were completely covered by cells, whereas only 2% were uncovered. At the same time, the SFM-culture C₃ contained only 20% confluent carriers and 40% cell-free ones. These data show clearly that those carriers which were not hit by at least one cell at the onset of the culture or on which the cells could not attach and adhere correctly stayed cell free during the whole culture, and this is also the most probable explanation for the differences in the final (virus inoculation) cell

Figure 1. Bioreactor cultures of Vero cells grown on microcarriers in SFM (culture C₃: —●—) and SCM (culture C₄: —□—) in a stirred tank reactor. The cultures have been infected with poliovirus (strain Sabin 1) after one or two washing steps with MDSS2 or PBS, respectively, and a medium change of 85.2% and 81.2%. The medium used for the virus production phase was enriched MDSS2 followed by a continuous addition of glucose, glutamine and histidine (C₃) from 14.75 h postinfection onwards and MDSS2 (C₄). The virus infection was done on 173.5 h (C₃) and 175.5 h (C₄). A: viable cells per ml; B: dilution rate (1/h); C: Cell distribution on microcarriers: culture C₄: confluent (= more than 95% of the available surface of the carrier is covered by cells, —□—), with cells (= microcarriers are covered by at least two cells and at most 95% of the available surface of the carrier is covered by cells, —□—), without cells (= less than 2 cells, —■—); culture C₃: confluent (—◆—), with cells (—◆—), without cells (—×—).

densities found between SFM and SCM microcarrier cultures.

Production of poliovirus in stirred tank reactor cultures of Vero cells and optimisation of the virus production medium

After medium change, which counted for 72–85%, and eventual washing steps (Table 1), the cells were infected at a MOI of 0.1–0.3 and incubated in normal or enriched MDSS2. The growth stopped between 0 h and 14 h postinfection, and the cell density decreased rather rapidly; for the cells grown in MDSS2 (culture C₃) this decrease was rather important between 14 h and 42 h postinfection, whereas most of viable cells (grown in DMEM+5%FCS (culture C₄)) were lost between 62 h and 86 h postinfection.

Culture C₁. This culture was entirely done in MDSS2. Sixtyone hours postinfection a titer of 6.2×10^8 TCID₅₀/ml was obtained, signifying that the overall specific virus production rate was 7.11 TCD₅₀/c.h (Figure 2A, Table 2). During the virus production phase, it appeared that several nutrients became limiting, glucose was exhausted at about 61 h postinfection; glutamine came down from 356 mg/l to 62–88 mg/l 37 h postinfection, and the methionine concentration stayed under 6.9 mg/l from 37 h postinfection onwards (Figures 2B–D). All other nutrients could be kept at non limiting levels or increased during the virus production phase.

Culture C₂. This culture was done in MDSS2 for biomass production. Because of the limitations observed during the virus production phase of culture C₁, MDSS2 was used for this culture, to which concentrated solutions of glucose and glutamine were added continuously from 36.5 h onwards. Although these conditions should improve virus production, a reduction in titer and production rate was observed. We obtained 1.36×10^8 TCID₅₀/ml (36.5 h postinfection), signifying an overall specific virus production rate of 3.5 TCID₅₀/c.h. (Figure 2A, Table 2). From Figures 2B–E it is evident that several limitations appeared during virus production phase. The concentration of glucose (Figure 2B) decreased down to 1.4 mM, a rather low concentration, indicating that the supplementation with additional glucose was started too late. No limitation was stated for glutamine (Figure 2C), however, limitations appeared with respect to methionine and histidine (Figures 2D–E). As for culture C₁ a residual

methionine concentration of <10 mg/l was stated from 36.5 h postinfection onwards. Very unexpectedly, the residual histidine concentration fell to 0 mg/l after 36.5 h postinfection. These results led to the run of culture C₃, which was planned to be performed avoiding any known nutrient limitation.

Culture C₃. The biomass production was done in MDSS2, whereas the virus production was performed in an enriched MDSS2. In order to avoid the limitations stated during the virus production phase of culture C₂, MDSS2 was enriched by methionine and cystine, which is not detectable by the amino acid analysis used. From 14.75 h postinfection onwards, glucose, glutamine, and histidine were continuously added.

These virus production conditions led to a correct titer of 6.3×10^8 TCID₅₀/ml, signifying an overall improved specific virus production rate of 17.9 TCID₅₀/c.h. (Figure 2A, Table 2). Using these conditions, no limitations could be detected by the methods used. The concentration of glucose (Figure 2B) and methionine (Figure 2D) stayed stable, whereas that of glutamine (Figure 2C) and histidine (Figure 2E) started to increase from 14.75 h postinfection onwards, due to the continuous addition.

Culture C₄. This reactor culture was performed as reference culture, whereby DMEM+5%FCS was used for biomass production. The virus production was performed in MDSS2 after having washed the cells twice with PBS in order to get rid of serum proteins. As the standard virus production conditions used by industry are not known, we have opted for MDSS2 because, in general, this medium is sufficient for proliferation/maintenance of Vero cells.

Unexpectedly, very high virus titers of about 2×10^9 TCID₅₀/ml were obtained, signifying an overall specific production rate of 18.9 TCID₅₀/c.h. (Figure 2A, Table 2). With respect to nutrients, limitations were stated only for glucose and glutamine, for which the residual concentrations were 0 and <22 mg/l, respectively, from 36.75 h onwards.

Discussion

Cell growth. Classical virus production processes are entirely or at least partly based on the use of serum containing medium (Montagnon *et al.*, 1981; Fabry *et al.*, 1989, for additional references see: Tomei and Issel, 1975), which has to be modified due to the problems

Figure 2. Virus production (Figure 2A) and the development of the concentration of selected nutrients (glucose (Figure 2B), glutamine (Figure 2C), methionine (Figure 2D), histidine (Figure 2E) for bioreactor cultures of Vero cells grown on microcarriers during the virus production phase: MDSS2 was used for biomass production of cultures C₁ (—■—), C₂ (—◇—), C₃ (—◆—); DMEM + 5% FCS was used for the biomass production of culture C₄ (—□—). For virus production, MDSS2 was used for cultures C₁ and C₄; MDSS2 with glucose and glutamine alimentation for culture C₂, and MDSS2 enriched for methionine and cystine with glucose, glutamine, and histidine alimentation for culture C₃.

associated with the use of serum (see: Introduction). The replacement of SCM by SFM very often leads to a detachment of the cells from the support and to growth in suspension (e.g. for CHO and BHK (Hayter *et al.*, 1992; Merten *et al.*, 1994)) whereas other cells, like MDCK or Vero, grow as adherent cells even in SFM (Cinnatl *et al.*, 1983; Merten *et al.*, 1994, 1996).

As a prerequisite for replacing SCM, the SFM has to assure all important characteristics of a good medium, like supporting optimal cell growth or protecting the cells against adverse culture conditions. Using the SFM MDSS2 for cell growth in replacing DMEM+5%FCS, we observed that the specific growth

rate of Vero cells was reduced by 12–26% in static and, in particular, in microcarrier based systems. Although there are several possible reasons for these differences, like the increased adverse effects of growth due to the absence of serum and/or the insufficient protection of the cells by protective polymers, like Pluronic or PEG (present MDSS2), it is more likely, that differences in the colonisation of the microcarriers by cells in SCM and SFM are the main reason.

In the case that the cell attachment conditions are not optimal (small cell to carrier ratio, adverse agitation conditions at the onset of the culture, inhomogeneous microcarrier-surface treatment by the producer, etc.),

Table 2. Comparison of poliovirus type 1 titers and overall specific virus production rates established for different virus production regimes in bioreactors

Culture	Medium for cell growth	virus production	TCID ₅₀ /ml ($\times 10^8$) max	TCID ₅₀ /c.h
Industry ¹	EMEM+5%FCS	?	1.39	2.87
C ₁	MDSS2	MDSS2	6.31	7.11
C ₂	MDSS2	MDSS2 ²	1.36	3.52
C ₃	MDSS2	MDSS2 ³	6.31	17.93
C ₄	DMEM+5%FCS	MDSS2	19.90	18.88

¹ ref. Montagnon *et al.* (1981, 1984): basis for calculations: cell density for infection: 1.35×10^6 c/ml and supposition that the maximum virus titer is obtained at 36 h postinfection. The composition of the virus production medium is unknown.

² from 36.5 h postinfection onwards continuous addition of glucose and glutamine.

³ the virus production medium was reinforced by methionine and cystine; from 14.75 h postinfection onwards: continuous addition of glucose, glutamine, and histidine.

the cell distribution on the microcarriers in SFM is less homogeneous than that in SCM. Because Vero cells do not colonize microcarriers devoid of cells very easily, most of the microcarriers without cells will never see any cell and are lost for the culture. So, an inhomogeneous distribution leads directly to a lower apparent specific growth rate as well as to a lower maximal cell density (compare with Hu *et al.*, 1985) as it was found for the cultures in SFM.

The maximal cell density obtained for the serum-free cultures was about 1.5×10^6 c/ml (Table 1); however, after optimisation of the attachment conditions (instead of an agitation at 40–50 rpm for 5 minutes with an interruption of 10 minutes, a slow continuous agitation at 30 rpm for the first 12–25 h for cell attachment followed by an augmentation to 45–50 rpm), we are now able to obtain about 3×10^6 c/ml ($\mu = 0.0158$ /h) in SFM-conditions by using Cytodex 1 microcarriers (not shown), approaching values found for the culture performed in SCM.

The perfusion during the biomass production phase is necessary because rather high cell densities can be obtained when 6.25 g/l microcarriers are covered with cells ($3\text{--}4 \times 10^6$ c/ml). Such a high cell density cannot be alimented in a strict batch culture where fore fed batch or better perfusion culture systems have to be used. In spite of this, the classical biomass production in industrial scale of 1000 l is based on a batch (or at most on a fed batch) system, where the culture is started at 1.5×10^5 c/ml (Montagnon *et al.*, 1984) by using 1.5 g/l Cytodex 1. After 6.5 days the cells ($1.35 \pm 0.35 \times 10^6$ c/ml) are infected with virus. This signifies that the average medium consumption rate is only 0.0086 ml/10⁶ c.h, which is very low with respect

to our cultures. For the SFM and the SCM cultures, the specific perfusion rates were 0.023–0.027 and 0.011–0.016 ml/10⁶ c.h, respectively, indicating that the cells were better alimented in the perfusion process than those in the actual industrial batch process. Summarizing, in order to increase the cell density it is necessary to use higher microcarrier concentrations as well as perfusion conditions for alimenting the cells correctly at a specific perfusion rate of 0.01–0.03 ml/10⁶ c.h. In this sense, by using perfusion conditions, Fabry *et al.* (1989) were able to produce 15×10^6 c/ml on 20 g Cytodex III microcarriers per l.

Virus production. This study had been performed by using the strain Sabin 1. It can be considered that if the SFM MDSS2 is sufficient for cell growth and virus production of this strain, it should also be useful for the production of the types 2 and 3, as it could be shown by Cinatl *et al.* (1993) using another SFM. The preliminary tests done in static cultures revealed no significant differences with respect to virus production between cells grown in SFM and SCM. A somewhat increased specific virus production rate was observed for the cultures done in SFM. Cinatl *et al.* (1993) could also show that there were only small differences between the virus titers obtained from Vero cells grown in SCM and SFM, however, they used this SFM only for static cultures in dishes and never for microcarrier cultures in stirred tank reactors.

With respect to serum-free reactor cultures for the production of poliovirus, there are practically no reported data available. The only suspension process, based on spinner cultures of lymphoblastoid cell lines, was described by Bjare and Råbb (1985). Although

titers of $10^7 - 10^{7.5}$ TCID₅₀/ml could be obtained for the three different types of poliovirus, this process was never used in larger scales than 20 l because the cell lines were not evaluated/accepted for the production of biologicals for human use.

The tests performed in bioreactors revealed that a production of poliovirus under serum-free conditions is feasible and, in general, better/more optimal than the classical production process in industry. The use of MDSS2 (culture C₁) for both, biomass as well as virus production, led to a virus titer of 6.3×10^8 TCID₅₀/ml, in spite of the occurrence of nutrient limitations, signifying a specific virus production rate of 7.11 TCID₅₀/c.h. These values are 4 and 2 times, respectively, higher than the values found for an industrial production process (average titers of 1.39×10^8 TCID₅₀/ml have been described for Sabin type 1 poliovirus by Montagnon *et al.* (1981), signifying that the specific virus production rate was about 2.87 TCID₅₀/c.h. (Table 2)). It is evident that concentration limitation of important or essential nutrients (as found for culture C₁) can lead directly to reduced growth and to a reduction or an interruption of the production of the biological. For instance, Zwartouw and Algar (1968) could show, that the presence of glucose was necessary for producing high yields of Semliki Forest virus. Similar results were presented by Ito *et al.* (1974) with respect to glutamine for the production of Sendai virus.

In order to avoid such limitations (glucose, different amino acids) cultures C₂ and C₃ have been performed. By resolving the glucose limitation detected in culture C₁, culture C₂ became limited in the histidine concentration, which directly led to a reduction in the virus production rate. By resolving all potential limitations associated with the amino acid and energy metabolism (use of an enriched medium for virus production (culture C₃)), high virus titers and very high virus production rates (17.9 TCID₅₀/c.h) were obtained.

The optimisation in two or more steps is well known in animal cell biotechnology. For instance, for optimizing the production of monoclonal antibodies in perfusion cultures, Büntemeyer (1988) could show that some intermediate optimisation steps had no positive effect on the production rate of monoclonal antibodies due to the appearance of new nutrient limitations.

The absence of detailed data from industrial production processes led us to perform culture C₄ where the biomass production was done in SCM and only SFM was used during the virus production phase. Although the culture (non-optimised) was limited for glucose already 37 h postinfection and to some extent

also for glutamine, the virus titers as well as the specific virus production rate were much higher than for all other cultures and the literature data presented in Table 2. The only valid explication for the differences found between C₄ and C₁ is a difference in the cellular metabolism due to the different conditions for biomass production. In culture C₄ there was an almost constant concentration of methionine during the virus production phase, whereas the concentration of methionine fell to very low levels (0–6.9 mg/l) during the virus production phase of C₁ (Figure 2D). The fact that culture C₄ produced rather high virus titers without any optimisation could be used as an argument for the development of a hybrid production process where the biomass production is done in a SCM, whereas only SFM is used for the virus production phase. However, the disadvantage is also evident: in order to get rid of the use of serum and therefore of the problems associated with use of serum, the whole process has to be performed in a SFM.

In principle, the results obtained with culture C₃ which was more or less optimized, should be better than those obtained with the non-optimized culture C₄. However, in both cases the same specific virus production rate was found. One reason might be that the cells grown in SFM and SCM are physiologically different (already mentioned), but there can be additional reasons. Culture C₃ was alimanted continuously with a concentrate of glucose, gluamine, and histidine during the virus production phase, and rather high residual concentrations of these substances were obtained, in particular with respect to glutamine (1.246 g/l) and histidine (224 mg/l). It is known that high amino acid levels (in particular of glutamine) lead to an increased release of HN₃, which can reduce or inhibit the production of virus (Jensen and Liu, 1961; Furusawa and Cutting, 1962). A further optimisation step (not done in this study) would be the alimentation of the culture with a substrate concentrate during the virus production phase, however, in a manner to avoid overalimention of glutamine as it was seen for culture C₃.

In conclusion, we could show that the production of high titer poliovirus (Sabin type 1) can be performed in a complete serumfree process, whereby considerably higher titers and higher specific production rates than in a standard batch process (Montagnon *et al.*, 1981, 1984) were obtained. The possibility of increasing the cell density using a perfusion process by keeping a high specific virus production rate (Fabry *et al.*, 1989, this paper) allows an optimal production process. Such a process leads directly to a reduction of the vessel

size by intensifying the reactor productivity. In any case, the data presented here are of great interest for the development of other virus production processes based on the use of Vero cells grown in SFM.

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