The Effect of Spent Medium Recycle on Cell Proliferation, Metabolism and Baculovirus Production by the Lepidopteran Se301 Cell Line Infected at Very Low MOI

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The aim of this paper was to study the effect of spent medium recycle on Spodoptera exigua Se301 cell line proliferation, metabolism, and baculovirus production when grown in batch suspension cultures in Ex-Cell 420 serum-free medium. The results showed that the recycle of 20% of spent medium from a culture in mid-exponential growth phase improved growth relative to a control culture grown in fresh medium. Although both glucose and glutamine were still present at the end of the growth phase, glutamate was always completely exhausted. The pattern of the specific glucose and lactate consumption and production rates, as well as the specific glutamine and glutamate consumption rates, suggests a metabolic shift at spent medium recycle values of over 60%, with a decrease in the efficiency of glucose utilization and an increase in glutamate consumption to fuel energy metabolism. Baculovirus infection provoked a change in the metabolic pattern of Se301 cells, although a beneficial effect of spent medium recycle was also observed. Both growth rate and maximum viable cell density decreased relative to uninfected cultures. The efficiency of glucose utilization was dramatically reduced in those cultures containing the lowest percentages of spent medium, whereas glutamine and glutamate consumption was modulated, thereby suggesting that infected cells were devoted to virus replication, retaining their ability to incorporate the nutrients required to support viral replication. Recycle of 20% of spent medium increased baculovirus production by around 90%, thus showing the link between cell growth and baculovirus production.

Keywords: Se301 cell line, baculovirus, spent medium recycle, metabolism, growth rate

Introduction

The in vitro infection of insect cells with baculoviruses is a powerful and versatile tool for the production of heterologous proteins of economic and biomedical interest [1, 28], virus-like particles for vaccination [41, 44, 54], and gene-therapy vectors [2, 29].

Such processes with the insect cell-baculovirus expression system involve the growth of insect cells to a required concentration, their infection with a recombinant baculovirus containing the gene of interest, and the incubation of infected cells to ensure expression of the recombinant gene [43, 44]. As such, an understanding of the factors that influence the productivity of proteins or the vector itself is required in order to develop efficient and attractive production processes to meet the increasing demand for such products. Some of these factors are well known, including the state and concentration of the cells upon infection and the availability of required nutrients [8, 14, 17, 24].

In terms of process productivity, protein yields are generally higher when cells are infected during the early or mid-exponential phase and at a cell concentration of less than 1 \( \times 10^6 \) cells/ml. Infecting the cells at later growth...
stages and higher cell concentrations leads to a dramatic decline in both protein yields [15, 47] and baculovirus production [8, 14, 37, 57]. Nutrient limitation rather than waste accumulation has been considered to be the main cause of this “cell density effect” [24]. Different strategies, including improving the medium composition, supplementation of key nutrients (glucose and glutamine), partial or total replacement of medium prior to or upon infection, and the use of fed-batch or perfusion cultures, have been used to overcome this decrease in product yield [13, 30].

The re-use of spent medium has also been proposed as an interesting approach in the context of large-scale culture since such a medium may contain substantial quantities of essential nutrients, especially when it has only been used to grow cells to a concentration below the stationary phase [58]. This approach has been used successfully in 100-L pilot-scale hybridoma cultures [48] and was found to be quite efficient in the insect cell-baculovirus expression system, with heterologous protein production values similar to those obtained in fresh medium [32, 58].

The recycling of spent medium could be particularly advantageous when cells are grown in serum-free medium. Despite the fact that such media have long been used to grow insect cells, the molecular mechanisms that enable them to proliferate in these media are unclear, although it has been shown that secreted autocrine factors (insulin-like peptides, soluble proteins, enzymes) are directly implicated in the regulation of cell proliferation [17, 18, 23, 32, 38].

The aim of the present study was to investigate the effects of spent medium recycle on Se301 cell proliferation, metabolism, and baculovirus production. The results strongly support the beneficial effect of serum-free medium recycle on cell growth and baculovirus production. A percentage of spent medium of 20% provided the best results in terms of growth and baculovirus production. Metabolism was also significantly affected by the proportion of spent medium in both uninfected and infected cells.

Materials and Methods

Cell Line, Culture Medium, Additives, and Maintenance

The host insect cell line Se301, originally isolated from Spodoptera exigua [27], was kindly donated by the Department of Virology at Wageningen University. Throughout this work the cells were grown in suspension culture in Ex-Cell 420 serum-free media (Sigma-Aldrich, Ref. 14420C) supplemented with 100 U/l penicillin-streptomycin (Sigma-Aldrich, Ref. P333) and 0.125 µg/ml amphotericin B (Sigma-Aldrich, Ref. A2942).

The protective additives against shear stress PVA (Sigma-Aldrich, Ref. P8136) and PVP (Sigma-Aldrich, Ref. P2307), both at a concentration of 0.2% (w/v), and the disaggregant DS (Sigma-Aldrich, Ref. 31404), at a concentration of 25 µg/ml, were used since they were found to offer optimal protective and disaggregant effects for this cell line with no toxic effects [4].

Cells were passaged every 4 days at a cell density of 5-6 × 10^9 cell/ml, maintained in an incubator with humidification at 27°C, and agitated at 90 rpm in an orbital shaker with an orbital diameter of 1.9 cm. The cell concentration was determined by cell counting in a hemocytometer under a light microscope and their viability assessed using the Trypan Blue dye-exclusion method. After every passage, the cell suspension was filtered through a 0.22 µm PES filter (Millipore, Ref. SCGPT02RE) to separate the cells from the spent medium. This spent medium was stored at 4°C and used in the experimental cultures without further treatment.

Experimental Cultures

To study the effect of spent medium recycle on Se301 growth and virus production, fresh and spent Ex-Cell 420 media were mixed in different proportions ranging from 0 to 100% of spent medium. No other additive or supplements were added.

The experiments were carried out in triplicate in 100 mL Erlenmeyer flasks with a working volume of 15 mL. Cultures were inoculated at 6 × 10^5 cells/ml, kept in an incubator at 27°C, and shaken at 140 rpm (Re = 5280). Cell samples were taken daily for cell density, viability, and nutrient and lactate determinations. Cell density and viability were determined as described above. Glucose, lactate, glutamine, and glutamate were determined using a YSI 2700 biochemistry analyzer (YSI Inc., Dayton, OH, USA).

For infection experiments, the budded form of a wild strain of the S. exigua multiple nucleopolyhedrovirus (SeMNPV) was used [4]. Cells from a culture in mid-exponential growth phase were inoculated at a concentration of 6 × 10^5 cells/ml with an MOI of 0.04. This MOI was used owing to the very well-documented benefits of using a very low MOI in production-scale bioreactors, since inoculation of the viral stock can be done directly from a well-characterized master bank [59]. The optimal time of harvest of the occlusion bodies (OBs) was 10 days. Cells were then lysed using ultrasound to release the OBs. A 1 mL culture sample was sonicated for 2 min on ice using a HIF200S sonicator (Hielscher Ultrasound GmbH, Teltow, Germany) with a 2-mm-diameter sonotrode. The OBs released after cell lysis were counted using a hemocytometer under a light microscope. Cell density and viability, glucose, lactate, glutamine, and glutamate were determined as described above for uninfected cultures.

Estimation of Specific Growth Rate and Specific Metabolic Rates

Specific growth rates (µ) were estimated by dividing the slope of the viable cell density (X_v) profiles by the average viable cell density during the exponential growth phase:

\[ \mu = \frac{1}{X_v} \frac{dX_v}{dt} \]
Similarly, specific glucose \( (q_{\text{Glc}}) \), lactate \( (q_{\text{Lac}}) \), glutamine \( (q_{\text{Gln}}) \), and glutamate \( (q_{\text{Glu}}) \) consumption and production rates were estimated by dividing the slopes of the concentration profiles by the average viable cell density during the growth phase:

\[
q_S = \frac{1}{X_c} \frac{dS}{dt}
\]

where \( S \) is the glucose, lactate, glutamine, or glutamate concentration. The apparent lactate yield coefficient from glucose \( (Y_{\text{Lac/Glc}}) \) was calculated according to the following equation:

\[
Y_{\text{Lac/Glc}} = \frac{q_{\text{Lac}}}{q_{\text{Glc}}}
\]

Results

Growth and Metabolism of Uninfected Se301 Cells

As an example of the experimental data obtained in uninfected Se301 cell cultures, the growth curves and metabolite profiles for two representative experiments, namely cultures containing 0% (fresh medium) and 100% spent medium recycle, are shown in Fig. 1.

Uninfected cells grew exponentially immediately after inoculation (Fig. 1A). However, both \( \mu \) and the maximum cell density \( (X_{\text{Vm}}) \) clearly depended on the percentage of spent medium (Fig. 2A). The duration of the growth phase was also affected by the percentage of spent medium (from 8 days in cultures containing 0% spent medium to 4 days in cultures containing 100%).

Glucose was not completely consumed during the growth phase in any case (Fig. 1B), with residual glucose concentrations of over 5 mM in cultures containing 0% spent medium at day 8 and about 10 mM in cultures containing 100% at day 4 being observed. Glucose was also consumed in the death phase and became exhausted after 10 days in all experiments. Lactate accumulation was proportional to glucose consumption (Fig. 1B), reaching final concentrations that were practically independent of the percentage of spent medium (between 18 mM in cultures containing 0% spent medium and 20 mM in cultures containing 100%).

Glutamine consumption was very limited (Fig. 1C), with residual concentrations at the end of the growth phase ranging from 5.3 to 4.8 mM in cultures containing 0% and 100% spent medium, respectively. Glutamate levels decreased gradually until it became exhausted after 8 days in cultures containing fresh medium and after 6 days in cultures containing 100% spent medium (Fig. 1C). From these data, glutamate seems to be the main limiting substrate of uninfected cultures.

In order to clarify the effect of spent medium on the proliferation and metabolism of Se301 cells, \( X_{\text{Vm}}, \mu \) and \( q_{\text{Glc}} \), \( q_{\text{Lac}} \), \( q_{\text{Glu}} \), and \( q_{\text{Gln}} \) were determined at different percentages of spent medium recycle (see Fig. 2). It can be seen from
Fig. 2A that $X_{Vm}$ increased (up to $2.12 \times 10^6$ cells/ml) for cultures containing 10% and 20% spent medium relative to the culture containing 0% spent medium ($1.9 \times 10^6$ cells/ml), followed by a gradual decrease to a value of $0.8 \times 10^6$ cells/ml for the culture containing 100%. The behaviour of $\mu$ was slightly different. Thus, this parameter increased for the cultures containing 10% and 20% spent medium (up to $0.21$ day$^{-1}$) and then remained constant up to a percentage of 60%, with values similar to that obtained in the culture containing fresh medium ($0.17$ day$^{-1}$). From 70% of spent medium, $\mu$ gradually decreased to a value of $0.078$ day$^{-1}$ for the culture containing 100%.

It can be seen from Fig. 2B, which shows the behavior of $q_{Glc}$, $q_{Lac}$, and $Y_{Lac/Glc}$, that both specific rates increased slightly (from about $2.5$ to $3.5 \times 10^{-17}$ mol cell$^{-1}$ s$^{-1}$ for $q_{Glc}$, and from $1.5$ to $1.7 \times 10^{-17}$ mol cell$^{-1}$ s$^{-1}$ for $q_{Lac}$) up to a spent medium percentage of 60%, with a constant $Y_{Lac/Glc}$ of about 0.5. Subsequently, $q_{Glc}$, $q_{Lac}$, and $Y_{Lac/Glc}$ increased significantly (up to $5 \times 10^{-17}$ mol cell$^{-1}$ s$^{-1}$, $3.5 \times 10^{-17}$ mol cell$^{-1}$ s$^{-1}$, and 0.75, respectively).

Fig. 2C shows the behavior of $q_{Gln}$ and $q_{Glu}$. Glutamine consumption was the lowest of all nutrients analyzed, with a maximum $q_{Gln}$ value of $0.19 \times 10^{-17}$ mol cell$^{-1}$ s$^{-1}$ attained in the culture containing 60% spent medium. This represents an increase of 30% with respect to the value observed in the culture containing fresh medium. Glutamate consumption remained constant at about $0.65 \times 10^{-17}$ mol cell$^{-1}$ s$^{-1}$ in cultures containing percentages of spent medium of up to 60%. For higher values, $q_{Glu}$ increased to over $0.8 \times 10^{-17}$ mol cell$^{-1}$ s$^{-1}$ in the culture containing 100% spent medium.

Growth, Metabolism, and Baculovirus Production by Infected Se301 Cells

The effect of spent medium recycle on BV production was assessed by infecting Se301 cultures at a CCI of $6 \times 10^5$ cell/ml and an MOI of 0.04. Evolution of the infected cultures is shown in Fig. 3. As in Fig. 1, only the growth curves and metabolite profiles for cultures containing 0% and 100% spent medium are shown.

A comparison of Fig. 3A with Fig. 1A clearly shows that cell growth is inhibited by baculovirus infection irrespective of the percentage of spent medium. Infected cells started growing exponentially after a lag phase of 24 h, reaching $X_{Vm}$ values about 30% lower than those for uninfected cells under similar conditions. A reduction of 2 days in the growth phase span of infected cells was also observed.

Glucose consumption and lactate accumulation (Fig. 3B) clearly decreased after viral infection. At the end of the growth phase, residual glucose concentrations were about 24 mM for cultures containing 0% spent medium and about 11 mM for cultures containing 100%. Lactate accumulation behaved similarly to glucose consumption, up to values of between 11 and 16 mM (roughly 40–50% lower than for uninfected cultures).
Glutamine consumption was similar to that observed in uninfected cultures (Fig. 3C), with residual concentrations at the end of the growth phase of 6.2 and 5.3 mM for cultures containing 0% and 100% spent medium, respectively. Glutamate consumption was also similar to that observed in uninfected cultures (Fig. 3C), although in this case it was never fully depleted. Concentrations at the end of the growth phase were 1.6 mM for the culture containing 0% spent medium and 0.75 mM for the culture containing 100%.
Similarly to uninfected cells, $X_{Vm}$, $\mu$ and $q_{Glc}$, $q_{Lac}$, $q_{Gln}$, and $q_{Glu}$ were calculated for infected cells at different percentages of spent medium recycle (see Fig. 4). Fig. 4A shows that, as in uninfected cultures, $X_{Vm}$ and $\mu$ reached maximum values in the culture containing 20% of spent medium ($1.5 \times 10^6$ cells/ml and 0.19 days$^{-1}$, respectively) and then subsequently gradually decreased.

Fig. 4B illustrates the effect of baculovirus infection on glucose and lactate metabolism. Thus, while $q_{Lac}$ was similar to that observed in uninfected cultures for spent medium recycle values of less than 60%, $q_{Glc}$ was clearly lower. This resulted in a considerable increase (over 100% in some cases) in $Y_{Lac/Glc}$.

The values of $q_{Gln}$ and $q_{Glu}$ were also different in infected cultures (see Fig. 4C) relative to uninfected cultures. Thus, $q_{Gln}$ increased monotonically from 0.07 to $0.2 \times 10^{-17}$ mol cell$^{-1}$ s$^{-1}$ for cultures containing 0% and 100% spent medium, respectively, whereas $q_{Glu}$ tended to decrease slightly as the percentage of spent medium increased, with values ranging between 1 and $0.8 \times 10^{-17}$ mol cell$^{-1}$ s$^{-1}$.

As far as baculovirus production is concerned, it can be seen from Fig. 5 that the percentage of spent medium clearly affected the final OB titer. A maximum value of around $5.3 \times 10^7$ OBs ml$^{-1}$ was measured in the culture containing 20% spent medium, which represents an increase of 90% with respect to the culture in fresh medium. The baculovirus concentration gradually decreased for cultures with larger percentages.

**Discussion**

The *Spodoptera exigua* Se301 cell line has shown great potential for the production of recombinant proteins [34] and baculoviruses [56] in attached cultures. However, this potential is dramatically reduced when Se301 is cultured in suspension due to problems adapting to suspension [34]. In previous works, we have succeeded in adapting Se301 cells to grow in suspension [5] and in commercially available serum-free media [4], thereby increasing their potential for use in bioprocess development. In this study, we present an analysis of the influence of spent medium recycle on the cell growth, metabolism, and baculovirus production by Se301 cells growing in suspension culture in serum-free medium.

The end of the exponential growth phase in batch cultures of the Se301 cell line was followed by a rapid decline in viable cells without a stationary phase. Similarly, the onset of the death phase was concurrent with both the accumulation of lactate and the exhaustion of glutamate. Lactate is a toxic metabolite, with inhibitory effects in a cell-line-specific manner, as shown by the reduced growth of BM5 cells at lactate concentrations of 8 mM [52] and the increase of apoptosis of High-Five cells at concentrations up to 12.5 mM [21], whereas Sf-9 cells do not accumulate lactate in significant amounts [10, 30]. Hence, the concentration reached at the end of the growth phase in this study (15 mM) could be high enough to affect the growth of Se301 cells. However, glutamate was completely exhausted by the end of the growth phase, and since glutamate catabolism exerts an important effect on the carbon and energy budgets of the cells [6], the rapid decline in cell density at the end of the growth phase is most likely caused by the exhaustion of glutamate.

Cultures with increasing proportions of spent medium were characterized by a smoother death phase (Fig. 1A), similarly to the results found by Ikonomou et al. [31] with Sf-9 and High-Five cells in a hydrosylate-containing medium as long as glucose was available, as in this study. According to these authors, this smoother death phase can be attributed to the antiapoptotic properties of the hydrolysate used (Primatone RL), which were initially noted in hybridoma cultures [50]. The analogous behavior of the cells in the present study may imply that the spent medium from Se301 cell culture could contain factors with similar properties.

The presence of autocrine or paracrine factors with antiapoptotic activity in spent medium from cultured mammalian cells is very well documented [45, 55].
Additionally, the presence of autocrine factors in the spent medium is also usually associated with the stimulation of proliferation of mammalian cells [39]. To the best of our knowledge, no data regarding the antiapoptotic activity of spent medium from cultured insect cells have been reported to date. Despite this, autocrine factors have been shown to be important for the proliferation of many lepidopteran cells in serum-free media [11, 12, 17, 23]. The positive correlation between spent medium recycle and enhanced cellular proliferation observed in this study ($X_{vm}$ and $\mu$ increased in cultures containing 10% and 20% of spent medium (Fig. 2A)) suggests the presence of autocrine factors that influence the proliferation of Se301 cells in this serum-free medium. Ikonomou et al. [32] observed similar growth rates, but a reduction in $X_{vm}$ for when using 50% of spent medium, a similar value to that found in the present study. In contrast, Kioukia et al. [35, 36] reported a decrease in $X_{vm}$ and $\mu$ for Sf-9 with increasing proportions of spent medium, which they attributed to the accumulation of inhibitory products.

The results of the present study suggest that autocrine factors exert a more significant influence on growth kinetics than on the stoichiometry, since the beneficial effect of autocrine growth factors in $X_{vm}$ was outbalanced by nutrient limitation at spent medium percentages higher than 20%, whereas the concentration of autocrine growth factors was sufficient to maintain growth under “optimal conditions” and to prevent the decrease in $\mu$ up to a percentage of 60%. This is particularly interesting since binding proteins of IGFs (insulin-like growth factors), which exert a mitogenic effect upon degradation, have been identified in spent medium from cultures of both Sf-9 and High-Five cells [3, 18]. More recently, matrix metalloproteinases have been shown to be involved in the release of IGFs from their binding proteins [25] and it has been speculated that binding proteins secreted by insect cells could be the target for extracellular metalloproteinases present in serum-free cultures of insect cells [23, 33].

It is well known that glucose is the preferred energy and carbon source for insect cells [19], although the efficiency of its use differs for different insect cell lines [10] and culture conditions [30], but with higher specific consumption rates than for mammalian cells. The $q_{Gl}$ value obtained in this study for Se301 cells growing in Ex-Cell 420 serum-free medium ($2.5 \times 10^{-17}$ mol cell$^{-1}$ s$^{-1}$) was similar to the maximum rates reported for Sf-9 [7, 14, 40] but lower than those reported for High-Five [7, 20, 30]. The behavior of $q_{mul}$, $q_{Lac}$, and $Y_{Lac/Gl}$, shown in Fig. 2B, together with that for $\mu$, indicates a metabolic shift with a change in the efficiency of glucose utilization. As discussed above, the beneficial effects of autocrine growth factors on $\mu$ were diluted above a spent medium recycle of 60%, thus suggesting a nutrient limitation that cells tried to compensate for by increasing glucose consumption, thereby leading to a decrease in the efficiency of glucose oxidation in the TCA cycle for energy production and biosynthesis and resulting in higher lactate production [8, 14]. This occurred in parallel to the increase in glutamate consumption (Fig. 2C) required to fuel the energy metabolism, entering the TCA cycle as $\alpha$-ketoglutarate, which is known to lead to less efficient glucose use [8]. The $q_{mul}$ values were similar to those found for Sf-9 cells, whereas $q_{Glu}$ values were about ten times lower than previously reported for Sf-9 cells [8, 14]. Glutamine consumption is clearly related to glutamate consumption since, as shown in Fig. 2C, $q_{Glu}$ decreased as $q_{mul}$ increased. Although it is well known that the amino acid consumption pattern of insect cells depends on the nutritional and growth status of the cell line concerned [16], additional experiments are required to determine whether this behavior is an adaptive response of Se301 cells to the specific conditions of these cultures or whether they constitutively express a higher preference for glutamate use rather than glutamine.

Baculovirus infection has a profound impact on the physiology and metabolism of the host cell, since the metabolic machinery of infected cells is fully devoted to virus replication [8, 42]. The results obtained in this study show a change in metabolic pattern after baculovirus infection. This change was evidenced in the reduction in $\mu$ and the decrease in $X_{vm}$ values of infected Se301 cells (Fig. 4A), as well as in energy (glucose) and amino acid (glutamine, glutamate) metabolism (Figs. 4B, 4C). The growth-promoting effect of spent medium was also observed in infected cultures. Thus, an increase in $\mu$ up to values similar to those for uninfected cultures was observed for 10% and 20% spent media, whereas the maximum $X_{vm}$ values were about 30% lower than those observed in uninfected cultures, thereby suggesting a greater effect of autocrine factors on kinetics than on stoichiometry. Despite this, baculovirus infection resulted in an overall reduction in both $X_{vm}$ and $\mu$ for spent medium recycle values higher than 20%, thus counteracting the positive effect of the autocrine factors.

A comparison of the metabolic rates shown in Figs. 2 and 4 reveals a decrease in the efficiency of glucose utilization for biosynthesis and energy production post-infection, especially in cultures containing lower percentages of spent medium. A similar behavior has previously been reported for other cell lines and baculoviruses [8, 14, 22, 42].
26]. However, other studies have shown that $q_{\text{ct}}$ increased after infection [46, 49, 53]. These contradictory results are probably due to different nutritional demands for different cell lines and culture conditions post-infection [26].

Glutamate consumption was higher at lower spent medium recycle values and lower at higher spent medium recycle values, whereas glutamine consumption was lower at lower spent medium recycle values and higher at higher spent medium recycle values. This variation in amino acid consumption in infected cultures suggests that infected cells retain their ability to incorporate the nutrients required to support the viral replication process rather than an overall reduction in nutrient consumption. This type of metabolic adaptation is very common and has been widely observed previously [9, 26].

Although most of the literature on baculovirus-insect cell system bioprocesses is related to the production of recombinant proteins rather than the baculovirus itself [30], the growing application of recombinant baculoviruses for gene therapy and the expected development of large-scale in vitro production of baculovirus-based biopesticides represent an important opportunity to broaden our understanding of this system [14, 26, 51]. Fig. 5 shows the link between growth and baculovirus production, with the OB titer being highest at a spent medium recycle of 20% (90% higher than in fresh medium). This recycle percentage is the same at which the maximum values of $\mu$ and $X_{\text{vm}}$ were reached. Hence, the autocrine factor affects baculovirus production in a similar manner to $\mu$ and $X_{\text{vm}}$.

In summary, this study shows that recycling of a high percentage of spent medium (20%) improves the cell growth and baculovirus production of Sc301 cells. This beneficial effect is thought to be due to autocrine factors present in the spent medium, although the presence of such factors has not been explicitly proved. After viral infection, Sc301 cells undergo metabolic adaptations, particularly important shifts in energy and amino acid metabolism. These findings may prove useful as regards the implementation of an economically efficient baculovirus-insect cell system on a production scale, which strongly depends on the detailed characterization of uninfected and infected cellular metabolism combined with infection kinetics and an analysis of medium composition and medium cost.

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**References**


