The use of an artificial neural network to model the infection strategy for baculovirus production in suspended insect cell cultures

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Abstract Since the infection strategy in the baculovirus-insect cell system mostly affects production of the vector itself or the target product, and given that individual infection parameters interact with each other, the optimal combination must be established for each such specific system. In this work an artificial neural network was used to model infection strategy, including the cell concentration at infection, the multiplicity of infection, the medium recycle, and agitation intensity, and to evaluate the relative importance of each factor in the baculovirus production obtained. The results demonstrate that this model can be used to select an optimal infection strategy. For the baculovirus-insect cell system used in this study, this includes low multiplicity of infection and agitation intensity, along with high cell concentration at infection and medium recycle. Our model is superior to regression methods and predicts baculovirus production more precisely, thus meaning that it could be useful for the development of feasible processes, thereby improving process performance and economy.

Keywords Baculovirus · Infection strategy · Modeling · Neural network

Introduction

The baculovirus-insect cell system is widely considered to be a robust and versatile technology for the production of a variety of products, including biopesticides, recombinant proteins, virus-like particles, and gene-therapy vectors (Dudognon et al. 2014; van Oers 2011). Such production processes typically involve two essential steps, with the cells initially being grown to a certain density before being infected with a baculovirus and incubated. After infection, the baculovirus takes control of the gene-expression machinery of the host cell to replicate itself, thereby triggering multilevel responses that lead to production of the target product (Contreras-Gómez et al. 2014).

From the perspective of large-scale, an understanding of the factors that influence production of the vector itself or the target product is of crucial importance for the development of solid and efficient processes to meet the increasing demand for such products. Indeed, a huge amount of effort has been expended over the last two decades to identify the key parameters governing such processes, including factors related to the specifics of the baculovirus-insect cell system (Hitchman et al. 2011; Mena and Kamen 2011), type of bioreactor (Agathos 2010), mode of operation (van Oers and Lynn 2010), feeding strategy (Ohki et al. 2012; Rausch et al. 2013), culture conditions (Contreras-Gómez et al. 2014), and infection strategy (Carinhas et al. 2009; Roldão et al. 2007).

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The infection strategy comprises the time of infection (TOI), the cell concentration at infection (CCI), the multiplicity of infection (MOI), and medium recycle (MR) or medium replacement at infection. All these factors interact with each other and can be manipulated to optimize the process. Although the influence of some of these parameters has been well established for different baculovirus-insect cell systems, the theoretical relationship between them has not yet been elucidated (Zhou et al. 2011). As such, the optimal combination of these parameters must be established for each individual system (Micheloud et al. 2009).

Traditionally, response surface methodology (RSM) has been a useful approach for the design and optimization of culture medium for baculovirus production from infected insect cells (Chan et al. 1998). However, to the best of our knowledge, RSM has never been used for quantitative assessment of the effects of environmental factors or infection strategy parameters.

In general, RSM is usually determined using multiple linear-regression analysis and may require a large number of cross-section parameters for better representation of the experimental data. In practice, the most widely used RSM is quadratic RSM. Once RSM has been applied, the final baculovirus concentration under various conditions within the range of variables used to develop the model can be predicted. The drawbacks of RSM arise from the fact that: (1) it is developed from linear and quadratic combinations of model variables for which linearity may or may not be justified, (2) a collinearity may exist between terms, and (3) the sensitivity analysis of the model output against each input parameter is inconvenient because of the cross-terms. However, it is well-known that artificial neural network-based models (ANNMs), which are highly nonlinear approximators, frequently outperform first- and second-order RSM, thereby leading to better mapping of nonlinear data (Geeraerd et al. 1998).

In light of the above, the main objective of this study was to develop an ANNM for predicting the effect of infection strategy (combined effects of CCI, MOI and RM) on occlusion body (OB) production in insect cell cultures infected in the mid-exponential growth phase (TOI). Since the model cell used in this

study (lepidopteran Se301 cell line) has been shown to be highly sensitive to shear stress (Beas-Catena et al. 2011), a parameter related to agitation intensity (Reynold number Re) was also included in the study. The prediction accuracy of the ANNM developed was compared to RSM to assess the ability of ANN to replace RSM. The ANNM was also applied to evaluate the relative importance of the four factors (CCI, MOI, MR, and Re) and used to generate a response surface for OB production.

Materials and methods

Cell line, culture medium, additives, and maintenance

The insect cell line Se301, which was kindly donated by the Department of Virology at Wageningen University (Wageningen, Nederland), was grown in suspension cultures in all experiments performed throughout this work. Insect cell Ex-Cell 420 serum-free medium (Sigma-Aldrich, St. Louis, MO, USA, Ref. A2942) was used after being supplemented with 100 U L⁻¹ penicillin–streptomycin (Sigma-Aldrich, Ref. P4333) and 0.125 µg mL⁻¹ amphotericin B (Sigma-Aldrich, St. Louis, MO, USA, Ref. A2942). PVA (Sigma-Aldrich, Ref. P8136) and PVP (Sigma-Aldrich, Ref. P2307), both at a concentration of 0.20% w/v, were used as protective additives against shear stress, and the disaggregate DS (Sigma-Aldrich, Ref. 31404) was used at a concentration of 25 µg mL⁻¹ to provide optimal protective and disaggregate effects. None of these additives induced toxic effects (Beas-Catena et al. 2013a).

Cells were routinely maintained in an incubator at 27 °C, agitated in an orbital shaker (1.9 cm orbital diameter) at 90 rpm, and passaged every 4 days to an inoculum cell density of 5–6 × 10⁵ cell mL⁻¹. Cell counting was performed using a hemocytometer, and viability was determined using the trypan blue exclusion method. After every passage, the spent medium was collected by centrifugation, filter-sterilized (0.22 µm PES filter, Millipore, Billerica, MA, USA, Ref. SCGPT02RE), and stored at 4 °C for use in further experiments.

Experimental cultures

In previous studies we have successfully adapted the *S. exigua* Se301 cell line to grow in suspension culture and serum-free media (Beas-Catena et al. 2011, 2013a). Cell proliferation, metabolism and baculovirus production have been investigated in cultures with different infection strategies (Beas-Catena et al. 2013a, b). The ranges for the different parameters investigated in the present work are based in those studies. Se301 cells in the mid-exponential growth phase were infected at different CCIs with the budded form of a wild strain of *Spodoptera exigua* multiple nucleopolyhedrovirus. Budded virus (viral titer) was determined prior to infection using the end point dilution assay (King and Possee 1992). Three low MOIs were used due to the well-documented advantage of using low MOIs in large-scale systems (Zhang et al. 2005). Ten days post-infection was previously found to be the optimal time of harvest for OBs (Beas-Catena et al. 2013b). Cells were then lysed to recover the OBs. Thus, a 1 mL culture sample was sonicated for 2 min on ice, using a HP200S sonicator (Hielscher Ultrasonics GmbH, Teltow, Germany). The OBs were then counted under a light microscope using a hemocytometer. To study the effect of spent medium recycle (MR), spent medium collected as described above was mixed with fresh Ex-Cell 420 medium in different proportions.

Cultures were kept in an incubator at 27 °C, agitated in an orbital shaker identical to that used for routine cell maintenance, at different rotational speeds, in order to attain different agitation intensities. *Re* was calculated according to García-Camacho et al. (2007). All experiments were carried out simultaneously in triplicate for each experimental condition in 100 mL shake flasks with a working volume of 15 mL.

Response surface methodology

The yield of OBs in *Spodoptera exigua* Se301 insect cell cultures was the response function investigated in this study. The optimal levels of four variables, namely CCI, MOI, RM, and *Re*, were determined. For this purpose, a response surface approach by using a set of experimental design (MD-CCD: central composite design of modified distance) was adopted to improve OB production. The statistical software

package Statgraphics Centurion® XVI (Statpoint Technologies Inc., Warrenton, VA, USA) was used to analyze the experimental design.

Each factor in the design was studied at different levels (see Table 1). For MD-CCD with four factors, a set of 40 experiments was carried out. A summary of the experimental design, with minimum and maximum ranges for the variables investigated and their values in actual and coded form, is provided in Table 1.

Upon completion of experiments, the OB yield was taken as the dependent variable or response (*Y*). A second-order polynomial equation was then fitted to the data using the multiple regression procedure. This resulted in an empirical model that relates the response measured to the independent variables of the experiment. For a four-factor system, the model equation is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \quad (1)$$

where *Y* is the predicted response, β_0 the intercept, β_i a linear coefficient, β_{ii} a squared coefficient, and β_{ij} an interaction coefficient. Statgraphics Centurion® XVI was then used to generate response surface graphs, using the above model to obtain the optimum levels of the variables.

Artificial neural network

Feed-forward back-propagation neural networks (FBNs) are particularly useful for capturing the highly nonlinear nutrient interactions in bioprocesses (López-Rosales et al. 2013; García-Camacho et al. 2016). The FBN input variables consisted of a $k \times s$ matrix with *s* (=40) columns for the levels of the four factors selected. The target output variable was OB production. The input and target variables were normalized using the following equations:

$$I_N = 2 \frac{I - I_{min}}{I_{max} - I_{min}} - 1 \quad (2)$$

$$y_N = 2 \frac{y - y_{min}}{y_{max} - y_{min}} - 1 \quad (3)$$

where *I* is the input variable, I_N the normalized value of the input, I_{min} the minimum value of *I*, I_{max} the maximum value of *I*, *y* the target variable (OB

Table 1 Experimental range and levels of the independent variables

Variables	Levels	Symbol coded	Range			
			Low actual	High actual	Low coded	High coded
CCI ($\times 10^{-6}$ cell mL $^{-1}$)	4	X ₁	0.4	1.3	−1	1
Re	3	X ₂	3815	13,655	−1	1
MOI (TCID ₅₀ cell $^{-1}$)	3	X ₃	0.05	0.2	−1	1
MR (%)	5	X ₄	0	40	−1	1

The ranges for the different parameters are based in previous studies (Beas-Catena et al. 2011, 2013a, b)

production), y_{min} the minimum value of y , y_{max} the maximum value of y , and y_N the normalized value of the target.

An FBN topology was selected, with BFGS quasi-Newton as the training algorithm. Overfitting to the training data was prevented by restricting the optimum number of nodes in the hidden layer (Maier and Dandy 2001), in accordance with the following criterion:

$$N \leq \frac{N^{TR}}{(N^I + 1)} \quad (4)$$

where N is the upper limit for the number of nodes in the hidden layer, N^{TR} the number of training samples, and N^I the number of inputs (i.e. factors). Equation (4) provided a value of 8 for N ($N^{TR} = 40$; $N^I = 4$).

The input layer consisted of four neurons (the four factors) while the target layer had a single neuron, i.e. OB production. A single hidden layer configuration with two neurons was selected to minimize the sum-of-squares error (SOS). Figure 1 shows the structure of the FBN. The FBN model for simulating OB production could be represented by the following set of equations:

$$y_N = \varphi_H \left(\sum_{j=1}^m Z_j \cdot \varphi_H \left(\sum_{i=1}^k W_{ij} \cdot I_i + b_{1j} \right) + b_2 \right) \quad (5)$$

$$\varphi_H = \tanh(x) = \frac{e^x - e^{-x}}{e^x + e^{-x}} \quad (6)$$

In the above equations, the connections between the nodes are represented by the weighted coefficients W_{ij} and Z_j ; b_{1j} and b_2 are the bias factors; I_i is the normalized value of the input i ; and φ_H is the hyperbolic tangent sigmoid transfer function that connects the hidden layer to the input layer and the

Fig. 1 Schematic representation of the feed-forward back-propagation neural network (FBN) model used in this study. The model encompassed four input nodes, one hidden layer with two nodes, and one output node. The connections between nodes, or neurons, were weighted with the coefficients W_{ij} and Z_j . The hidden layer and the output layer were connected to the input layer and the hidden layer, respectively, using a hyperbolic tangent sigmoid transfer function (Eq. 5)

output layer to the hidden layer. The normalized output data from the model (y_N) were denormalized to obtain the actual output values of OB production; thus:

$$\frac{C_t}{C_0} = 0.5(y_N + 1)(y_{max} - y_{min}) + y_{min} \quad (7)$$

The risk of overfitting was low because the number of connections between nodes in the FBN was smaller than the total number of data values in the training set. Nonetheless, the “early stopping by cross-validation” methodology was applied to prevent overfitting. Thus, the input data were randomly divided so that 70% of samples were assigned to the training set, 15% to the test set and the remaining 15% to the validation set. The sum-of-squares errors (SOS) and correlation coefficients (R) for the training, testing and validation datasets were mainly used to evaluate the FBN model.

Table 2 ANOVA of the quadratic model for OBs

Source	Sum of squares	Degrees of freedom	Mean square	F-Ratio	<i>p</i> value
A:CCI	38.8856	1	38.8856	75.97	0.0000*
B:Re	3.15802	1	3.15802	6.17	0.0201*
C:MOI	2.89031	1	2.89031	5.65	0.0255*
D:MR	4.29379	1	4.29379	8.39	0.0077*
AA	0.571329	1	0.571329	1.12	0.3009
AB	4.07575	1	4.07575	7.96	0.0092*
AC	1.80756	1	1.80756	3.53	0.0719
AD	4.61335	1	4.61335	9.01	0.0060*
BB	0.299496	1	0.299496	0.59	0.4515
BC	0.0178117	1	0.0178117	0.03	0.8535
BD	1.35974	1	1.35974	2.66	0.1157
CC	0.917746	1	0.917746	1.79	0.1926
CD	1.22048	1	1.22048	2.38	0.1351
DD	0.851043	1	0.851043	1.66	0.2091
Total error	12.7971	25	0.511883		
Total (corr.)	88.4851	39			

$R^2 = 0.8554$; adj. $R^2 = 0.7744$; standard error of est. = 0.71546; mean absolute error = 0.46718; Durbin–Watson statistic = 2.21063 ($P = 0.7242$); lag 1 residual autocorrelation = -0.132546

Statistically significant factors ($p < 0.05$) have been marked with an asterisk (*)

The FBN was trained on a MATLAB[®] platform R2012b (MathWorks, Natick, MA, USA).

During the FBN training process, the SOS between the experimental data and the corresponding predicted data was calculated and then propagated back through the network in each cycle. The algorithm adjusted the weightings between the input layer, the hidden layer and output neurons in order to reduce the error. This procedure was repeated until the error between the experimental data and the predicted values satisfied the error criteria. The weight (W_{ij} and Z_j) and bias (b_{1j} and b_{2j}) values in Eq. (5) were obtained after the training phase of the FBN model had been completed. The three-layered configuration of the FBN model is depicted in Fig. 1.

Sensitivity analysis

A global sensitivity analysis (GSA) supplies information about the relative importance of the variables used in a neural network. The GSA is defined as the ratio of the FBN error with a given input omitted to the FBN error with the input available (VSR). If the ratio is 1 or less, the FBN actually performs better if

the variable is omitted entirely (Ahmadi and Golian 2010).

Results and discussion

To develop a realistic model of a process, a previous knowledge and understanding of the process itself and the process parameters under investigation are a must. In recent works we have reported the successful adaptation of the lepidopteran insect cell Se301 to growth in agitated suspension culture (Beas-Catena et al. 2011) and in agitated serum-free suspension culture (Beas-Catena et al. 2013a). Additionally, it has been shown that infection strategy highly influences cell growth and baculovirus production (Beas-Catena et al. 2013b). However, the quantitative relationships among different parameters of infection (CCI, MOI, and MR) and environmental factors (*Re*) remain unclear and need to be elucidated to potential development of large-scale process for producing OBs in Se301 agitated serum-free cultures. To do this, the use of an ANNM is discussed below.

Fig. 2 **a** Main effects plot (estimated change in the response when each factor is moved from its low level to its high level); **b** interaction plot. An interaction between factors occurs when the change in response from the low level to the high level of one variable differs from the change in response at the same two levels of a second variable. Any discrepancy between the two lines marked with + and – can be attributed to a significant interaction between the variables in question

Response surface modeling and analysis

Several response surface models have been proposed for optimization of bioprocesses using baculovirus-infected insect cells (Sävenhed 2001) and other cells (Chang and Zandstra 2004; Mandenius and Brundin 2008). In this work, a MD-CCD experimental design with 40 experiments was performed according to Table 1. Previous studies (see above) allowed us to select the most appropriate ranges for the variables shown in Table 1. The results of the second-order response surface model fitting in the form of ANOVA are shown in Table 2. The R^2 statistic indicates that the

model as fitted explains 85.5% of the variability in OB production. The adjusted R -squared statistic also indicates a moderate significance of the model. The Durbin–Watson (DW) statistic indicates that there is no serial autocorrelation in the residuals at the 5.0% significance level (p value > 0.05). Table 2 shows that six effects have p values of less than 0.05, thus indicating that they are significantly different from zero at the 95.0% confidence level. It can be seen that all linear effects were significant, with CCI having the largest effect (F -ratio = 75.97; p value = 0.0000). Only the interactions CCI- Re and CCI-MR were also relevant. The main effects and interaction plots shown in Fig. 2 are consistent with these results. The lines in Fig. 2a indicate the estimated change in OB yield as each factor is moved from its low level to its high level, with all other factors held constant at a value midway between their lows and their highs. Clearly, the four factors significantly affect the response. Figure 2b highlights the relevance of the interactions CCI- Re and CCI-MR with respect to the remaining interactions.

As none of the squares effects were significant, the quadratic regression equation, as calculated using Statgraphics Centurion^R XVI, was reduced to the following expression:

$$\begin{aligned}
 OBs = & 1.5757 + 5.10022 \times CCI + 3.64745 \times 10^{-4} \\
 & \times Re + 8.08211 \times MOI + 5.54957 \times 10^{-2} \\
 & \times MR - 2.63559 \times 10^{-4} \times CCI \times Re \\
 & + 7.53931 \times 10^{-2} CCI \times MR
 \end{aligned}
 \quad (8)$$

From quadratic Eq. (8) of the model, the combination of factor levels which maximizes the OB yield to around 10.63×10^7 OB mL⁻¹ was calculated as CCI = 1.3×10^6 cells mL⁻¹, $Re = 3815$, MOI = 0.10 TCID₅₀/cell, and MR = 40%. The 3D response surface and the 2D continuous contour plots are displayed in Figs. 3a–f. Each contour curve represents an infinite number of combinations of two factors, with the other two maintained at their respective optimal level. The predicted maximum OB production is indicated by the red colored elliptical surface in the contour diagram. This is obviously in close agreement with the model prediction. A straightforward way to visualize this is provided in Fig. 4, in which a 3D-contour plot for the estimated response surface is displayed for the statistically significant interactions as shown in Table 2 of the ANOVA. It can

Fig. 3 Response surface and contour plot showing the effect of the interactions **a** CCI-MR, **b** CCI-Re, **c** CCI-MOI, **d** Re-MOI, **e** Re-MR, and **f** MOI-MR on OB production

Fig. 4 3D contours of estimated response surface for interactions CCI-Re-MR (at MOI = 0.1)

Fig. 5 Scatter plot and regression for observed versus predicted values for OB production. **a** RSM model; **b** FBN model

be seen that the optimal values of the factors maximizing OB yield are located in the outer corner of the cube. Thus, a multifactorial statistical approach

Fig. 6 Distance-weighted least-squares plot of artificial neural network-based predicted values for OB production for different levels of **a** Re and CCI, **b** MOI and CCI, **c** MR and Re, **d** MOI and Re, **e** MR and CCI, and **f** MR and MOI

Table 3 Relative importance of inputs in the FBN artificial neural network model for OBs

Item	CCI	Re	MR	MOI
VSR	5.32	2.72	2.03	1.27
Rank	1	2	3	4

that considers the interaction of independent factors affecting the production of OBs in agitated batch cultures provides a compelling basis for modeling the nonlinear nature of the OB response in a short-term experimental set.

FBN modeling and analysis

ANN modeling has demonstrated to be more accurate to optimize bioprocess conditions when compared to RSM, and has emerged in the last years as an attractive tool for non-linear empirical model development (Aehle et al. 2010; Marique et al. 2001; Silva et al. 2012; Tokatli et al. 2009; Valletta et al. 2016). The experimental design used in the CCD method and shown in Table 1 was also used as the data set for the ANN modeling. A FBN with four neurons in the input layer and one neuron in the output layer was chosen. The BFGS (Broyden–Fletcher–Goldfarb–Shanno) training function was chosen and the structure including one hidden layer with only two neurons, which had the minimum SOS value between the predicted outputs and experimental responses of the test set (SOS = 0.5733) and validation test (SOS = 0.1532), was selected. The SOS for the training set was 0.0872. Thus, the selected FBN model had a 4–2–1 topology, i.e. an input layer with four neurons, a hidden layer with two neurons, and an output layer with one neuron (see Fig. 1). The correlation coefficients between the model-predicted and experimental results for the training, test and validation sets were 0.9289, 0.9326, and 0.9944, respectively. The small SOS and the high correlation coefficients for the training set indicated that the ANN model has an outstanding approximation ability. Furthermore, the satisfactory values for the SOS and correlation coefficients for the test and validation sets suggested that the FBN model also has a good generalization capacity.

A comparison between the RSM and FBN models is shown in Fig. 5. The statistical parameters revealed that FBN outperformed the RSM model in terms of describing the response (OB production) for the investigated inputs. Regression analyses such as RSM are dependent on predetermined statistical significance levels, and less significant terms are usually not included in the model. In contrast, all data are potentially used with ANN methods, thus making the models more accurate (Korany et al. 2012). Baş and Boyacı (2007) implied that the main limitation of RSM is the use of a second-order equation to model the process, whereas the ANN model has a universal approximation capability that can approximate almost any kind of nonlinear function.

The relative importance of the input variables was determined using all the data (training, testing and validation) to calculate the overall VSR. The VSRs obtained for the model output (i.e. OB production), with respect to levels of CCI, *Re*, MOI and MR are shown in Table 3. The OB response was more sensitive to CCI, followed by *Re*, MR and MOI in that order. It is important to note that the sensitivity analysis revealed that the most important secondary variable in the OB production model was *Re*, whereas an ANOVA for the RSM model considered the effect of *Re* to be statistically insignificant. This discrepancy clearly shows that the influence of these four factors on the OB response depends upon the statistical method. However, appropriate mathematical or statistical models remain necessary to extract appropriate conclusions regarding the relative importance of each factor on OB production.

Based on the FBN model, three-dimensional response surface graphs were generated to represent the variation in OB production at different input levels (Fig. 6). These distance-weighted least-squares plots may be useful for understanding the complete inter-relationships between factors and response and could be used to optimize OB production. The optimal levels of the factors that maximize OB yield are similar to those obtained using the RSM model.

Conclusions

It is well known that optimal infection strategy largely depends on the cell line, the physiological state of the cells, and the baculovirus, as well as on the medium and the mode of operation of the bioreactor. However, the theoretical relationship

between different infection strategy parameters remains unknown. The results of this research revealed that the experimental data from RSM can be used to build an ANN model to establish an optimal infection strategy for baculovirus production. In this study the optimal strategy includes low *Re* and MOI and high CCI and MR. The ANN model can predict the baculovirus production more accurately than polynomial regression methods such as RSM. It will be of great help in the process of scale-up and will contribute to improve the process performance and economy, which are critical in the production of low-value products as baculovirus-based pesticides.

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