Biochemical Engineering Journal 35 (2007) 48–55

Biochemical Engineering Journal

www.elsevier.com/locate/bej

Optimization of the *Bacillus thuringiensis* var. *kurstaki* HD-1 δ-endotoxins production by using experimental mixture design and artificial neural networks

Guilherme A. Moreira [a,b,1], Gabriela A. Micheloud [b], Alejandro J. Beccaria [b,*], Héctor C. Goicoechea [a,*]

[a] *Laboratorio de Desarrollo Analítico y Quimiometría (LADAQ), Cátedra de Química Analítica I, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, CC 242 (S3000ZAA), Santa Fe, Argentina*

[b] *Laboratorio de Fermentaciones, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, CC 242 (S3000ZAA), Santa Fe, Argentina*

Received 4 September 2006; received in revised form 28 November 2006; accepted 19 December 2006

Abstract

An experimental mixture design coupled with data analysis by means of both response surface methodology (RSM) and artificial neural networks (ANNs) followed by multiple response optimization through a desirability function, was applied to the production of δ-endotoxins from *Bacillus thuringiensis* var. *kurstaki*. The composition of a culture medium was defined by testing three regional effluents: milky effluent, beer wastewater and sugar cane molasses. Both RSM and ANNs accomplished the goal pursued in this work, by predicting the optimal mixture of the effluents. ANNs provided more reliable results due to the complexity of the models to be fitted. The optimal selected blend was: 74%, 26% and 0%, respectively for each the above-mentioned effluents.

Keywords: *Bacillus thuringiensis* var. *kurstaki*; Modelling; Mixture design; Artificial neural networks (ANNs); Multiple response optimization

1. Introduction

The use of biological agents is a convenient strategy for urban, agriculture and forestry plague control. Different strains of *Bacillus thuringiensis* (Bt) are commonly employed, which is the most popular microorganism, with the 90% of the biopesticides world market [1,2]. Bt is a Gram-positive, aerobic, spore-former bacterium, with the ability to produce, during the sporulation phase, parasporal crystals named δ-endotoxins. These crystals are formed by proteins which possess the interesting quality of being toxic only against target insects [3]. Parasporal crystals and spores of Bt var. *kurstaki* constitute the active principle of commercially available products for the control of many lepidopteran larvae in agriculture and forestry [4,5]. The mechanism of action involves the solubilization of parasporal crystals and the activation of the released proteins in the target insect midgut. Then the active toxin links to specific cell receptors and forms channels trough the cell membrane. It causes the cell death and finally the insect death. In addition, the spores present in the product formulation can germinate, forming more Bt cells that contribute to kill the insect target and to maintain the infection in field [3].

The Bt industrial production for commercial purposes is mainly done by submerged fermentation processes. The cost of components employed in industrial fermentation media for Bt biopesticide production is 45% of the overall cost of the raw materials employed [1]. From an economical point of view, different alternative production processes have been described as promising: solid substrate fermentation [6,7], culture supernatant re-cycle [8] and effluents employment [9,10]. The latter alternative also generates a new concept on the ecological impact: the possibility of producing a useful biopesticide together with the effluent treatment [11,12]. Indeed, several works have shown that Bt could be isolated at high frequency

* Corresponding authors.
E-mail addresses: abeccari@fbcb.unl.edu.ar (A.J. Beccaria), hgoico@fbcb.unl.edu.ar (H.C. Goicoechea).
[1] Present address: Centro Politécnico, Laboratório de Processos Biotecnológicos, Universidade Federal do Paraná, Conselho Universitário, Jardim das Américas, 81531-990-Curitiba, PR, Brazil.

1369-703X/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2006.12.025

from effluents [13,14]. Besides, different kinds of effluents have successfully been employed as culture media for Bt biopesticides production [9,15,16].

There are a large number of techniques available to design culture media. They can vary from the traditional one-variable-at-a-time method [17,18] to more complex statistical and mathematical techniques involving experimental designs such as full and partial factorials, Plackett-Burman, Hadamard matrix and central composite designs [19–21], followed by optimization techniques such as response surface methodology (RSM), artificial neural networks (ANNs), fuzzy logic, genetic algorithms (GA) [22–26] and particle swarm optimization (PSO) [27,28], among others. An interesting review of the strategies used in the optimization of fermentation media can be found in the literature [29]. Regrettably, no multipurpose technique is known to be applicable to all situations. Consequently, sometimes it becomes necessary to screen several approaches to find the one which provides the best result in a particular case.

The aim of this work was to define the composition of a culture medium by testing three regional effluents which present high economical impact: milky effluent, beer wastewater and sugar cane molasses, through a mixture design. Remarkably, this class of designs is seldom used for fermentation optimization, although it has been commonly employed in other areas such as industrial, chemical, engineering, agricultural and food sciences. However, mixture designs have proved to be very efficient in biological sciences [30,31]. On the other hand, the rational Bt media optimization was only aimed for classical media culture, applied in lab scale [32,33].

The goals herein pursued were to maximize the quantity of parasporal crystals and spores, while minimizing the remaining vegetative cells in batch culture of Bt var. *kurstaki* HD-1 by using multiresponse optimization. Three regional effluents were tested: milky effluent, beer wastewater and sugar cane molasses. The latter one is a by-product of sugar refinery, commonly employed as carbon source in culture media used for many industrial fermentation. On the other hand, milky effluent and beer wastewater are industrial wastes with considerable biochemical oxygen demand. Previous results in our group have shown these effluents as promising ingredients for Bt culture medium (data not published).

Both RSM and ANNs approaches were tested to deal with the problem of formulating the culture medium. To the best of our knowledge, ANNs have generally been applied to fermentative processes monitoring, but not for culture media optimization for the microorganism herein studied [34–37]. In addition, no applications of ANNs have been published neither for optimization of experimental mixture designs nor for multiresponse optimization. As will be shown, both RSM and ANNs accomplished the goal pursued in this work by predicting the optimal mixture of the effluents. Probably due to the complexity of the models to be fitted, ANNs provided more reliable results.

2. Theory

Two approaches were tested in order to find the optimal proportion of all components (effluents) in the culture medium: (a) fitting a polynomial model trough the response surface methodology, and (b) application of ANNs. In both circumstances, multiple response optimization through the desirability function was performed.

2.1. Fitting a polynomial model

The polynomial used in the present work has some terms modified from the complete polynomial expression generally used in RSM. It allows to eliminate the constraint originated in the correlated variables, and was introduced by Scheffé in 1963 [38]. Eq. (1) shows the canonical form of the *special cubic* model which corresponds to a linear or a quadratic model if only a part of it is used for the fitting:

$$y = \sum_{i=1}^{q} \beta_i x_i + \sum\sum \beta_{ij} x_i x_j + \sum \sum_{i<j<k}^{q} \sum \beta_{ijk} x_i x_j x_k \quad (1)$$

where the parameter β_i represents the expected response to the pure mixture $x_i = 1, x_j = 0, j \neq i$. The term given by $y = \sum_{i=1}^{q} \beta_i x_i$ represents the response when blending is strictly additive, and there are no interactions among the components of the mixture, i.e., the linear model. The term $\beta_{ij} x_i x_j$ represents the excess response over the linear model due to the interaction between two components, and this effect is often called synergism (or antagonism). The cubic term $\beta_{ijk} x_i x_j x_k$, accounts for the effect of ternary blending among the components in the interior of the simplex [39].

2.2. Application of ANNs

The ANN modelling is a powerful chemometric tool for processing information, which simulates some properties of the human brain, especially developed to model non-linear data. The so-called multilayer feed-forward networks [40,41] are often used for prediction as well as for classification. In the present work we used ANNs that consist of three layers of neurons or nodes, which are the basic computing units: the input layer with a number of active neurons corresponding to the predictor variables in regression, and one hidden layer with a number of active neurons. The input layer corresponds to the number of studied factors and the hidden layer number is optimised during training. The output layer has just one unit. The neurons are connected in a hierarchical manner, i.e., the outputs of one layer of nodes are used as inputs for the next layer and so on. In the hidden layer the sigmoid function $f(x) = 1/(1 + e^{-x})$ is used, and the output of the hidden neuron j, O_j, is calculated as

$$O_j = f\left[\sum_{i=1}^{m} (s_i w_{ij} + w_{bj})\right] \quad (2)$$

In Eq. (2) s_i is the input from neuron i in the layer above, to neuron j in the hidden layer, w_{ij} the connection weights between neurons i and j, w_{bj} the bias to neuron j and m is the total number of neurons in the layer above.

Linear functions are used both in the input and output layers. In the present work, learning is carried out through the

back-propagation rule [40]. The number of hidden layers and of neurons in each hidden layer must be selected to achieve a satisfactory fitting ability of the network, associated to a satisfactory predictive ability. If the number of hidden layers or neurons in the hidden layer(s) is too high, the network – although it will reach a great modelling ability – will lose the ability to generalise and to predict. This is known as overfitting.

It is important to stress that ANNs trained with this rule have a remarkable advantage: there is no need to know the exact form of the analytical function on which the model should be built. Furthermore, neither the functional type nor the number of parameters in the model need to be given [40]. This is the main difference from the RSM, which requires fitting one of the models presented in Eq. (1).

2.3. Multiresponse optimization

The responses predicted by RSM or ANNs are used for generating a function that assigns a value ranging from 0 to 1 to each mixture of compounds. This function, referred to as *partial desirability function for the response i* (d_i) [42,43], would be equal to 0 when the predicted response does not fulfil the predefined requirements, and 1 when the predicted value completely satisfies them. The methodology consists in building one- or two-sided functions, which depend on whether each of the m responses has to be maximized or minimized, or has an allotted target value. The procedure involves creating a function for each individual response d_i and finally obtaining a global function D that should be maximized choosing the best conditions of the designed variables. When the goal is a maximum, the desirability curve will be defined by the Eq. (3):

$$d_i = \left[\frac{Y_i - \text{Low}_i}{\text{High}_i - \text{Low}_i}\right]^{wt_i}, \quad \text{Low}_i < Y_i < \text{High}_i \tag{3}$$

where Y_i is the predicted response using the fitted model (Eq. (1)), High_i and Low_i are the highest and the lowest values obtained for the response i respectively and wt_i is the weight. Weights give emphasis to upper or lower bounds, or to a target value. With a weight of 1, the d_i will vary from 0 to 1 in a linear way while approaching to the desired value. Weights greater than 1 give more emphasis to the goal, whereas weights lower than 1 give less emphasis to the goal (in both cases, d_i varies in a non-linear way).

On the other hand, if the goal is a minimum, the desirability is defined as Eq. (4):

$$d_i = \left[\frac{\text{High}_i - Y_i}{\text{High}_i - \text{Low}_i}\right]^{wt_i} \tag{4}$$

Alternatively, for goal as a target, the desirability ramps are created like a maximum on the way up, and a minimum on the way down. Finally, for a goal within a range, the desirability will be defined by the following equations:

$$d_i = 0 \quad \text{for} \quad Y_i \leq \text{Low}_i \tag{5}$$

$$d_i = 1 \quad \text{for} \quad \text{Low}_i < Y_i < \text{High}_i \tag{6}$$

In the present report we chose weights equal to 1 for all the five responses.

The d_i functions are then combined to obtain a *global desirability function D*, which should be maximized choosing the best conditions of the designed variables. This function can be represented by Eq. (7):

$$D = (d_1^{r_1} \times d_2^{r_2} \times d_3^{r_3})^{1/\sum_{i=1}^{i=3} r_i} \tag{7}$$

where D is the value of the global desirability function, d_1, d_2 and d_3 are the partial desirability functions computed for each response and r_i is the relative importance assigned to each response. Relative importance r_i is a comparative scale for allotting emphasis to each d_i in the expression of the function D.

Finally, the mixture of the three effluents that predicts the highest value of D is selected as the best blend of components to be present in the culture medium being developed.

3. Materials and methods

3.1. Bacterial strain

Bt var. *kurstaki* HD-1 was used in this study. It was kindly provided by Dra. Graciela Benintende, IMYZA, Instituto Nacional de Tecnología Agropecuaria, Argentina. The bacterial strain was grown on Tryptic Soy Agar slants (Britania, Argentina) and stored at 4 °C.

3.2. Substrates and culture media design

Sugar cane molasses, reduced-fat milk and beer wastewater were employed as substrates. A volume of sugar cane molasses (Melrico, Argentina) was suspended in demineralised water in order to obtain a 30° Brix suspension. A suspension of 2.624 g L^{-1} of powder reduced-fat milk (Nestle, Argentina) in demineralised water was employed as milky effluent substitute. It is equivalent to 1915.5 mg L^{-1} of biochemical oxygen demand [44]. Beer wastewater was collected at the inlet of a beer wastewater treatment plant at Compañía Industrial Cervecera, Santa Fe, Argentina. This effluent had a chemical oxygen demand of 3473 mg L^{-1}; pH 10.2 and suspended solids of 1.0 mg L^{-1}.

Effluents proportion was analysed by using a simplex lattice $\{3, 2\}$ augmented with the overall centroid and axial points. This design has 10 points, with four of these points in the interior of the simplex [45]. Additional replicates and a random point were added in order to increment the number of experiments for modelling purposes (see Table 1). A control culture was done employing TSB as culture medium.

3.3. Inoculum preparation and culture conditions

A loopful of stored slants was used to inoculate 250 mL Erlenmeyer flasks containing 20 mL of sterilized TSB (Tryptic Soy Broth, 30 g L^{-1}; Britania, Argentina). After 4 days of incubation on a rotary shaker at 200 rpm (shaking diameter 20 mm) and 30 ± 1 °C, the biomass was harvested by centrifugation (3000 × g for 15 min). The supernatant was discarded and the

Table 1
Experimental design (simplex lattice mixture) used to optimize the parasporal crystal production and responses obtained

Run	Volume (mL)			Concentration ($\times 10^6$ mL^{-1})		
	Artificial milky effluent	Beer wastewater	Sugar cane molasses	Parasporal crystals	Spores	Remaining cells
1	20.0	0.0	0.0	3.5 (0.6)	16.1 (2.9)	2.6 (0.8)
2	0.0	0.0	20.0	58.9 (8.2)	58.4 (4.4)	25.8 (7.2)
3	6.7	6.7	6.7	10.1 (2.3)	25.2 (2.3)	2.9 (0.9)
4	3.3	3.3	13.3	40.9 (7.3)	20.7 (5.4)	59.9 (8.9)
5	0.0	20.0	0.0	25.6 (3.0)	21.3 (4.0)	11.2 (2.4)
6	13.3	3.3	3.3	247.0 (20.0)	77.6 (9.0)	4.6 (12.0)
7	0.0	10.0	10.0	3.8 (0.9)	2.8 (1.8)	3.1 (0.8)
8	10.0	10.0	0.0	5.8 (0.9)	13.1 (4.4)	9.4 (2.0)
9	3.3	13.3	3.3	4.3 (0.7)	8.8 (1.1)	2.8 (1.4)
10	6.7	6.7	6.7	22.6 (5.1)	43.8 (6.3)	4.1 (1.7)
11	0.0	0.0	20.0	73.3 (12.7)	116.0 (12.0)	104.0 (17.0)
12	10	0.0	10.0	4.8 (1.0)	6.2 (0.5)	6.2 (0.7)
13	14.0	6.0	0.0	447.0 (44.0)	329.0 (35.0)	10.7 (3.0)
Control	–	–	–	117.0 (20.0)	211.0 (53.0)	49.4 (10.0)

Values in parenthesis are standard deviations of measurements.

pellet was suspended in 2.6 mL of PBS (phosphate buffer saline: KH_2PO_4 0.6 g L^{-1}, Na_2HPO_4 0.78 g L^{-1}, NaCl 8.8 g L^{-1}; pH 7.3). This suspension contained 1.6×10^9 spores mL^{-1}. A 0.2 mL of this suspension was employed to inoculate 250 mL Erlenmeyers flasks, each containing 20 mL sterilized (121 °C, 15 min) designed medium as described above. The pH of each mixture was previously adjusted to 7.00. The flasks were incubated as the inoculum flask, for 4 days. After this incubation period, samples of each culture were drawn for parasporal crystals, spores and remaining cells count. These culture setting values (i.e., shaking speed and volume culture to volume flask ratio) were previously optimised in our laboratory. In this way, we are confident that sufficient oxygen transfer rate and mixing conditions for each mixture are obtained. Also, the harvest of the culture at fourth day, allow a complete growth and differentiation of Bt. Indeed, in controls, the batch culture is completed in 24–48 h. On the other hand, long period of time is not convenient for the profitability of this kind of bioprocess.

3.4. Analytical

Counts were done by Breed method. Briefly, a 2 μL of PBS-washed sample dilution were distributed on a 0.28 cm^2 on a slide. Samples were fixed by flame and then stained with a solution of violet crystal (5.0 g L^{-1}) during 1 min. After cleaning with tap water and drying at room temperature, samples were counted employing a calibrated microscopy. Each sample was processed in duplicate.

Design Expert™ version 7.0.3 trial (Stat-Ease Inc., Minneapolis, USA) was used for performing experimental design and data analysis. A MATLAB [46] routine that was kindly provided by Dr. Alejandro Olivieri (Universidad Nacional de Rosario, Argentina), was used for ANNs applications.

4. Results and discussion

Table 1 shows the yield of each culture. The highest spore yield obtained in culture #13 is comparable with values reported for Bt var. *kurstaki* grown in wastewater sludge as batch culture in shake flask [13,47]. However, spores yields in shake flask are slightly lower than those reported for bioreactor cultures [47,48] probably due to better control of pH and dissolved oxygen [47,49]. Results obtained in culture #13, also shows the better conversion of total cells formed in the culture (spores plus remaining vegetative cells) to spores (97%). These obtained values are higher than the ones obtained in a control culture in TSB. On the other hand, culture #6 showed the best ratio of parasporal crystal to total cell formed in the culture, followed by culture #13.

When experimental data were analyzed through the model presented in Eq. (1), it was possible to fit different models (linear, quadratic, or cubic) for each response that was dependent on medium composition. The model selected for each response was the highest order polynomial where the terms are significant and the model is not aliased. The response surface plots corresponding to the fitting performed for all the three responses (parasporal crystal and spores production and remaining cells) in the experimental design is shown in Figs. 1–3, in which the surfaces depend on the polynomial model fitted for every one of them (see below). As can be seen, similar plots were obtained for the two first responses, indicating a high correlation among these dependent variables. Another interesting finding can be made observing Fig. 3, it is that the minimum of remaining cells is not flattered by sugar cane molasses. The fitted models and their corresponding statistical parameters can be seen in Table 2. As can be noted, a cubic model was selected for all the three responses. The rather high complexity of the response surface model was probability necessary due to strong interactions between the components. Table 2 summarizes the *F*- and the *P*-values obtained for the fitted models: *F*-Model, *P*-Model, and the *F*- and *P*-values corresponding to the lack of fit (LOF) test, *F*-LOF and *P*-LOF. *P*-Model values should be less than 0.05 for the model to be significant (that is, not due to noise). On the other hand, *P*-LOF > 0.05 is desirable, meaning the lack of fit is not significant relative to the pure error. As can be observed from Table 2, only for spores a good fit is

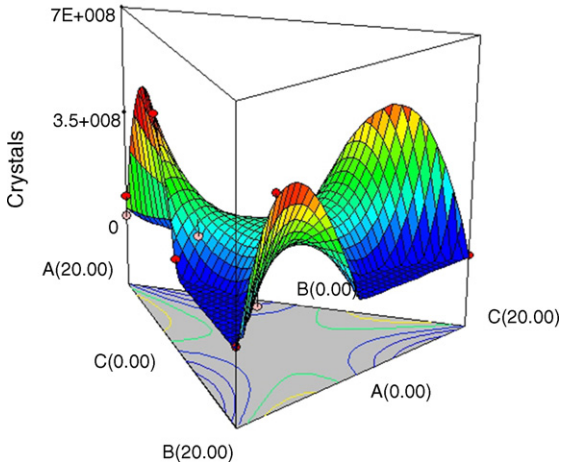

Fig. 1. Response surfaces obtained for parasporal crystals production as a function of the three effluents being optimized (A: milky effluent; B: beer wastewater; C: sugar cane molasses).

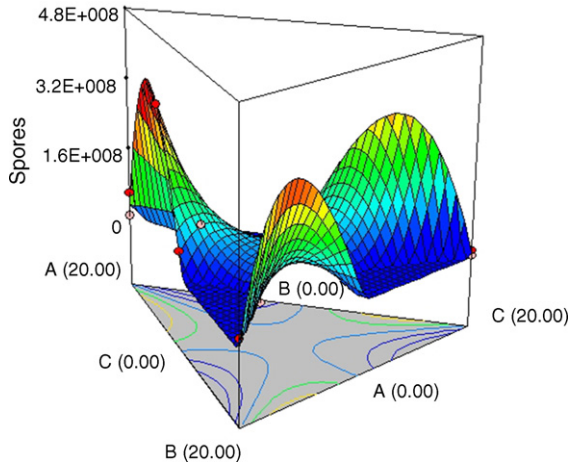

Fig. 2. Response surfaces obtained for spores production as a function of the three effluents being optimized (A: milky effluent; B: beer wastewater; C: sugar cane molasses).

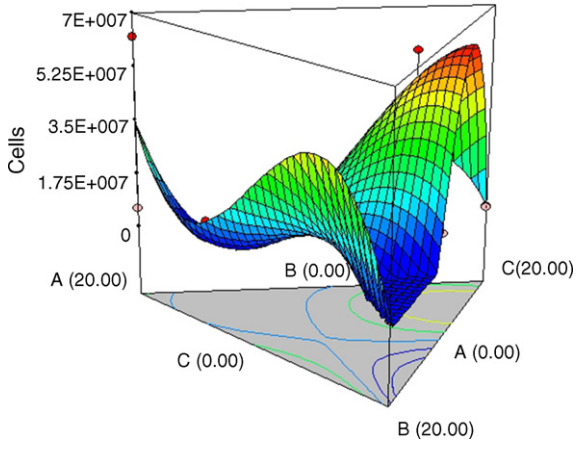

Fig. 3. Response surfaces obtained for remaining vegetative cells as a function of the three effluents being optimized (A: milky effluent; B: beer wastewater; C: sugar cane molasses).

Table 2
Statistical results obtained for the model and the lack of fit test

Statistical parameter	Parasporal crystals	Spores	Remaining cells
Fitted model	Cubic	Cubic	Cubic
F-Model	4.45	16.72	0.57
P-Model[a]	0.126 (not significant)	0.022 (significant)	0.773 (not significant)
F-LOF	17.43	1.38	0.49
P-LOF[a]	0.0529 (not significant)	0.326 (not significant)	0.556 (not significant)
R^2	0.888	0.969	0.632

[a] Conclusion between parenthesis.

obtained ($R^2 = 0.969$, *P*-Model < 0.05 and *P*-LOF > 0.05), while for the other two responses, although the lack of fit is not significant (*P*-LOF > 0.05 in both occasions), the models are not good enough (*P*-Model > 0.05). Consequently, poor reliability can be expected from these models. This complexity could be explained not only by the strong interactions between the components, but also by the high noise level.

Despite of the poor fitting results, the analysis through the desirability function was performed in order to get an approximation to the optimum effluents mixture. The goal was to establish which effluent mixture produces the highest values of both parasporal crystal and spore concentration while minimizing the remaining vegetative cells. Fig. 4 shows the plot corresponding to the *D* function. The optimal value found was $D = 0.979$, which corresponds to the optimal blend (mL): artificial milky effluent 15.3, beer wastewater 4.4 and sugar cane molasses 0.3. The corresponding predicted response values (mL^{-1}) are: parasporal crystals 4.5×10^8; spores 3.3×10^8; remaining vegetative cells 6.4×10^6. The global desirability function was calculated assigning a $r_i = 1$ (Eq. (7)). It is important to note that also reasonably good *D* values were obtained for several mixtures containing more sugar cane molasses, fact that agree with the surfaces of Figs. 1–3.

Due to the low reliability on predicting an optimal blend of effluents by means of RSM, artificial neural networks were

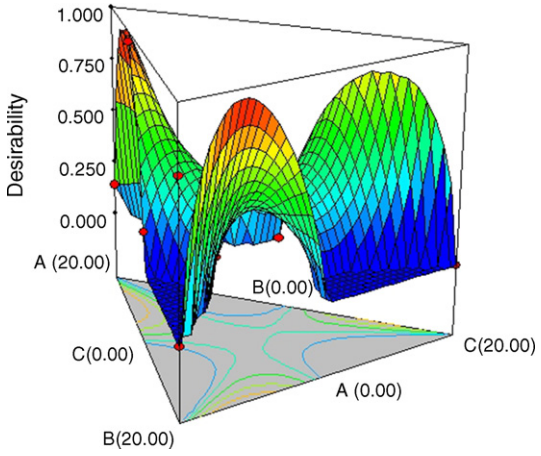

Fig. 4. Response surface obtained for the global desirability function computed using the Scheffé model (A: milky effluent; B: beer wastewater; C: sugar cane molasses).

Table 3
Architecture and statistical parameters of the selected ANNs

	Parasporal crystals	Spores	Remaining cells
Net architecture	3/3/1	3/3/1	3/3/1
Parameters/data	16/39	16/39	16/39
Epochs	5000	5000	5000
Learning rate	0.4	0.5	0.45
Momentum coefficient	0.6	0.5	0.55
REP (%)[a]	3.7	6.3	5.1
R^2	0.983	0.979	0.834

[a] REP $(\%) = 100/\bar{y}_{act}\left[(1/I)\sum_1^I (y_{act} - y_{pred})^2\right]^{1/2}$ where y_{act} are the experimental values for each response, $\bar{y}_{act}$ the corresponding average value for the 13 experiments, and y_{pred} are the model predicted values.

trained to explore this experimental space. The general architecture of the ANNs used in this work consisted in three layers of neurons: an input layer of three neurons (the combination of the three effluents in the medium), a hidden layer of neurons and an output layer of one neuron (each response). Several different architectures for each response were optimized by trial and error, varying the number of the neurons of the hidden layer. Finally one ANN was selected for modelling each response. Table 3 shows the architecture for each ANN (the numbers between brackets indicate how many active neurons are employed in each layer), the relative error prediction (REP%) and other figures of merit obtained when ANNs are trained: parameters-data ratio (the number of parameters being adjusted should no exceed the number of data), number of epochs, learning rate and momentum coefficient. The REP% values are indicative that ANNs were trained up to an experimental error level (see Table 3). Lower values of REP% should conduct to overtraining and consequently a bad predictive ability. On the other hand, actual versus ANN-predicted values were employed to calculate R^2 in order to compare the ability of ANN against the RSM methodology. The These parameters are also displayed in Table 3. A considerable improvement of the R^2 can be observed, especialy for remaining cells (ca. 30%).

After training the ANNs, the best models were used for predicting the responses of 5151 different simulated mixtures of the three variables, which exhaustively covered the entire experimental space. The predicted responses were then used to calculate their associated d_i and D values (see Section 2). The global desirability function was calculated assigning a $r_i = 1$ (Eq. (7)), as for RSM. The optimal value found was $D = 0.896$, which corresponds to the mixture (mL): artificial milky effluent 14.8 (74%), beer wastewater 5.2 (26%) and sugar cane molasses 0.0 (0%). The corresponding predicted response values are (mL^{-1}): parasporal crystals 4.1×10^8, spores 2.9×10^8 and remaining vegetative cells 9.0×10^7. Once more, as when the desirability was previously analysed for the RSM methodology, reasonably good D values were obtained for several mixtures containing more sugar cane molasses. As can be seen, these latter values agree well with those obtained by RSM, although one could say that they are more reliable due to the better statistical parameters obtained for ANNs model. Interestingly, the D value for the RSM method was better than the corresponding one to ANNs, although both of them can be considered excellent considering three responses are being simultaneously optimised.

An interesting observation can be made when analysing the obtained result, and comparing it with the one proportioned for culture #13. This latter culture gave the highest yield of parasporal crystals and spores, with an acceptable number of remaining vegetative cells. The culture #13 composition is almost equal to the optimum blend suggested by the application of ANNs. However, a new experiment was performed to check the optimization. The effluent mixture was set following the conditions recommended by the ANN optimization. The result of this experiment was: parasporal crystals 4.5×10^8, spores 3.4×10^8 and remaining vegetative cells 8.5×10^7. As can be seen, high concordance with the theoretical value was obtained.

These results show the relevance of the milky effluent as a blend component. This fact agrees well with results published by Desai et al., in which defatted milk powder was used as secondary nitrogen source in culture media for bioinsecticidas species of *Bacillus* [50]. This component resulted promising for toxicity production in *Bacillus sphaericus* culture. Finally, it is important to remark that milky effluent is a bulk waste in our region, place where soy cultures are also developed. From a self-sufficient point of view, our results could encourage the installation of anti lepidopteran Bt var. *kurstaki* strain production process, employing milky effluent in the formulation of a culture medium to control a relevant soy plague. However, the improvement obtained when optimal beer effluent was added, shows the importance of same components in the brewer waste. Between these, ethanol and residual sugar could be good carbon and energy sources that improve the carbon to nitrogen ratio when milk effluent is employed. On the other hand, same yeast-derived components, could act as growth factors for Bt. In fact, yeast extract is mainly employed in industrial fermentation of Bt, specially because it supplies growth factors [50,51].

5. Conclusion

An optimal mixture of effluents was defined by RSM, although the fitted model presented low reliability. Conversely, ANN trained with the same experimental results allowed us to select a mixture of the three effluents that is consistent with the estimation made by RSM, while the results were more reliable, in view of the statistical parameters computed for both models.

In the case exposed here, ANNs proved to be a more suitable approach than RSM for defining the formulation of an improved culture medium used in a productive bioprocess. Apparently, ANNs are able to better model the rather high complexity of the data probability due to strong interactions between the components. Nevertheless, ANNs call for more sophisticated software than fitting a polynomial.

In this work, employing rational combinations of different effluents was possible to obtain the optimal mixture that allow not only Bt var. *kurstaki* growth, but differentiation and parasporal crystals production. The spores yielding was similar to that reported by other authors who developed the Bt biopesticides production technology in effluents. However, complementary studies should be made to evaluate the formulation predicted by

the optimization such as the efficiency and potency of the products in target larvae. Also, the redefinition of parameters of the raw materials employment in media formulation could be important with the aim of increase the δ-endotoxins production. The study could be enhanced with a new screening of combinations of other effluents by using this methodology.

Acknowledgements

Universidad Nacional del Litoral and CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) are acknowledged for financial support. G.A. Moreira thanks AUGM for a fellowship. G.A. Micheloud thanks CONICET for a fellowship.

References

[1] G.E. Rowe, A. Margaritis, Bioprocess design and economic analysis for the commercial production of environmentally friendly bioinsecticidas from *Bacillus thuringiensis* HD-1 *kurstaki*, Biotechnol. Bioeng. 86 (2004) 377–388.

[2] N. Zouari, S.B.S. Ali, S. Jaoua, Production of delta-endotoxins by *Bacillus thuringiensis* strains exhibiting various insecticidal activities towards lepidoptera and diptera in gruel and fish meal media, Enzyme Microbial Technol. 31 (2002) 411–418.

[3] A. Aronson, Sporulation and δ-endotoxin synthesis by *Bacillus thuringiensis*, Cell. Mol. Life Sci. 59 (2002) 417–425.

[4] R.K. Hynes, S.M. Boyetchko, Research initiatives in the art and science of biopesticide formulations, Soil Biol. Biochem. 38 (2006) 845–849.

[5] A. Navon, *Bacillus thuringiensis* insecticides in crop protection-reality and prospects, Crop Protect. 19 (2000) 669–676.

[6] T.T. Adams, M.A. Eitman, B.M. Hanel, Solid state fermentation of broiler litter for production of biocontrol agents, Bioresource Technol. 82 (2002) 33–41.

[7] C. Hongzhang, X. Fujian, T. Zhonghou, L. Zuohu, A novel industrial-level reactor with two dynamic changes of air for solid-state fermentation, J. Bios. Bioeng. 93 (2002) 211–214.

[8] C.L. Luna, G.R. Silva, E.M.M.M. Rios, *Bacillus thuringiensis* var. *israelensis* production involving re-use of the supernatant, Biotechnol. Lett. 26 (2004) 143–145.

[9] A.S. Vidyarthi, R.D. Tyagi, J.R. Valéro, R.Y. Surampalli, Studies on the production of *B. thuringiensis* based biopesticides using wastewater sludge as a raw material, Water Res. 36 (2002) 4850–4860.

[10] M.L. Tirado Montiel, R.D. Tyagi, J.R. Valéro, Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticides, Water Res. 35 (2001) 3807–3816.

[11] P. Kumar, R. Chandra, Detoxification of distillery effluent through *Bacillus thuringiensis* (MTCC 4714) enhanced phytoremediation potential of *Spirodela polyrrhiza* (L.) Schliden, Bull. Environ. Contam. Toxicol. 73 (2004) 903–910.

[12] V. Sachdeva, R.D. Tyagi, J.R. Valéro, Production of biopesticides as a novel method of wastewater sludge utilization/disposal, Water Sci. Technol. 42 (2000) 211–216.

[13] S. Mohammedi, S. Bala Subramanian, S. Yan, R.D. Tyagi, J.R. Valéro, Molecular screening of *Bacillus thuringiensis* strains from wastewater sludge for biopesticide production, Process Biochem. 41 (2006) 829–835.

[14] E. Mizuki, M. Maeda, R. Tanaka, D.W. Lee, M. Hara, T. Akao, S. Yamashita, H.S. Kim, T. Ichimatsu, M. Ohba, *Bacillus thuringiensis*: a common member of microflora in activated sludges of a sewage treatment plant, Curr. Microbiol. 42 (2001) 422–425.

[15] H.L. Lee, P. Seleena, Fermentation of a Malaysian *Bacillus thuringiensis* serotype H-14 isolate, a mosquito microbial control agent utilizing local wastes, Southeast Asian J. Trop. Med. Public Health 22 (1991) 108–112.

[16] I.O. Moraes, D.M.F. Capalbo, R.O. Moraes, Bacterial insecticide production by *Bacillus thuringiensis*, Food Biotechnol. 4 (1990) 282–292.

[17] A.V. Pundle, H. Siva Raman, Medium optimization for the production of penicillin V acylase from *Bacillus sphaericus*, Biotech. Lett. 16 (1994) 1041–1046.

[18] K. Furuhashi, M. Takagi, Optimization of a medium for the production of 1,2-epoxytetradecane by *Nocardia corallina* B-276, Appl. Microbiol. Biotechnol. 20 (1984) 6–9.

[19] P.M.L. Castro, P.M. Hayter, A.P. Ison, A.T. Bull, Application of a statistical design to the optimization of culture medium for recombinant interferon-gamma production by Chinese hamster ovary cells, Appl. Microbiol. Biotechnol. 38 (1992) 84–90.

[20] R.G. Silveira, T. Kakizono, S. Takemoto, N. Nishio, S. Nagai, Medium optimization by an orthogonal design for the growth of *Methanosarcina barkeri*, J. Ferm. Bioeng. 72 (1991) 20–25.

[21] T. Thiel, J. Bramble, S. Rogers, Optimum conditions for growth of cyanobacteria on solid media, FEMS Microbiol. Lett. 61 (1989) 27–31.

[22] N.A. Jacometti, C. Furtado, M.C. Teixeira Duarte, S. de Albuquerque, C. Mello, J. Kenupp Bastos, Improvement of trypanocidal metabolites production by *Aspergillus fumigatus* using neural networks, Microbiol. Res. 160 (2005) 141–148.

[23] P. Teissier, B. Perret, E. Latrille, J.M. Barillere, G. Corrieu, A hybrid recurrent neural network model for yeast production monitoring and control in a wine base medium, J. Biotechnol. 55 (1997) 157–169.

[24] S. Jin, K. Ye, K. Shimizu, J. Nikawa, Application of artificial neural network and fuzzy control for fed-batch cultivation of recombinant *Saccharomyces cerevisiae*, J. Ferm. Bioeng. 81 (1996) 412–421.

[25] R.F. Wilkinson, R. Ming, B. Anderson, T.D. Bunch, K.L. White, The use of neural networks in developing novel embryo culture media-formulations, Theriogenology 45 (1996) 41–49.

[26] S.G. Prapulla, Z. Jacob, N. Chand, D. Rajalakshmi, N.G. Karant, Maximization of lipid production by *Rhodotorula gracilis* CFR-1 using response surface methodology, Biotechnol. Bioeng. 40 (1992) 965–970.

[27] A.R. Cockshott, B.E. Hartman, Improving the fermentation médium for *Echinocandin B* production. Part II. Particle swarn optimization, Process Biochem. 36 (2001) 661–669.

[28] W.-Q. Lin, J.-H. Jiang, Q. Shen, G.-L. Shen, R.-Q. Yu, Optimized block-wise variable combination by particle swarm optimization for partial least squares modeling in quantitative structure-activity relationship studies, J. Chem. Inf. Model. 45 (2005) 486–493.

[29] M. Kennedy, D. Krause, Strategies for improving fermentation medium performance: a review, J. Ind. Microb. Biotechnol. 23 (1999) 456–475.

[30] K.-M. Lee, D.F. Gilmore, Formulation and process modeling of biopolymer (polyhydroxyalkanoates: PAHs) production from industrial wastes by novel crossed experimental design, Process Biochem. 40 (2005) 229–246.

[31] C. Didier, M. Etcheverrigaray, R. Kratje, H.C. Goicoechea, Crossed mixture design and multiple response analysis for developing complex culture media used in recombinant protein production, Chemom. Intell. Lab. Syst. 86 (2007) 1–9.

[32] Z. Tokcaer, E. Bayraktar, U. Mehmetoglu, G. Özcengiz, N.G. Alaeddinoglu, Response surface optimization of antidispteran delta-endotoxin production by *Bacillus thuringiensis* subsp. *israelensis* HD 500, Process Biochem. 41 (2006) 350–355.

[33] R.R. Farrera, F. Pérez-Guevara, M. de la Torre, Carbon:nitrogen ratio interacts with initial concentration of total solids on insecticidal crystal protein and spore production in *Bacillus thuringiensis* HD-73, Appl. Microbiol. Biotechnol. 49 (1998) 758–765.

[34] K.M. Desai, S.K. Akolkar, Y.P. Badhe, S.S. Tambe, S.S. Lele, Optimization of fermentation media for exopolysaccharide production from *Lactobacillus plantrarum* using artificial intelligence based techniques, Process Biochem. 41 (2006) 1842–1848.

[35] R.K.I. Anderson, K. Jayaraman, Impact of balanced substrate flux on the metabolic process employing fuzzy logic during the cultivation of *Bacillus thuringiensis* var. *galleriae*, World J. Microbiol. Biotechnol. 21 (2005) 127–133.

[36] L. Valdez-Castro, I. Baruch, J. Barrera-Cortés, Neural networks applied to the prediction of fed-batch fermentation kinetics of *Bacillus thuringiensis*, Bioproc. Biosyst. Eng. 25 (2003) 229–233.

[37] Q. Zhang, J.F. Reid, J.B. Litchfield, J. Ren, S.W. Chang, A prototype neural network supervised control system for *Bacillus thuringiensis* fermentations, Biotechnol. Bioeng. 43 (1994) 483–489.
[38] H. Scheffé, Simplex-centroid design for experiments with mixtures, J. R. Stat. Soc. B 25 (1963) 235–263.
[39] G. Dingstad, F. Westad, T. Naes, Three case studies illustrating the properties of ordinary and partial least squares regression in different mixture models, Chemom. Intell. Lab. Syst. 71 (2004) 33–45.
[40] J. Zupan, J. Gasteiger, Neural Networks in Chemistry and Drug Design, 2nd ed., Wiley-VCH, Weinheim, 1999.
[41] F. Despagne, D.L. Massart, Neural networks in multivariate calibration, Analyst 123 (1998) 157R–178R.
[42] G. Derringer, R. Suich, Simultaneous optimization of several response variables, J. Qual. Technol. 12 (1980) 214–219.
[43] R.H. Myers, D.C. Montgomery, Response Surface Methodology, Process and Product Optimization using Designed Experiments, John Wiley & Sons, New York, 1995.
[44] A.J. Calamante, S.R. Serra, C.H. Lombardi, J.G. Gonzales, Guía de funcionamiento, operación y control de un sistema de barros activados a escala de laboratorio, Ingeniería Sanitaria (AIDIS) 4 (1980) 19–25.
[45] J.A. Cornell, A comparison between two ten-point designs for studying three-component mixture systems, J. Qual. Technol. 18 (1986) 1–15.
[46] MATLAB 6.0, The MathWorks Inc., Natick, Massachusetts, USA, 2000.
[47] A. Yezza, R.D. Tyagi, R.D. Valéro, R.Y. Surampalli, Correlation between entomotoxicity potency and protease activity produced by *Bacillus thuringiensis* var. *kurstaki* grown in wastewater sludge, Process Biochem. 41 (2006) 794–800.
[48] R.D. Tyagi, Producing Bt biopesticides from wastewater sludge, Ind. Bioproc. 27 (2005) 9.
[49] K. Lachhab, R.D. Tyagi, J.R. Valéro, Production of *Bacillus thuringiensis* biopesticides using wastewater sludge as a raw material: effect of inoculum and sludge solids concentration, Process Biochem. 37 (2001) 197–208.
[50] S.Y. Desai, Y.I. Shethna, Production & formulation of *Bacillus thuringiensis* var. *israelensis* & *B. sphaericus* 1593, Indian J. Med. Res. 93 (1991) 318–323.
[51] Y. Içgen, B. Içgen, G. Özcengiz, Regulation of crystal protein biosynthesis by *Bacillus thuringiensis*. II. Effects or carbon an nitrogen sources, Res. Microbiol. 153 (2002) 605–609.