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

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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.

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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

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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 $\beta_{ij}x_ix_j$ 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 $\beta_{ijk}x_ix_jx_k$, 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

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