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A new and general model to describe, characterize, quantify and classify the interactive effects of temperature and pH on the activity of enzymes

M.A. Prieto*, J.A. Vazquez & M.A. Murado

Consejo Superior de Investigaciones Científicas (CSIC)

*Corresponding Author:
E-Mail: michaelumangelum@gmail.com (M.A. Prieto)
Tel.: +34654694616

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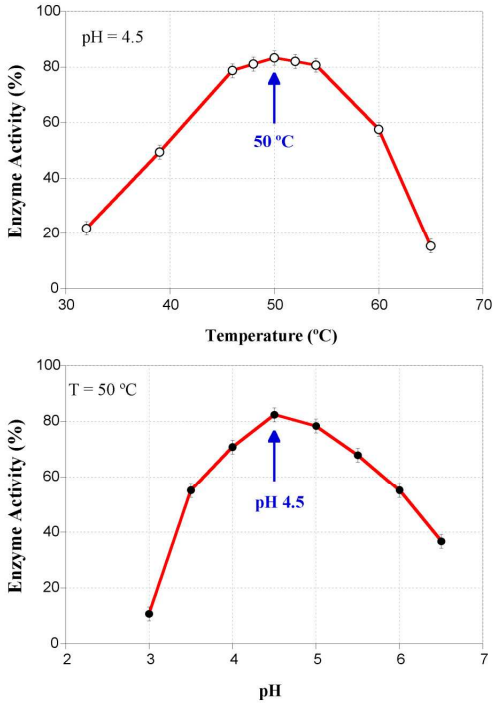	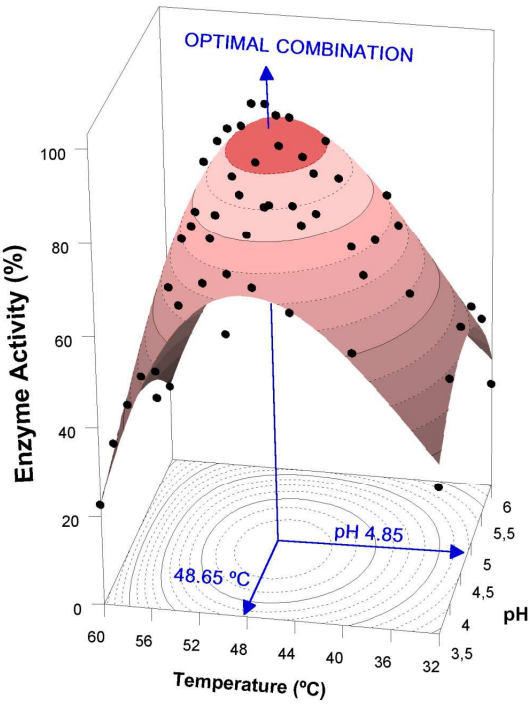

The common approach *vs* modern approach to analyze effects of temperature and pH on the activity of enzymes

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ABSTRACT

We suggest a new and general model to describe the effects of temperature (*T*) and pH on the catalytic activity of enzymes. Despite the abundance of models to describe those effects, the current proposals are unsatisfactory, except for specific experimental cases in which the interactive mechanism between the two variables doesn't exist. For both variables, our solution analyses the activated and deactivated phases of an enzyme as phenomena of different nature. The system is described with independent probability functions. The interactive effects between *T* and *pH* are introduced with simple auxiliary functions. These functions describe the variations induced by each variable in the parameters that define the effects of the other. The structure of the resulting equation is in theory and practice very regular, which facilitates its, use and it is highly descriptive in different scenarios with or without interactive effects. The model was tested on three different enzymatic systems which are specifically designed to produce data for the evaluation of the effect of *T* and *pH* on the enzyme activity (*A*). Afterwards, our model was validated using results from other authors. Briefly, the authors found that: 1) other available models that were compared with our proposal were inefficient and in all cases our model provided the only statistically consistent solution; 2) in four cases, the enzymatic activity could only be explained, if interactive effects are accepted; 3) synergy and antagonism concepts for the interaction between *T* and *pH* were describe and classified; and 4) our solution is universal and independent of the structure of an enzyme and the reaction concerned.

Keywords: enzyme engineering, industrial biotechnology, mathematical modeling, pH and temperature effects

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1. INTRODUCTION

Enzymes catalyze nearly all chemical transformations in cells. Therefore, the understanding of the mode in which enzymes responds to different conditions is fundamental for the comprehension of cellular functions. The availability of data on enzyme concentrations, activities and regulatory responses in the cell, organism or system is the key for any serious study on systems biology. An important feature in many cellular and regulatory studies is, how enzymes respond to changes in *T* and *pH*. There are many physiological and regulatory reasons why enzymes have specific *T* and *pH* ranges of activity. Knowing the way in which they respond to different conditions is critical.

The hypothesis which supports the effect of *T* on the catalytic rate of an enzyme relies on two thermal properties: (1) the activation energy and (2) the enzyme's thermal stability. With increasing *T*, the energy of the substrate and enzyme also increases. Therefore, more collisions between the substrate and the enzyme's active site are expected to occur, causing more enzyme-substrate complexes and finally more product compounds will be formed. When enough energy is supplied, the intermolecular attractions between the polar groups, as well as the hydrophobic forces between the non-polar groups within the enzyme's protein structure may be disturbed, changed or broke-down which will cause a re-structure of the active site's shape and so, its capacity to catalyze. In the case of *pH*, the main argument points out that the amino acid content of the enzyme's active site, which is responsible for the interaction among them and with the substrate, influences the catalytic process. Changes in *pH* may not only affect the shape of an enzyme but, also the properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis. In addition, the effect of *pH* could cause denaturation of the enzyme rather than protonation and deprotonation of specific catalytic groups. The complex reactions concerning both variables generally cause fundamental changes as function of the temperature and ionic strength in the microenvironment around the enzyme's active site. This affects the structure, stability and solubility of the enzyme and makes it difficult to find a general equation to describe *T* and *pH* satisfactorily.

Nevertheless, several mathematical alternatives have been proposed to obtain valuable information. The enzyme activity as a function of *T and pH* commonly varies following bell-type profiles. Therefore the formal definition of the available models is based on functions that are able to describe those profiles. Next, the current alternatives and its applications are described:

(a) From an empirical perspective, practical benefits for microbiological safety and industrial enzymatic processes are needed. For example, a practical benefit common in many fields of study, when optimizing the response of an enzyme, is the need to know the maximum activity (A_m) value as a function of *T* and *pH*. The typical procedure is performed by analyzing one variable to a fixed value of the other one (near to the optimum), and to select by graphical or mathematical analysis the apparent A_m [1–4].

(b) From mechanistic and phenomenological principles, for theoretical interests in enzymology and fields such as microbiology and metabolic responses. The changes caused by *T and pH* on *A* have been repeatedly treated from a enzymatic [5–10] and microbiological [11–17] standpoint, in the last case, under the assumption that the growth rate is limited by the activity of a single enzyme. The structure of most of these approaches is related to the Arrhenius (*T* dependent) and Henderson-Hasselbalch (*pH* dependent) equations, separately or as a product in a bivariate description. Their application has been used for specific problems of enzyme kinetics [7,9,18,19], thermal stability [10,20–22], microbial kinetics [23], reconstruction of metabolism in humans [24] or others such as poikilothermic [25,26] (with a

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model that was subsequently modified by Schoolfield et al. [14] to reduce the correlation between the parameters), to improve, with genetic techniques, the natural resistance to environmental conditions for industrial purposes [27,28], or to study the adaptation of microorganisms to extreme conditions [29,30]. Other approaches are based on more phenomenological functions, such as those applied to the description of microbial growth and its inhibition by temperature [13,15,16,31–33]. In this sense, a mechanistic base, close to nature, does not seem to guarantee a better fit than those achieved by phenomenological functions [34].

However, none of the cited approaches considers the possibility to describe interactive effects between the variables *T* and *pH*. Any approach that avoids to explore the option of effects between the variables is inappropriate [5,7,11,35,36], because the combined effect of *T* and *pH* exhibit the existence of synergistic and antagonistic interactions [8], in which the variation of the effects of one variable depends on the value of the other one, and vice versa. In this sense, the inclusion of interactions is only attempted at the end of empiricism by applying routable factorial designs [35], which provide a bivariate approach with multiplicative quadratic polynomials [5,37,38]. However, its usefulness is limited because, in the best case, the paraboloid, defined by these equations, describes only a small domain around the optimum.

In this work, we adopt an intermediate position between the mechanistic and phenomenological perspective, and we propose that the rise and decline of enzyme activity for any of the two variables obeys probability functions whose parameter values can be modified by the values of the other variable according to very simple relations. This approach allowed us to describe our results and others (with broad experimental domains), obtaining high statistical accuracy and consistency with an immediate convergence during the curve-fitting procedure. Our new and general model not only provided descriptions that cannot be achieved with other models, but also significantly improved the results taken from bibliographic material. Also, we believe that with its application, we have developed a new way to characterize, describe and classify the combined effect of *pH* and *T* on the enzyme activity.

2. MATERIAL AND METHODS

Six enzymes were studied. The experimental results regarding the activity of three enzymes (one of them versus two different substrates) were produced in our laboratory, under conditions of initial rates. The data of the other three enzymatic reactions were obtained from relevant results from the bibliography.

2.1. Enzymes and procedure applied for the data specifically obtained in our laboratory

To ensure that the data is free from controversial aspects, the initial conditions for evaluating the joint effect of *T* and *pH* on the enzymatic activity were determined following the analysis described by Prieto et al. [34]. Briefly, first, the effective range of *A* for *T* and *pH* were obtained by studying the two variables separately. Afterwards, a suitable ratio between the substrate and enzyme was selected by carrying out a kinetic assay, measuring the enzyme activity with different enzyme concentrations with a constant substrate concentration in the final solution at the cardinal conditions of *T* and *pH* detected previously. Finally, an analytical time was selected at a point in which the product formation rate would continue to show a linear profile. Thus, we ensure that the effect of other variables such as time or enzyme concentration will be adequately selected to avoid influences on the activity results of the combined action of *T* and *pH*. The system conditions and the enzymatic commercial products used to test the model are described next.

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2.1.1. Alcalase

Alcalase® 2.5 L is a protease with a broad spectrum of activity and is used in various industries. Eighty independent experiments (performed in triplicate and averaged) of the combined effect of *T* and *pH* on enzyme activity were measured at *pHs* from 6.0 to 13.0 in steps of 1.0 (with 0.075M Britton-Robinson universal buffer) and at temperatures from 30.0 to 80.0 ºC with different interval steps (with casein as substrate). The enzyme activity was tested following the procedure described above using the following selected conditions: an analytical time of 20 min and enzyme/substrate ratio of 1.5 mL/Kg of a final substrate solution of 8 mg/mL. The reaction was stopped with 5% trichloroacetic acid (w/v) and the activity was computed as function of the amount of tyrosine released by a colorimetric method [39].

2.1.2. Esperase

Esperase® 8.4 L is a protease used in laundry and automatic dishwasher detergent formulations to remove protein-based stains. A total of 176 independent experiments (performed in triplicates and averaged) of the combined effect of *T* and *pH* on enzyme activity were measured at *pHs* from 4 to 12 in steps of 1 (with 0.075M Britton-Robinson universal buffer) and at temperatures from 30 to 90°C with different interval steps (with casein as substrate). The enzyme activity was tested following the procedure described above using the following selected conditions: an analytical time of 20 min and enzyme/substrate ratio of 6.0 mL/Kg of a final substrate solution of 8 mg/mL. The reaction was stopped with 5% trichloroacetic acid (w/v) and the activity was computed as function of the amount of tyrosine released by a colorimetric method [39].

2.1.3. Glucanex

Glucanex® 200G is a β1,3-glucanase used for hydrolyzing the oligosaccharides from yeast cell walls in order to obtain β-glucans, to control wine spoilage yeasts, protoplast preparation and as a biocontrol agent against plant pathogenic fungi. The combined effect on enzyme activity was measured at several *pHs* (from 3.5 to 6.0 in steps of 0.5 with 0.02M citric/phosphate buffer) and at different incubation temperatures (from 32.0 to 60.0 ºC at different interval steps for each substrate) with curdlan (100% β1,3-glucan links) and laminarin (66.6 % β1,3-glucan links) as substrates (54 and 66 independent experiments performed in triplicates and averaged, respectively). For both reactions, the enzyme activity was tested following the procedure described above using the following selected conditions: an analytical time of 15 min and enzyme/substrate ratio of 1:10 (w/w) in the final solution (knowing that we use a solution of 250 µg enzyme/L). The reaction was stopped adding the DNS reagent and the activity was computed as function of the amount of reduced sugars measured by a colorimetric method [40].

In all cases, the experimental data were expressed as the percentage of the maximum concentration of product formed and then framed in the [0,1] range.

2.2. Obtained data from bibliographic material

The model's descriptive accuracy was verified using results from other authors (taken from the published figures by means of *GetData Graph Digitizer 2.24*), selected in such a way that they implied different methods, substrate and time domains. In all cases, the experimental data collected was expressed in a [0,1] range whereby, the value 1 is the maximum concentration of the product found.

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2.3. Numerical and statistical methods

All the numerical and statistical methods were applied directly in a Microsoft Excel spreadsheet:
- *Fitting procedure:* simulated and experimental results were adjusted to the proposed models by non-linear least squares methods (quasi-Newton), using Solver complement [41].
- *Parametric estimations:* performed by incorporating the 'SolverAid' macro [42] for estimating the confidence intervals.
- *Model selection criteria:* to compare the models reviewed from the bibliography to predict the joint effect of *pH* and *T* against the new model developed using selection criteria such as Akaike Information Criterion Corrected (*AICc*), Bayesan Information Criterion (*BIC*), Residual Information Criterion (*RIC*), Mallows' Cp (*Cp*), Adjusted Coefficient of Determination (R^2_{adj}), and Akaike's Final Prediction Error (*FPE*); to evaluate the appropriateness of equations based on their goodness of fit, complexity, overfitting and generalizability [34], and the selection criteria leave one out cross-validation (*LOO-CV*) and finally, Monte Carlo cross-validation (*MCCV*) to evaluate the data predictiveness of the models [43].

3. RESULTS

We will present the proposed model approach, demonstrate its benefits against other available models using the specific activity behavior of the enzyme Alcalase as a function of *T* and *pH*. In addition, we will propose a new way to quantify, characterize and classify the interactive mechanisms between *T* and *pH*; test the model to analyze different enzyme systems specifically designed to produce data free of common controversial aspects; validate the model using results from other authors; and provide facts that explain clearly, why other available models fail to describe the combined effect of *T* and *pH* over *A*.

3.1. The proposed approach

The model will be presented under an ideal enzymatic behavior with one single active site. Afterwards, the possibility to analyze more than one active site is discussed.

3.1.1. Modeling in the absence of interactions

First, we established that each of the two considered variables (*V*) plays a dual effect on the enzyme activity. Thus, a variable *V* acts as an activator in a given interval, producing an increase in enzyme activity (*A*) from a null value up to an asymptotic maximum (A_m=1) and acts as a deactivator (in another given interval) decreasing *A* from the maximum asymptotic to the null value. However, both events overlap, around the maximum A_m.

In this sense, an equation that can describe a variety of asymptotic profiles is the Weibull mass distribution function [44,45]. It generates different characteristic profiles from the first-order to the sigmoidal ones which are typically involved in many complex processes such as autocatalytic or allosteric cooperatives. Thus, we can formulate the activating process as follows:

$$A_a^V = A_m \left\{ 1 - \exp\left[-\ln 2 \left(V / \tau_a^V \right)^{\alpha_a^V} \right] \right\} \qquad [1]$$

where *A* is the enzyme activity. The superscript *a* denotes activation, the subscript *V* the variable cardinal values (*T* or *pH*), A_m the asymptotic maximum, τ_a^V a parameter that corresponds to *V* when *A* is A_m /2 and α_a^V a parameter that together with the other parameters (A_m and τ) is related

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to the slope of the generated profile. If the asymptotic maximum is known, the response can be standardized to the interval [0,1] as follows:

$$A_a^V = 1 - \exp\left[-\ln 2\left(V/\tau_a^V\right)^{\alpha_a^V}\right] \text{ or briefly } A_a^V\left(\tau_a^V, \alpha_a^V\right) \quad [2]$$

In similar terms, we can formulate that the deactivating (*d*) processes follow a Weibull decreasing function as follows:

$$A_d^V = A_m \exp\left[-\ln 2\left(V/\tau_d^V\right)^{\alpha_d^V}\right] \text{ or if } A_m = 1 \quad A_d^V = \exp\left[-\ln 2\left(V/\tau_d^V\right)^{\alpha_d^V}\right] \text{ or } A_d^V\left(\tau_d^V, \alpha_d^V\right) \quad [3]$$

Since the process displays the interactive product of the corresponding part that remains activated by the part that is deactivated, the resulting enzymatic activity for each variable (*R*) can be written as:

$$R(V) = A_a^V\left(\tau_a^V, \alpha_a^V\right) \times A_d^V\left(\tau_d^V, \alpha_d^V\right) \quad [4]$$

If in equation [4] the term *V*, that denotes the variable, is replaced by *T* or *pH*, the obtained models describe individually the effect of these variables on the resulting enzyme activity. Therefore, on the assumption of no interactions, the effects of both variables are statistically independent. Thus their combined action can be expressed as the product of the functions that describe their individual effects by the following expression:

$$R(T, pH) = \left[A_a^T\left(\tau_a^T, \alpha_a^T\right) \times A_d^T\left(\tau_d^T, \alpha_d^T\right)\right] \times \left[A_a^{pH}\left(\tau_a^{pH}, \alpha_a^{pH}\right) \times A_d^{pH}\left(\tau_d^{pH}, \alpha_d^{pH}\right)\right] \quad [5]$$

which, if the response is standardize to the interval [0,1], involves a total of eight parameters, two for each of the four independent additive processes.

3.1.2. Modeling in the presence of interactions

Modeling in the presence of interactions requires admitting that each variable can modify the parameter values of the other variable. Therefore, each parameter (θ) is modified by a biparametric hyperbolic (*H*) term as a function of the values of the other variable. This is achieved if the term *H* multiplies each θ as follows:

$$\theta_\omega^{V_1} H_\omega^{V_2} = \theta_\omega^{V_1} \frac{\left(1 + m_\omega^{V_1} V_2\right)}{\left(1 + n_\omega^{V_1} V_2\right)} \text{ being } V_{1,2} = T, pH \text{ , } \omega = a, d \text{ and } \theta = \tau, \alpha \quad [6]$$

Where *m* and *n* are the parameters of the term *H*, θ in the presence of interactions corresponds to the starting value of the hyperbolic function when V_2=0 (θ_0) and in the absence of interactions (*m*=*n*=0) is just a parameter, V_2 represents the variable that modifies the parameters and V_1 the variable governed by the modified parameters. Then, the term *H* should be able to model situations in which the value of any affected variable would increase or decrease as function of the other variable.

When such interactive modification [6] is replaced by model [5] for all parameters (τ and α), the initial amount of parameter combinations would be 24 in the most complex scenario (8 for the

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basic form and 16 for the possible parametric modifiers). Fortunately, the solutions can be considerably simplified because when the data is standardized to the maximum possible response in the [0,1] range, the value of the parameter A_m is always 1, thus the changes in the position parameters (τ) causes a variation of slopes, satisfying functional settings without admitting independent variations of the shape parameters (α). Therefore, the possible interactions of α can be suppressed (8 parameters). In fact, in all the enzyme reactions studied, it was sufficient to consider the following simplified version of the initial model with 16 possible parameters (8 from the basic form and 8 modifiers of the parameters τ):

$$R(T,pH)=\left[A_a^T\left(\tau_a^T H_a^{pH},\alpha_a^T\right)\times A_d^T\left(\tau_d^T H_d^{pH},\alpha_d^T\right)\right]\times\left[A_a^{pH}\left(\tau_a^{pH} H_a^T,\alpha_a^{pH}\right)\times A_d^{pH}\left(\tau_d^{pH} H_d^T,\alpha_d^{pH}\right)\right] \quad [7]$$

In addition, the total final number of possible parameters can also be reduced, because the interactive effects over the parameters τ are proportional (m=0) or inversely (n=0) proportional to the value of the variable that modifies them. In this sense, the most complex cases that we found in our experiments, involved a maximum of two uniparametric modifier H terms (10 parameters, 8 from the basic form plus 2 modifiers from the H terms).

When considering all the hyperbolic terms H=1, all the coefficients m and n are equalized to zero, and equation [7] is transformed to equation [5]. Thus, equation [7] can be used as general model to describe the resulting enzyme activity in the absence or presence of interactive effects between T and pH. For clarification reasons when we would need to mention the model without interactions we will use model [5] and in the presence of interactions model [7] will be applied.

3.2. Determination of rate parameters, response regions and cardinal values of interest

3.2.1. Parametric rate values

From each of the four processes of model [7] with or without interactions, other interesting values such as the average rate (r) and the maximum rate (R) of the joint action of T and pH can be computed. If interactions were present, the response would be a function of the variable that perturbs the response, as follows:

$$r_\omega^{V_1}=\frac{\alpha_\omega^{V_1}\ln 2}{2\left(\tau_\omega^{V_1}H_\omega^{V_2}\right)} \quad \text{where: } V_{1,2}=T,pH \text{ and } \omega=a,d \quad [8]$$

$$R_\omega^{V_1}=\frac{\alpha_\omega^{V_1}}{\left(\tau_\omega^{V_1}H_\omega^{V_2}\right)}(\ln 2)^{1/\alpha_\omega^{V_1}}\,G^G\exp(-G) \quad \text{where: } G=\frac{\alpha_\omega^{V_1}-1}{\alpha_\omega^{V_1}} \quad [9]$$

If the response lacks of interactions, the output would be a continuous single value for each of the regions governing the superposition processes present in model [7] and therefore, the interactive hyperbolic terms must be equalized to one ($H_\omega^{V_2}=1$).

3.2.2. Response regions

If the distribution of the experimentally obtained data is well designed, covering as much as possible the variable ranges, and if the model used, fits consistently to this set of data, we could simulate and predict the response at any range of the two variables. In addition, using 2D or 3D contour graphs, tools that connect with a line the points where the model has the same enzyme

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activity, we are able to map the response illustratively as a function of *T* and *pH.* Therefore, any desired response region and cardinal variable value can be numerically determined effortless from the simulation or can be found graphically by analyzing the contour graph.

3.2.3. Cardinal variable values: Minimum, medium, maximum, or any other desired value of the cardinal variables

Once the model is established through simulations, we are able to reproduce precisely a map of the enzymatic activity at any *T* and *pH* value. However, authors are always looking for analytical expressions to extract the values of interest directly from the response, among other things because the values obtained analytically present the interval of confidence. This may seem irrelevant as long as the model that simulates the response presents a highly consistent fit to the experimental data. In this sense, there is an alternative way that would allow us to obtain the cardinal variable (*T* and *pH*) values for the *A* response regions at the initial, medium, maximum or other percentages of interest.

The Weibull distribution equation, used to describe the activated (*a*) [2] and deactivated (*d*) [3] phases of an enzyme under any of the two variables (*V*), can, in both cases, be modified as follows:

$$A_a^{V_1} = 1 - \exp\left[-\ln\left(\frac{100-x}{100}\right)\left(\frac{V_1}{\tau_{a,x}^{V_1} H_a^{V_2}}\right)^{\alpha_a^{V_1}} \right] \text{ and } A_d^{V_1} = 1 - \exp\left[-\ln\left(\frac{100-x}{100}\right)\left(\frac{V_1}{\tau_{d,x}^{V_1} H_d^{V_2}}\right)^{\alpha_d^{V_1}} \right] \qquad [10]$$

where *x* is the value of the response (in percentage) at which we want to obtain a value of the cardinal variable. Thus the relation $\tau_{\omega,x}^{V_1} H_{\omega}^{V_2}$ will provide a function that describes the behavior of the V_1 branch (*a* or *d*) as a function of V_2 at the response percentage *x*. Other parameters and notations remain with the same meaning as noted previously.

Therefore, in absence of interactions ($H_{\omega}^{V_2} = 1$), the calculation of any cardinal value of the response is directly obtained through the parameter $\tau_{\omega,x}^{V_1}$ by simply changing the *n* value. To find analytically the cardinal variable values under the presence of one or more interactions, we have to find the intersection points between the following combined functions: $\tau_{a,x}^{T} H_a^{pH}$ *vs* $\tau_{a,x}^{pH} H_a^{T}$; $\tau_{a,x}^{T} H_a^{pH}$ *vs* $\tau_{d,x}^{pH} H_d^{T}$; $\tau_{d,x}^{T} H_d^{pH}$ *vs* $\tau_{a,x}^{pH} H_a^{T}$; and $\tau_{d,x}^{T} H_d^{pH}$ *vs* $\tau_{d,x}^{T} H_d^{pH}$. Their intersections represent the analytical form that will provide the four cardinal variable values at any percentage *n* of the response. Note, that the determination of the intersections is simple because these $\tau_{\omega,x}^{V_1} H_{\omega}^{V_2}$ functions are constant lines (absence of interactions), linear (decreasing or increasing) or hyperbolic (decreasing or increasing).

The cardinal values can be very helpful. For example, if we want to classify the range of activity of enzymes as function of the ionic strength (acidophile, neutrophile, alkaliphile) and temperature (psychrophile, mesophile, thermophile), by computing the initial with *n*=1% we will obtain the cardinal variable values that will allow researchers to be much more precise when defining the activity range of enzymes.

3.3. Mode of action of *T* and *pH* on the activity of an enzyme: characterization, description and quantification of null, synergistic and antagonistic interactive effects.

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The presence or absence of interactions denotes two different response modes to the changes caused by the joint action of *T* and *pH* to one or more active sites of an enzyme. In the absence of interactions, the changes caused by the variables *T* and *pH* takes place at different physical-chemical levels, acting independent from each other. Such a response mode is then defined as null action or additive action (AA) mode, because a simple additive superposition of the four independent processes occurs. The existence of interactions translates changes caused by *T* and *pH* at the same point on the physical-chemical structure of an area at the active site of an enzyme, response mode that must be defined as interactive action (IA).

Furthermore, when interactions are present (IA mode), or in other words, in the model [7], one variable alters at least one parameter of the equation governing the other variable. The approach developed allows a purely phenomenological mode, but highly versatile for defining the interactive effects between *T* and *pH* as positive (synergistic) and negative (antagonistic) for each of the interactive processes. Table 1 shows a summary of the proposed classification system.

To quantify the nature and the intensity of the synergistic or antagonistic interactions once an explicit algebraic model for a response to *T* and *pH* is established, only a comparison between the response corresponding to the null interaction (AA mode: absence of interactions) and the interactive (IA mode: presence of interactions) response hypotheses seem necessary. The difference could be summarized in just a single numerical value, an index that clearly would help and become useful for different purposes. The best alternative could be to compute the percentage relative unit of volume (*RUV*) between the volume of the surface produced by the additive action (SV_{AA}) and the volume of the surface in the IA mode (SV_{IA}) as follows:

$$RUV = \frac{SV_{IA} - SV_{AA}}{SV_{IA}} \times 100 \text{ ; being } \quad SV = h_i h_j \sum_{i=x_0}^{x_f} \sum_{j=y_0}^{y_f} f\left(V_i V_j\right) \phi_{i,j} \qquad [11]$$

in which V_i and V_j are the dependent variables (*T* and *pH*), the *x* and *y* values denote the desired range of the variables for the initial (subscript *0*) and final (subscript *f*) values, h_i and h_j are the interval sets and $\Phi_{i,j}$ is the product of the nested composite trapezoidal rule coefficients. Therefore, positive and negative values of *RUV* will describe the predominantly synergistic and antagonistic interaction effects in percentage, in the joint action of both variables.

The reader must be aware that the usefulness of this index from a theoretical and practical perspective is questionable. In fact, neither the difference nor the quotient between the typical responses in null interactions and any interactive situation remains constant throughout the domain of the independent variables. Therefore, to quantify an interactive response, the hyperbolic modifications of τ_ω^V parameters must also be taken into account, when defining such a response. When a response is synergistic or antagonistic in the full domain, we will define it as synergistic or antagonistic in a strict sense. When a response is globally found synergistic or antagonistic, but can be composed of opposite effects at different regions, we will defined it as partially synergistic or antagonistic.

3.4. Further considerations

The general agreement is that the effect caused by *T* and *pH* on *A* produces one defined and unique optimum. However, there are cases in which enzymes, as a function of one variable, may only present one branch (activation/deactivation) or would present more than one defined peak of activity. Supposing that some of these behaviors are not an error caused by studying the effect

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of the variables individually at a fixed value of the other, the proposed equation [7] will still be fully functional and will be able to solve these situations with some necessary adaptations.

For instance, the issue related to the lack of effect of one variable on the deactivating or activating part of the model. When fitting such a response, the parametric relation of the corresponding variable to the particular sub-function that controls that part of the global model [7] can be found to be approximately equal to 1, or in other words: $A_{\omega}^{V_1}\left(\tau_{\omega}^{V_1} H_{\omega}^{V_2}, \alpha_{\omega}^{V_1}\right) \cong 1$. Consequently, those parameters involved in this particular sub-function will show a non-statistical significant result. In other words, our own results will force us to delete this sub-function. Only then, the results of the fit will be statistically consistent to describe the effect of the enzyme as function of *T* and *pH*.

When more than one peak per variable is found, we can theorize several different combined circumstances. For example, if the changes in *pH* cause two clear peaks at different *T*, probably due to the amino acid composition of the active site, we would have to add an additional decomposition phase to model [7]: $A_d^{pH}\left(\tau_d^{pH} H_d^{T}, \alpha_d^{pH}\right)$. If the enzyme has more than one active site, the accumulative result of the response may be identical to those with one active site, or could present different peaks, even more than two. For complex cases, we suggest to add logically sub-functions without interactions until the response is fitted relatively well, and then authors should include interactions to find the proper solution. This is also applicable to any other combination within an enzymatic reaction.

3.5. Comparison of fittings among available models: Alcalase as a case study

Next, we will exemplify and compare the model developed against those examples available. As a case study, we have deliberately selected the data obtained from Alcalase. To compare the effectiveness of the models we will only focus on the fitting results to the experimental data and its competence to predict the response. Any other comparison in relation to the advantages of our proposal previously described will be avoided.

At the introduction section, we have reviewed the available models from the literature to describe the enzymatic activity under different *T and pH* conditions. In Table 2, we present the most relevant models (M). First, we describe regular models with empirical forms (polynomials) whose parameters do not have any physical meaning (M1 and M2). Then, we show other models (M3 to M7) that have a theoretical or phenomenological foundation in other fields of knowledge (*i.e.* microbial growth) and their possible application to our problem is based on the analogy between the effects of the variables involved. Finally, we assess the developed structured model to study specifically the combined effect of different *T and pH* on the enzymatic reaction (M8).

The raw results obtained for the case of the enzyme Alcalase are shown in the first part of Figure 1 in a 2D contour graph. A progressive increase of *A* as *T* and *pH* increase can be observed, until it reaches a *pH* value between 9 and 10 and temperatures around 55 to 60 ºC, where *A* sharply decrease to zero occurs. In the second and third part of Figure 1, we present the comparative results between the current models and our new model developed (in both versions: without [5] and with [7] interactions). To illustrate the differences, three basic graphical criteria are used: the ability to simulate the changes of *A*; the capacity to predict the results obtained (based on R^2 coefficient); and the residual distribution as a function of each of the variables. From this visual analysis the following conclusions are derived: 1) equations M1, M2, M4, M5, M6 and M7 are not able to simulate a low *A*, obtaining negative responses rather than values close to zero or zero; 2) models [7] and [5] present the best statistical results with values of R^2= 0.9889 and

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0.8418 respectively, followed by M8 with 0.8416. Note that all other models showed a R^2 lower than 0.80. Thus, the predictiveness of the models can be ordered as follows: model [7] >>> model [5] > M6 > M7 > M4 > M5 > M2 > M1>> M3; 3). Only model [7] shows a residual distribution for each variable free of autocorrelation, randomly scattered around zero and equally distributed at the studied range.

If we look at the number of non-significant parameters (α=0.05), in many cases large confidence intervals are found. Only the models [7] and [5] had all the parameters statistically significant at a 95 % level of confidence (Table 3). Results of fittings for the other equations were not shown. Furthermore, when the model selection criteria described in the material and methods section are used to rank the properties of all models, similarly results to those described above for the R^2 analysis (data not showed) are found. In all cases, models [7] and [5] ranked first and second respectively and furthermore, model [7] was always far more efficient than any of the other models.

Figure 2A and 2B show, in a 2D representation, the fitting results of models [7] and [5] to the experimental profiles of the combined effect of *T* and *pH* on the Alcalase activity. Marked differences in the hydrolysis of casein by Alcalase are found. The model without interactions is not able to predict correctly the experimental results in many different areas of the response and, as we previously showed in Figure 1, it produces dispersion in the correlation between observed and predicted activity. The maximum of the resulting theoretical activity in the absence of interactions is shifted from a value of A_m=0.93 at T=57.4 ºC and pH=9.0 to A_m =0.95 at T=60.3 ºC and pH=9.3. However, in practice probably is more interesting the differences of the resulting activity between both hypothesis in the range of low *T* and high *pH* or low *pH* and high *T*, which makes remarkably better model [7] to predict the *A* at the full range of *T and pH*.

Figure 2C shows the interactive parametric behavior, other interesting values ([8], [9] and [10]) and a 3D representation of the fitting results produced by model [7]. It becomes clear from all of these different perspectives (Figure 1, Figure 2 and Table 3), that the problems are markedly reduced when accepting that increasing values of *pH* lower the position parameter corresponding to thermal denaturation and increasing values of *T* reduce the same parameter in the alkaline denaturation process (both scenarios with high statistical significance). Although such interactions seem to be a simple formulation, the consequences of these events were quite complex, as illustrated on the change of the isolines between figures where the interactions were accepted or rejected (Figure 1).

In general, the conditions that maximize the resulting activity of an enzyme does not maximize its stability. Thus, often for optimizing the performance of any enzymatic reaction, a compromise it is required. A fact that makes the overview provided by model [7] crucial. The combination of all the above arguments indicates that model [7] is the only relevant approach for predicting the join effect of *T* and *pH* on the Alcalase activity.

3.6. Other data specifically obtained to test the model

3.6.1. Esperase

The hydrolysis of casein by the commercial enzymatic product Esperase, although with less intensity than in the case of Alcalase, could be explained with two statistically significant interactive effects at high *pH* values combined with low and high temperatures (Figure 3 and Table 3). In the first part of Figure 3, the interactive effects caused by both variables are clearly represented in the simulated contour graph (equation [7] with parametric results in Table 3).

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Temperature decreases the value of the position parameter corresponding to the *pH* deactivating function ($\tau_d^{pH} H_d^{T}$) while *pH* reduces the value of the position parameter in the temperature activating function ($\tau_a^{T} H_a^{pH}$).

3.6.2. Glucanex

The hydrolysis of laminarin by the commercial enzymatic product Glucanex could be explained satisfactorily without admitting interactions between the effects of *T* and *pH* (Figure 3 and Table 3). Thus, equation [7] with all hyperbolic terms H=1 produces statistically significant parameter estimates with an adequate sensitivity, a high correlation between observations and predictions and unbiased residues. In this regard, it has to be mention, that in some cases the assumption of some kind of interactions between both variables ($H \neq 1$) led to produce minimum improvements with better fittings but, caused a lack of statistical significance of some parameter estimations of the basic model or/and the hyperbolic coefficients (*b* or/and *c*) considered. Therefore, these improvements should be rejected because, it may be reflecting only a portion of the experimental error. To confirm the lack of interactive effects, the enzyme was checked with the substrate curdlan. The differences between the glycoside linkages laminarin and curdlan created differences in accessibility to the active center which, in turn, may interact with the effects of the variables studied, the results obtained with curdlan (not showed due to redundancy) reproduced the behavior of the enzyme when the substrate was laminarin.

3.7. Validation with data from other authors: phitasa, polyphenol oxidase and invertase cases

Even if the effect of *T* and *pH* has been studied in many diverse enzymatic reactions, the available data in the bibliography lacks, in many occasions, a full range of study in at least one of the variables [19,46] or, the effect is mixed with other variables such as time and enzyme/substrate ratio [18,47] or, the data was presented only in a 3D graph which makes its digitization impossible [48]. Thus, after an extensive search, only three examples could be used to test the general applicability of the model proposed. Fortunately, two of them contained data from studies that had proposed a model to specifically describe the effect of *T* and *pH*. So, we hope that the reader will consider them as complementary key data, rather than a lack of experimental effort.

The examples selected involve different enzymatic reactions. The first one uses a wide range of *T* and *pH* on the activity of phytase from different origins and its data is analyzed based on a developed model in a two-step estimation procedure [7]. The second one uses the enzyme polyphenol oxidase for the development of a mathematical model [9] that in a two-step estimation procedure describes the effects of pH, temperature, substrates and enzyme concentrations on the initial rate of the sinapine transformation. Finally, the third case [49] studies the activity of invertase to breakdown sucrose to produce sucrose-inverted sugar, a mixture of glucose and fructose, used extensively in the food industry.

In similar terms to the previous examples analyzed, the experimental results from other authors [7,9,49] (Figure 3 and Table 3) confirmed, the descriptive accuracy of our approach. The interactive effects on the enzyme activity of polyphenol oxidase and invertase revealed statistical significant interactions between *T and pH*.

In the case of the enzyme phytase, no interactions between *T* and *pH* were found. This was also the only case where the inclusion of the interactive terms did not improve the predictability of

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the model without interactions. In the case of Glucanex the same hypothesis was accepted. due to the lack of statistical significance of any other hypothesis accepting interactive mechanisms, fact that was related to high experimental error or/and poor experimental design. If we avoid the statistical significance analysis of the parameters involved various alternative would be found displaying improvements compared to the non-interactive hypothesis. The enzyme exhibits a strong asymmetry as function of *T* rising gentle on the activating side and decreasing abruptly on the deactivating branch, while as a function of *pH* a pronounced symmetry is found.

Considering the set of results obtained from this six enzymatic reactions (seven, when including the hydrolysis of curdlan), it can be concluded that model [7] improves the results of other alternatives, not only by its ability to describe the interactive effects, but also for its superior accuracy and generality in the absence of interactions. In fact, the accuracy of other models in the cases Glucanex and phytase was not only lower, but also dependent on the enzyme concerned.

3.8. What is the problem with the current available models and what model [7] is capable to do that the others are not?

There are two main reasons that make other models weak in comparison with the one we have developed.

The first one is associated with the fact that authors try to model the activating and deactivating phases produced by the effect of one variable with a single bell profile (or U-shaped) equation [50]. The parameters in those equations do not have the capacity to control independently the shape of one branch from the other due to the relationships between the parameters which makes it difficult to adapt the profile to the heterogeneity of the real cases. Thus, only approximations to reality are found, when the activity of the enzyme is free from interactions between the effects of *T and pH*. In our model, we have proposed an independent analysis of each of the activating and deactivating phases of the active site of an enzyme with a S-shaped equation, which allows us to control the behavior precisely in any of the activating and deactivating phases and to extract very detailed information of each of the processes.

The second one is related to the lack of mathematical expressions that would allow including interactions between the two variables. In our model, we have accepted that the parameters that affect one variable vary, depending on the values of the other one, resulting in interactions [51]. From a theoretical perspective, this is always accepted, but not from its mathematical translation. The reasons for avoiding the inclusion of interactions into the structure of those models, are perhaps because: (*a*) a wrong interpretation of interactions, occasionally confused with combining the effect of *T* and *pH* in a single function; or (*b*) most models contain a high number of parameters (occasionally fitting of one variable at the time) [7,9] with very diverse numerical values, and if we add the need for auxiliary functions that would allow to define the reciprocal interactive effects onto the parameter values, the approximations becomes impractical and its statistical validation problematic.

To support our arguments, Figure 4 shows six simulated cases (C1 to C6) that illustrate clearly the above two problems within the available models and the advantages of our proposal. C1-C3 of Figure 4 are cases in which the individual effects of each variable are simulated with purely bell profiles that are multiplied between each other to reproduce the joint effect of *T* and *pH* over *A*. If we look at Table 2, the mathematical expressions available in the best scenarios (M3 and M8) are only bell profiles for each variable, multiplied by each other to obtain the joint effect on *A*. Therefore, even if we combine those profiles in many different ways (C1-C3 of Figure 4), the

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joint response will always be the simple result for the multiplicative effects of the individual profiles. If we look carefully at the 2D plot of the joint effect, we will realize that by multiplying different bell profiles, we only cause vertical or horizontal changes around the maximum response. However, when we look at the other three simulated cases produced with our general model [7] (cases C4 to C6 of Figure 4), readers should clearly see that different effects that modify the parameters τ_{ω}^{V} by a hyperbolic term H_{ω}^{V} would cause some additional diagonal changes of different angles, which are real interactive effects between *T* and *pH* over *A*.

More specifically, C4 of Figure 4displays a simple case in which the *pH* reduces the rate at which the *T* activating phase increases ($\tau_a^T H_a^{pH}$), antagonistic in a strict sense, twisting diagonally the activity map at temperatures and pHs below the maximum. C5 shows a more typical antagonistic (strict sense) case, in which *T* and *pH* reciprocally interact with each other at the deactivating phases increasing the inactivation rate of the active site, which causes the diagonal effect that can be seen at the figures that represent the joint action *T* and *pH*. Finally, C6 shows a similar case to the previous one with reciprocal antagonistic effects at the deactivating phases, but with an additional synergistic effect at the activating temperature phase. The ability to perform an interaction between the variables clearly differentiates model [7] from the other ones. On one hand, these interactive effects will create responses that exists, but that will never be described by the models available. On the other hand, if we analyze the response of those other models with our general model [7], we always find simple responses without interactions ($H_{\omega}^{V}=1$).

3.9. A new standardized format to summarize the effects of *pH* and *T* over A

Once, our approach has been found undoubtedly better than any other and has been successfully applied to specific cases produced in our laboratory and to other relevant cases present in the bibliography, a standard format to present the results is proposed. This standard format should include the full name of the enzyme and substrate, initial conditions of the enzymatic test performed, a clear map of the data obtained experimentally and predicted with the model, statistical information about the fitting results, relevant cardinal variable values (minimum, optimal and maximum of the responses regions), the type of interactive mode of action and, if present, the quantity and domain of the interaction. In Figure 5, we believe, we were able to bundle all of this information for each of the enzymes tested, in a simple and visually manner. Readers should see this standardized template as a box completed only with the minimum requirements to present their data and should feel free to incorporate as much information as they consider relevant.

DISCUSSION

The descriptive (and combined) effects of *T* and *pH* on the enzymatic activity are a difficult problem because, the causes behind activation and deactivation by both variables dependent on processes of different nature. In the activating branches of enzymes, it is possible to apply general physical-chemical laws which concern the effect of temperature on the rate of chemical reactions, or in the case of pH, concern the influence of the degree of dissociation of water on the ionic state of the system, which in a large number of enzymatic reactions (hydrolysis) affects the affinity between the substrate and the active center of the enzyme. However, in the subsequent decline in activity, the possible laws that can be used are a disorganization process of a highly structured enzyme molecule, a process that depends on a more diverse set of phenomena with chaotic dynamics and profiles, much less predictable compared to the laws governing the activating phase. This may explain the difficulties of developing models that generalize in a

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unifying mode the activating and declining stages of the enzyme activity. Moreover, the fact that, at least in the cases studied here, the significant interactions were found on the deactivating stages which appears to support the previous view.

Our approach proposed is able to describe the heterogeneous responses of enzymes as function of *T* and *pH* variables, by applying simple phenomenological principles: first, the problem is described in terms of the joint probability of four statistically independent phenomenological equations; second, regardless of the mechanisms involved, each of these four equations varies increasingly or decreasingly asymptotically between the values 0 and 1; and third, variables may interfere in the mechanisms involved between each other, a fact that is mathematically reflected by allowing one variable to alter the parameter values that govern the equations of the other one.

The assumption of dual asymptotic effect of *A* under *T* and *pH* is consistent with the phenomenological behavior of an enzyme, it is the consequence of the interaction of infinite possible mechanistic reactions. Thus, if we accept the theoretical possibility of these four separate processes, undoubtedly, model [7] has a conceptual and formal regularity that significantly solves generally all possible enzymatic responses. When the same problems are confronted, using any of the models previously described [34] for enzymatic responses that lack interactions, always less acceptable results are found compared to those found with model [7] and for those enzymatic cases that present interactive responses between *T* and *pH,* model [7] always approximates *A* remarkably better than any other one. In addition, the parameters governing model [7] are associated with specific kinetic characteristics in terms of rate of the processes, gradients and independent variable values for certain system states. Therefore, we wish to define this model as universally applicable rather than a finalized quantitative exercise, because it explains the foundation events independently of the reaction concerned and of the enzyme structure.

The mechanisms that affect the activity of an enzyme by the variables *T* and *pH* are complex. The approach proposed here provided more accurate descriptions than any of the models mentioned in the previous section for all enzymatic reactions studied, and with or without the necessity to accept interactive effects. The data experimentally obtained as well as the data collected from the bibliography ensures that the analysis here proposed is reliable. In all cases, the enzyme activity was measured extensively in a large and meticulous matrix of experimental conditions, covering the full dynamic space of activation and declining by heat and *pH* configuration.

Once the model is accepted, the next steps would lead us to insert our model into other available knowledge, to control, in a joint mode, the activity of an enzyme as function of other relevant variables such as time and enzyme concentration.

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FIGURE CAPTIONS

Figure 1: Comparative analysis to fit the behavior of the effects of *T* and *pH* on the *A* of Alcalase. The first part shows in a 3D contour graph the changes of *A* obtained for Alcalase. On the second and third part, three graphical criteria (simulation, prediction and residual distribution) are used to compare the capabilities between the current models (Table 2) and the new general models developed. Note, that in the simulation graphical criteria, a dark line shows the border at which the simulation switches from a positive *A* to a negative one. Note that for the prediction graphs the X-axis (predicted data) had to be adjusted to negative values to show the complete distribution for all cases.

Figure 2: Sections A and B show in a 2D representation the fitting results of models [7] (solid line) and [5] (dotted line) to the experimental profiles of the combined effect of *T* and *pH* on Alcalase activity. Section C shows a 3D representation of the fitting results, the interactive parametric behavior of $\tau_{\omega}^{V_1} H_{\omega}^{V_2}$ and other interesting values ([8], [9] and [10]) derived from model [7].

Figure 3: Fitting results of model [7] to the enzyme systems specifically designed to produce data free of common controversial aspects (Esperase and Glucanex) and the validation results from other authors (polyphenol oxidase, phytase and invertase) selected to validated the approach. For each enzyme, we are showing the 2D map of the response regions, prediction of the data and parametric perturbations caused by the interactions between the variables *T* and *pH*.

Figure 4: Simulated cases that illustrate the problems of the available models and the advantages of our proposal (model [7]) to predict the interactive joint effects on the enzyme activity as a function of *T* and *pH*.

Figure 5: Standardized format to summarize the effects of *T* and *pH* on *A*.

TABLE CAPTIONS

Table 1: Proposed classification system for the response modes of the joint action of *T* and *pH* on the activity of an enzyme.

Table 2: Relevant models (M) taken from the scientific bibliography to describe the enzymatic activity under different *pH* and *T* conditions.

Table 3: Joint effects of temperature and *pH* on specified enzymatic activities, as described by model [7]. For Alcalase, both alternatives of modeling (*a*, *b*) are shown. Confidence intervals are presented as percentage of the parametric estimate values. Notice that independent variables are used as natural values. Consequently, the effects of the interaction terms are very relevant, despite their low absolute magnitudes.

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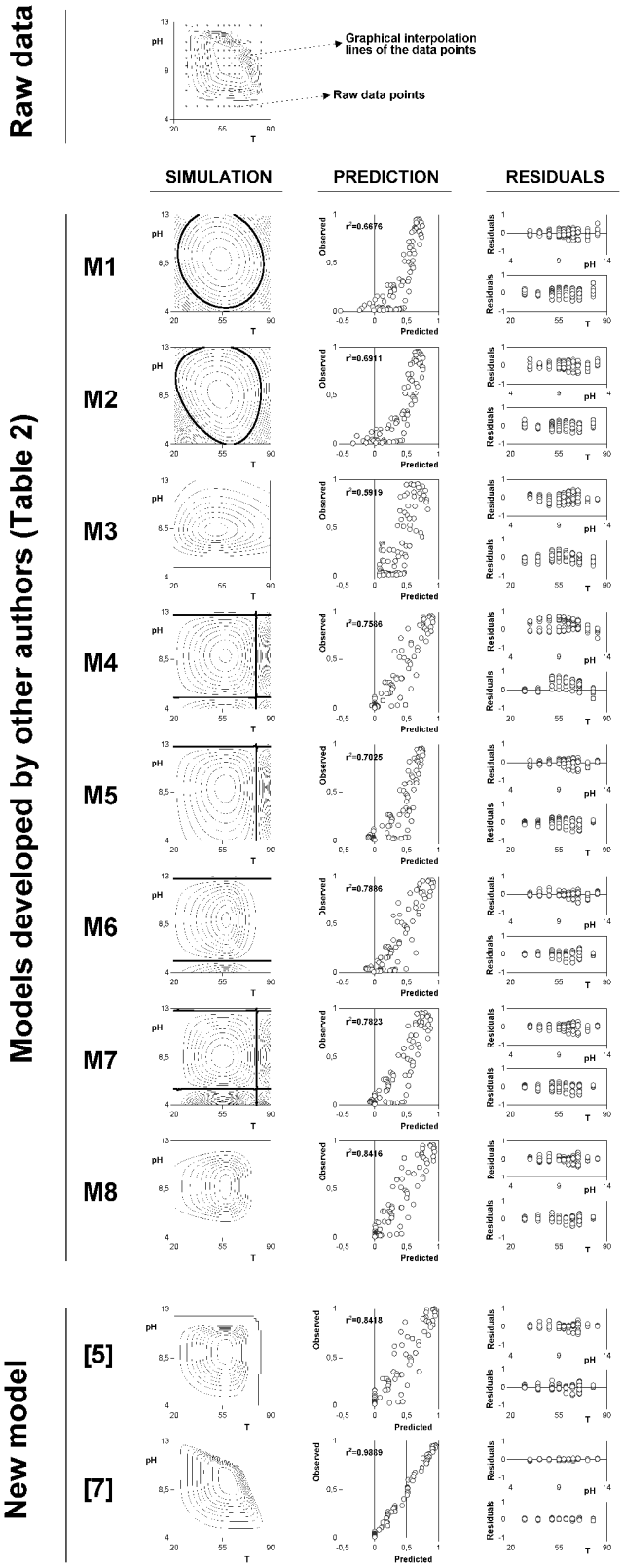

Figure 1: Comparative analysis to fit the behavior of the effects of *T* and *pH* on the *A* of Alcalase. The first part shows in a 3D contour graph the changes of *A* obtained for Alcalase. On the second and third part, three graphical criteria (simulation, prediction and residual distribution) are used to compare the capabilities between the current models (Table 2) and the new general models developed. Note, that in the simulation graphical criteria, a dark line shows the border at which the simulation switches from a positive *A* to a negative one. Note that for the prediction graph the X-axis (predicted data) had to be adjusted to negative values to show the complete distribution for all cases.

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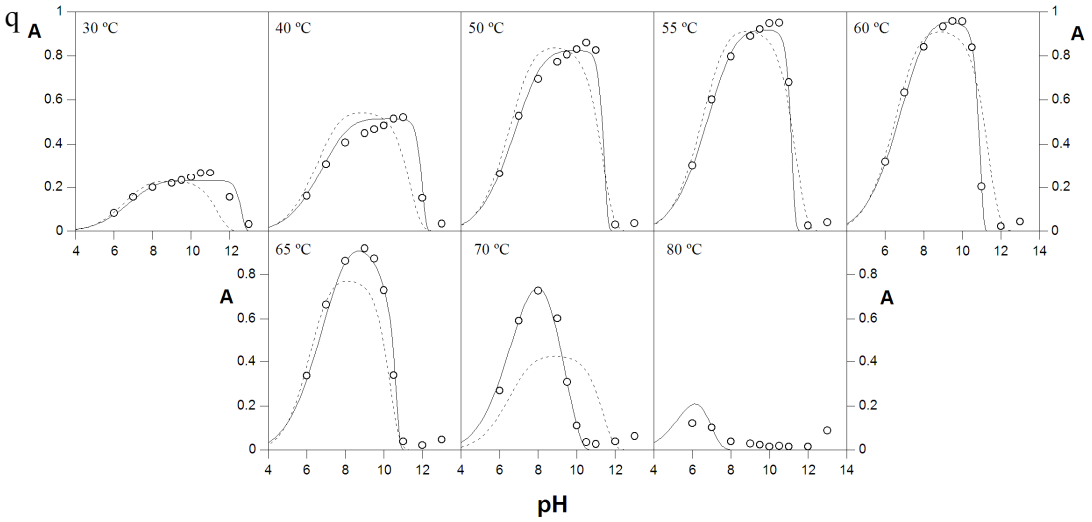
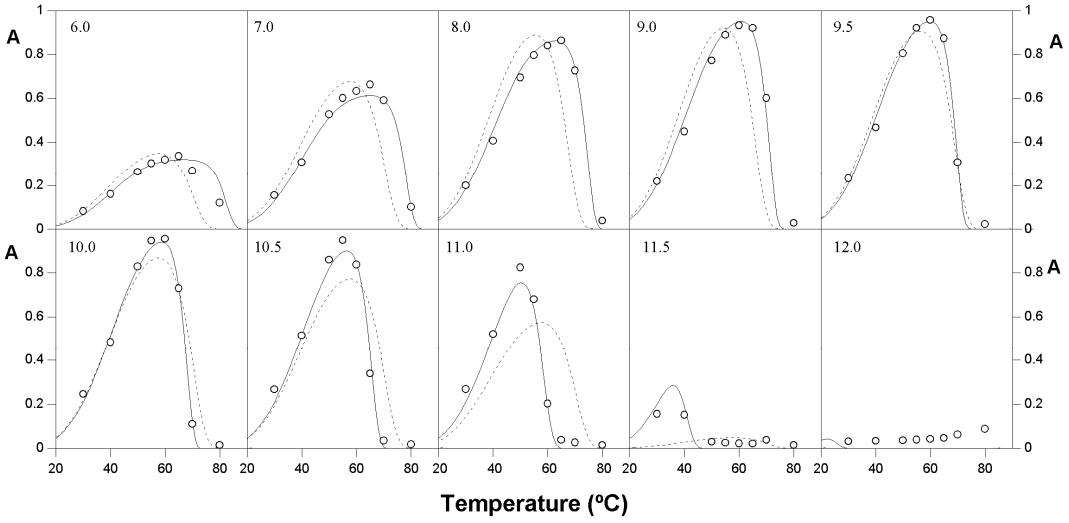
A: ***Activity as a function of pH***



B: ***Activity as a function of T***



C: ***3D representation and main parameters***

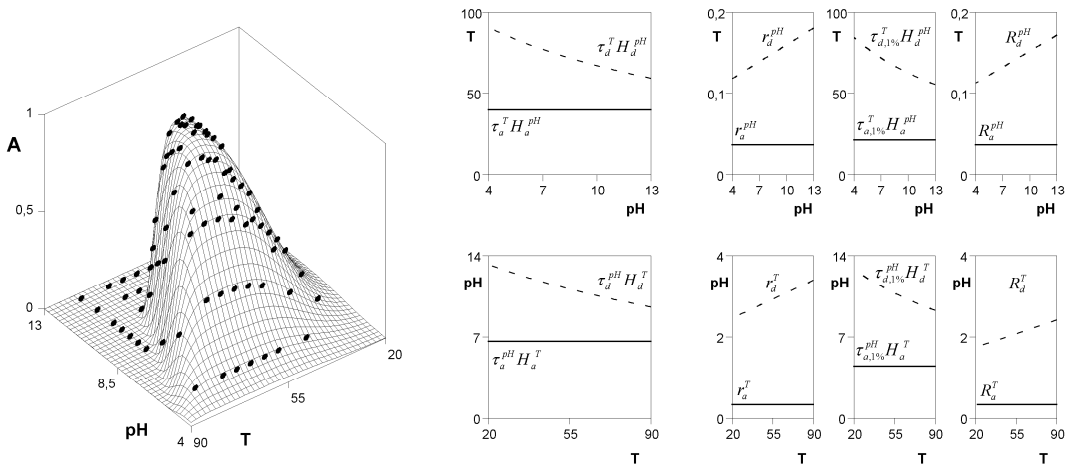


Figure 2: Sections A and B show in a 2D representation the fitting results of models [7] (solid line) and [5] (dotted line) to the experimental profiles of the combined effect of *T* and *pH* on Alcalase activity. Section C shows a 3D representation of the fitting results, the interactive parametric behavior of $\tau_{\omega}^{V_1} H_{\omega}^{V_2}$ and other interesting values ([8], [9] and [10]) derived from model [7].

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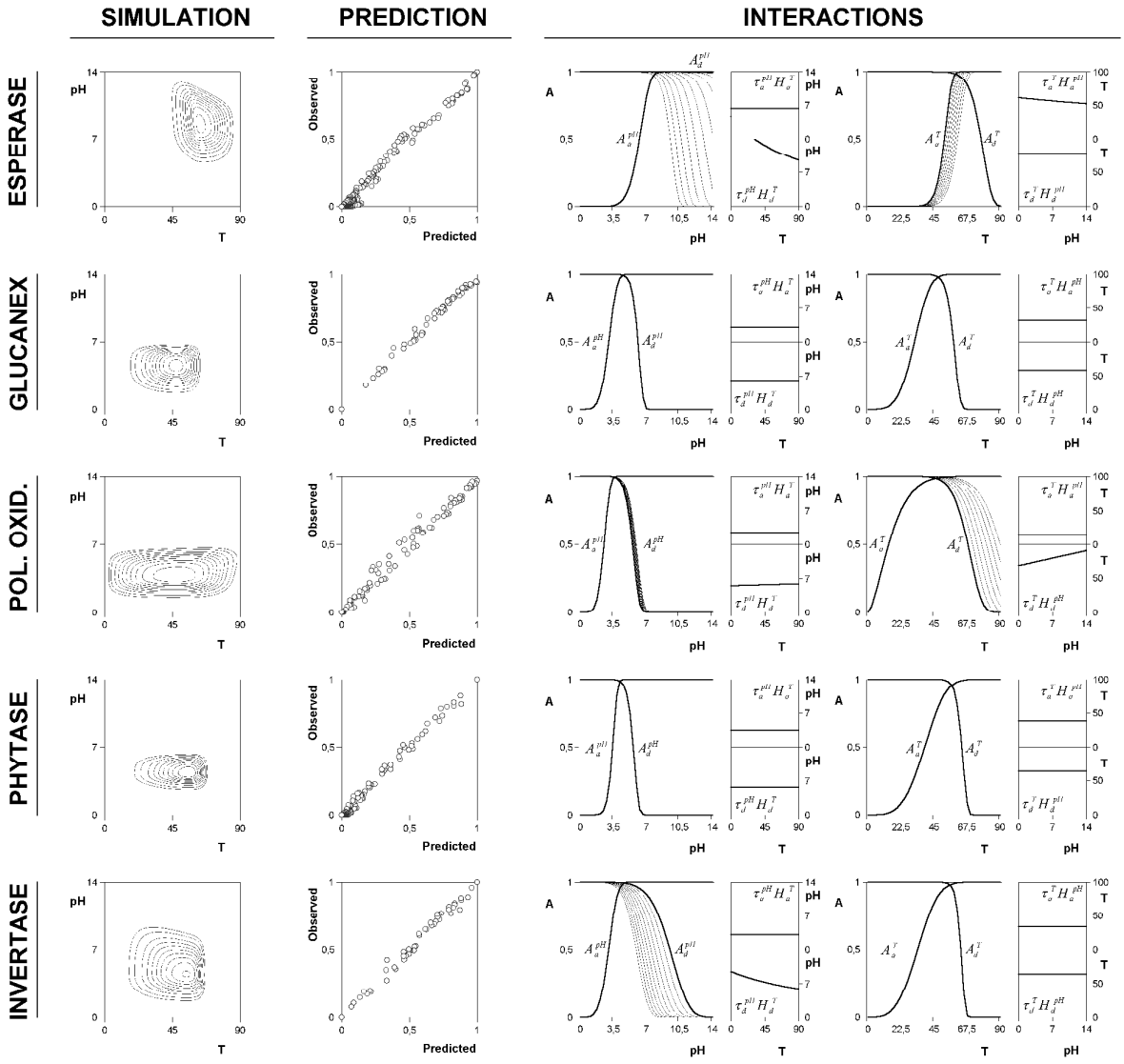

Figure 3: Fitting results of model [7] to the enzyme systems specifically designed to produce data free of common controversial aspects (Esperase and Glucanex) and the validation results from other authors (polyphenol oxidase, phytase and invertase) selected to validated the approach. For each enzyme, we are showing the 2D map of the response regions, prediction of the data and parametric perturbations caused by the interactions between the variables *T* and *pH*.

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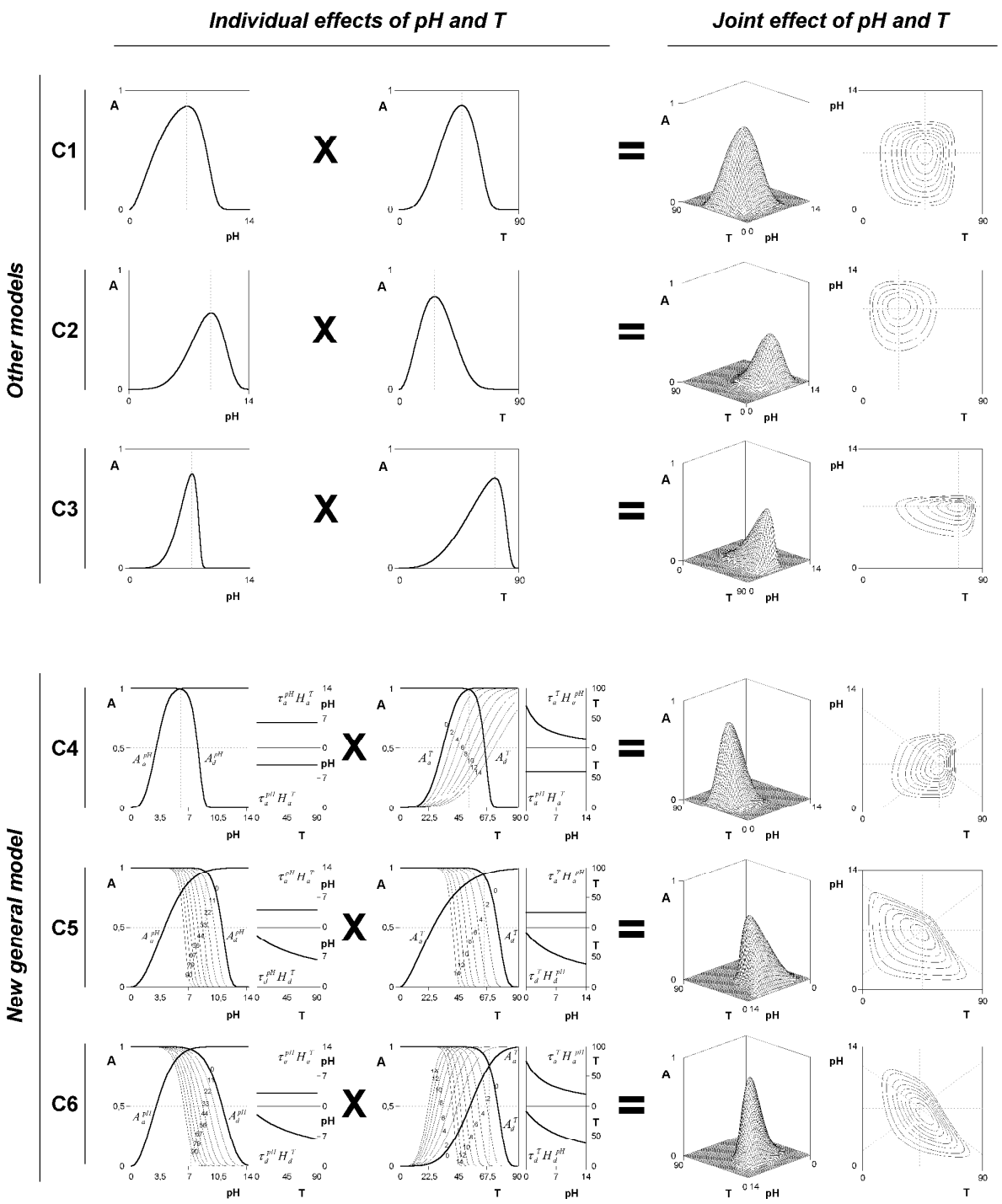

Figure 4: Simulated cases that illustrates the problems of the available models and the advantages of our proposal (model [7]) to predict the interactive joint effects on the enzyme activity as a function of *T* and *pH*.

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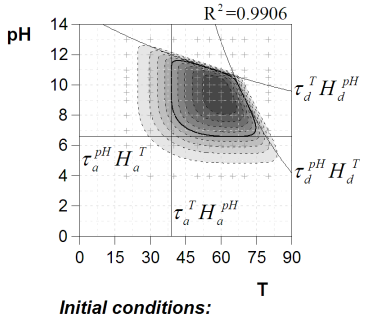
ALCALASE



Initial conditions:

t = 20 min
S = casein
E/S = 1.5 mL/Kg

Cardinal values:

	min	opt	max
T:	*19.1*	*60.4*	*84.2*
pH:	*4.4*	*9.2*	*12.8*

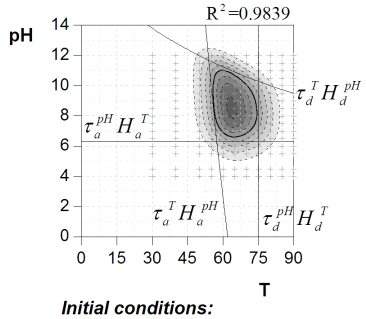
Opt. A = 41%

Mode of action:

$$\text{IA} \begin{vmatrix} R\downarrow\left(pH \xrightarrow{I} A_T^d\right) \\ R\downarrow\left(T \xrightarrow{I} A_{pH}^d\right) \end{vmatrix}$$

Strictly antagonistic (-3.2 %)

ESPERASE



Initial conditions:

t = 20 min
S = casein
E/S = 6.0 mL/Kg

Cardinal values:

	min	opt	max
T:	*43.7*	*63.4*	*84.8*
pH:	*4.7*	*8.3*	*12.7*

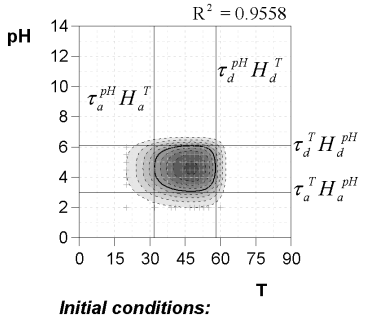
Opt. A = 81%

Mode of action:

$$\text{IA} \begin{vmatrix} R\downarrow\left(pH \xrightarrow{I} A_T^d\right) \\ R\uparrow\left(T \xrightarrow{I} A_{pH}^a\right) \end{vmatrix}$$

Partially synergistic (1.3%)

GLUCANEX



Initial conditions:

t = 15 min
S = laminarin
E/S = 0.1 g/g

Cardinal values:

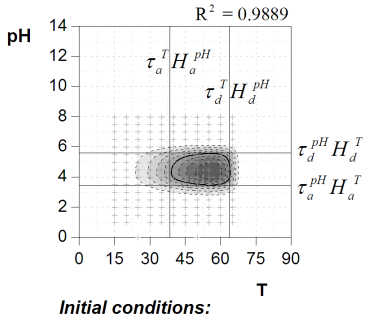
	min	opt	max
T:	*16.2*	*47.7*	*63.5*
pH:	*2.2*	*4.5*	*6.1*

Opt. A = 44%

Mode of action:

AA

PHYTASE



Initial conditions:

t = 10 min
S = phytate
E/S = ¿?

Cardinal values:

	min	opt	max
T:	*18.1*	*55.5*	*68.2*
pH:	*2.4*	*4.2*	*6.1*

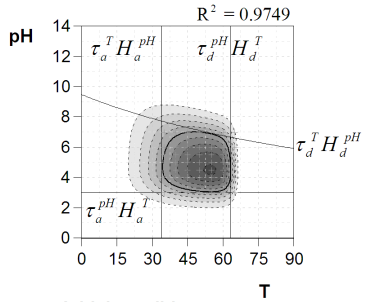
Opt. A = 4.9%

Mode of action:

AA

A

INVERTASE



Initial conditions:

t = ¿? min
S = ¿?
E/S = ¿?

Cardinal values:

	min	opt	max
T:	*16.1*	*54.3*	*66.2*
pH:	*1.9*	*4.4*	*9*

Opt. A = ¿?

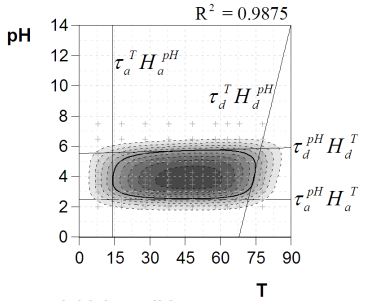
Mode of action:

$$\text{IA} \left| R\downarrow\left(T \xrightarrow{I} A_{pH}^d\right) \right.$$

Stricly antagonistic (-1.3 %)

A

POLI. OXID.



Initial conditions:

t = ¿? min
S = ¿?
E/S = ¿?

Cardinal values:

	min	opt	max
T:	*4.5*	*47.1*	*85.2*
pH:	*1.8*	*3.7*	*6.1*

Opt. A = 45%

Mode of action:

$$\text{IA} \begin{vmatrix} R\uparrow\left(pH \xrightarrow{P} A_T^a\right) \\ R\downarrow\left(T \xrightarrow{I} A_{pH}^d\right) \end{vmatrix}$$

Partially synergistic (0.2 %)

Figure 5: Standardized format to summarize the effects of *T* and *pH* on *A*.

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TABLES

Table 1: Proposed classification system for the response modes of the joint action of *T* and *pH* on the activity of an enzyme.

MODE OF ACTION OF THE RESPONSE	INTERACTION SUBDIVISION				INTERACTION ABREVIATION
	Interactive variable	***Sub-function modified in V2***	***Hyperbolic modification***	***Effect on the resulting activity (R)***	
Additive action (AA)	Lack of relevance because the response is defined as free from interactive mechanisms				AA
Interactive action (IA)	V1	Activated (*a*)	Proportional (*P*)	R ↓ (antagonist)	IA: $R\downarrow\left(V_1 \xrightarrow{P} A^a_{V_2}\right)$
			Inversely (*I*)	R ↑ (synergist)	IA: $R\uparrow\left(V_1 \xrightarrow{I} A^a_{V_2}\right)$
		Deactivated (*d*)	Proportional (*P*)	R ↑ (synergist)	IA: $R\uparrow\left(V_1 \xrightarrow{P} A^d_{V_2}\right)$
			Inversely (*I*)	R ↓(antagonist)	IA: $R\downarrow\left(V_1 \xrightarrow{I} A^d_{V_2}\right)$

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Table 2: Relevant models (M) taken from the scientific bibliography to describe the enzymatic activity under different *pH* and *T* conditions.

CLASIFICATION	ABREVIATION	MODELS	REFERENCES
Regular models	**M1**	$A = b_0 + b_1 T + b_2 pH + b_{12} TpH + b_{11} T^2 + b_{22} pH^2$	5,37,38
	M2	$A = b_0 + b_1 T + b_2 pH + b_{12} TpH + b_{11} T^2 + b_{22} pH^2 + b_{112} T^2 pH + b_{122} TpH^2$	
Models used in other fields of knowledge	**M3**	$A = p\left(T - T_{\min}\right)^{n_1} \left(pH - pH_{\min}\right)^{n_2} \exp\left[-a_1\left(T - T_{\min}\right) - a_2\left(pH - pH_{\min}\right)\right]$	52
	M4	$A = p\left(T - T_{\min}\right)^2 \left\{1 - \exp\left[a_1\left(T - T_{\max}\right)\right]\right\}\left(c_0 + c_1 pH + c_2 pH^2\right)$	53
	M5	$A = p\left(T - T_{\min}\right)^2 \left\{1 - \exp\left[a_1\left(T - T_{\max}\right)\right]\right\}\left(pH - pH_{\min}\right)^2 \left\{1 - \exp\left[a_2\left(pH - pH_{\max}\right)\right]\right\}$	15
	M6	$A = \dfrac{A_r \dfrac{T}{T_r} \exp\left[\dfrac{H_r}{R}\left(\dfrac{1}{T_r} - \dfrac{1}{T}\right)\right]}{1 + \exp\left[\dfrac{H_1}{R}\left(\dfrac{1}{T_1} - \dfrac{1}{T}\right)\right] + \exp\left[\dfrac{H_2}{R}\left(\dfrac{1}{T_2} - \dfrac{1}{T}\right)\right]}\left(c_0 + c_1 pH + c_2 pH^2\right)$	14,25,26
	M7	$A = A_m \dfrac{\left(T - T_{\min}\right)^2 \left(T - T_{\max}\right)}{\left(T_{opt} - T_{\min}\right)\left[\left(T_{opt} - T_{\min}\right)\left(T - T_{opt}\right) - \left(T_{opt} - T_{\max}\right)\left(T_{opt} + T_{\min} - 2T\right)\right]} \dfrac{\left(pH - pH_{\min}\right)\left(pH - pH_{\max}\right)}{\left[\left(pH - pH_{\min}\right)\left(pH - pH_{\max}\right) - \left(pH - pH_{opt}\right)^2\right]}$	11
Models developed to study the combined effect of the T and pH	**M8**	$A = A_m \dfrac{k_{sr} \exp\left[\dfrac{E_s}{R}\left(\dfrac{1}{T_r} - \dfrac{1}{T}\right)\right] \exp\left[-k_{dr} t \exp\left[\dfrac{E_d}{R}\left(\dfrac{1}{T_r} - \dfrac{1}{T}\right)\right]\right]}{1 + \dfrac{H^+}{K_{EH}} + \dfrac{K_w}{K_{EOH}} \dfrac{1}{H^+}}$	7,19

NOTATIONS: A, is the enzymatic activity; A_m, is the asymptotic value of A; T and pH are the variables, when they are written with the subscripts they are cardinal parameters values of the variables (*min*, below which no activity occurs; *max*, above which no enzymatic activity occurs; *opt,* cardinal value at which the enzyme activity is optimal); The parameters written with *b* and *c* notations, and ordered with regular numerical values denote coefficients of polynomial structures; The parameters written with *a* and ordered with regular numerical values are exponential coefficients; The parameters written with *n* and ordered with regular numerical values are potential coefficients that makes more versatile the descriptive capacities of the equation; p, is a pre-exponential parameter; T_r, is the reference temperature in *Kelvin* degrees; H_r is the enthalpy at the reference temperature T_r; R, is the ideal gas constant (8.314 J mol^{-1} K^{-1}); A_r enzymatic activity to a reference temperature T_r; H_1, H_2, T_1 and T_2 are the enthalpies and temperatures that corresponds to the 50% drop for excess (1) and defect (2) in enzymatic activity respectively; t, is the reaction time, k_{sr} is the specific reference rate for the enzymatic process (min^{-1}), k_{dr} is the specific reference rate for the deactivation enzymatic process (min^{-1}), E_d is the activation energy for the catalytic process (J mol^{-1}), E_s is the deactivation energy for the catalytic process (J mol^{-1}), H^+ is the pH value with the expression $H^+ = 10^{-pH}$, K_w is the water dissociation constant, and K_{EH} and K_{EOH} are the equilibrium constants of the protonation and hydroxylation reactions respectively.

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Table 3: Joint effects of temperature and *pH* on specified enzymatic activities, as described by model [7]. For Alcalase, both alternatives of modeling (*a*, *b*) are shown. Confidence intervals are presented as percentage of the parametric estimate values. Notice that independent variables are used as natural values. Consequently, the effects of the interaction terms are very relevant, despite their low absolute magnitudes.

TYPE OF ENZYME	basic part of model [7]								interactions part of model [7] on the parameter τ								STATISTIC	
	T				pH				pH→T				T→pH					
	A_a^T		A_d^T		A_a^{pH}		A_d^{pH}		H_a^{pH}		H_d^{pH}		H_a^T		H_d^T			
	τ_a^T	α_a^T	τ_d^T	α_d^T	τ_a^{pH}	α_a^{pH}	τ_d^{pH}	α_d^{pH}	m_a^T	n_a^T	m_d^T	n_d^T	m_a^{pH}	n_a^{pH}	m_d^{pH}	n_d^{pH}	R^2	R^2_{adj}
Illustrative case study analyzed in detail on the text																		
alcalase [a]	38.78±1.4	3.98±5.1	69.04±0.3	17.07±16.4	6.40±0.4	6.62±11.6	11.05±0.3	27.64±4.5	--	--	--	--	--	--	--	--	0.841	0.831
alcalase [b]	39.57±2.2	3.93±10.8	118.6±8.9	27.45±19.5	6.61±1.8	5.93±15.6	14.85±3.4	63.31±30.6	--	--	--	0.076±21.5	--	--	--	0.006±13.5	0.990	0.989
Other experimental results obtained in our laboratory																		
glucanex	31.80±1.9	3.98±11.1	58.01±0.4	16.47±9.4	3.00±3.7	4.54±24.4	6.11±3.8	13.77±19.7	--	--	--	--	--	--	--	--	0.962	0.955
esperase	61.98±3.6	12.42±6.5	74.93±0.8	10.10±7.8	6.32±1.3	7.30±10.5	17.88±10.4	13.33±12.1	--	0.01±36.2	--	--	--	--	--	0.01±26.8	0.982	0.983
Relevant data from other authors at the scientific bibliography																		
phytase	38.51±1.4	3.84±5.3	63.89±0.3	23.65±11.7	3.42±0.8	9.20±8.3	5.61±0.6	13.39±9.3	--	--	--	--	--	--	--	--	0.989	0.988
polyf. ox.	14.07±4.5	1.51±7.9	67.92±3.8	9.72±8.6	2.50±1.2	5.49±8.1	5.52±2.3	10.52±8.3	--	--	0.023±46.2	--	--	--	0.0008±62.9	--	0.987	0.984
invertase	34.21±3.7	3.22±24.0	63.77±0.7	27.41±27.1	3.04±3.2	4.38±18.5	9.26±19.1	5.72±30.5	--	--	--	--	--	--	--	0.01±77.5	0.974	0.973

In the case of alcalase the subscripts *a* denotes the fitting results for the model [5] (without interactions) and *b* fitting results for the model [7] (with interactions).

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