Comparison of several mathematical models for describing the joint effect of temperature and pH on glucanex activity

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ABSTRACT

The aim of the present work was to evaluate with different statistical criteria the suitability of nine equations for describing and optimizing the simultaneous effect of temperature and pH on glucanex activity using two characteristic polysaccharides (curdlan and laminarin) as substrates. The most satisfactory solutions were found with an empirical equation constituted with parameters of practical interest (Rosso model), and a hybrid model between the Arrhenius equation and the mathematical expression generated by the protonation-hydroxylation mechanism (Tijskens model). The joint optimal values of pH and temperature calculated with the Rosso model were obtained at 4.64 and 50ºC with curdlan and 4.64 and 48ºC using laminarin as substrate.

Keywords: glucanex, enzymatic activity, curdlan, laminarin, mathematical modelling, pH and temperature effects.

1. INTRODUCTION

Describing the combined effect of temperature and pH on the enzymatic reaction rate is a frequent problem that is not always solved satisfactorily. When they are studied independently, the two variables produce well-known rate profiles that increase to a maximum point which is then followed by a drop in activity.[1,2,3] The results from experiments repeatedly demonstrate that the profiles obtained for the independent variables are linked to each other, generally in a non-additive interaction.[1,2,3] The correct modeling of these experimental profiles is especially important when a rigorous and predictive quantification of the maximum enzymatic activity, and minimum, optimum and maximum pH and temperature values are necessary, for example, in the case of an enzyme reactor that is controlled by software.

The Arrhenius model provides a possible resource for modelling temperature when it is applied to the three rates involved in the process: the substrate transformation rate and the enzyme denaturation rate at high and low temperatures. This approach was used to describe the metabolism of poikilotherm organisms in which the restrictive factor of the rate is defined by only one enzyme.[4,5] The equation obtained was later reformed by Schoolfield et al.[6] who inserted a reference rate at a pre-established temperature and redefined the parameters (six in both mathematical expressions) in order to reduce the correlation among them. In spite of the formal basis of this last approach, the empirical equation proposed by different authors[7,8,9] and modified by Zwietering et al.[10] generated the best fits (with only four parameters) when several mathematical models for assessing the effect of temperature on the bacterial growth of *Lactobacillus plantarum* on a conventional culture medium MRS were compared.[10] The same model led to satisfactory fits for describing *Salmonella* growth on soudjouk-style fermented semi-dry sausage.[11] In enzyme kinetics, the Arrhenius equation has been used extensively to model the effect of temperature on the increase in reaction rate,[12,13,14] on the deactivation constant[15,16] and on the thermal stability of enzymes.[17,18]

The requirements for modelling pH are similar or larger than those defined by the Arrhenius equation for temperature. The most formal description is based on an acid-base dissociation mechanism.[1,2,3] The use of this resource provides good fit results as well as parameters with a clear physical meaning and in some cases for defining the optimal intervals of enzyme activity.

Glucanex is a multicomponent enzyme preparation consisting of several isoenzymes that contain β-1,3 glucanase activity,[19] that is used commonly for hydrolyzing the oligosaccharides from yeast cell walls in order to obtain β-glucans,[20] to control wine spoilage yeasts, protoplast preparation and as a biocontrol agent against plant pathogenic fungi.[21,22,23] Nevertheless, there are no studies describing the kinetic characteristics of this enzyme, such as the combined effect of temperature and pH on glucanex activity, and there are no data on the optimum temperature and pH in the literature.

The aim of the present work was to evaluate and compare the suitability of nine equations for describing the combined effect of pH and temperature on the catalytic activity of a commercial glucanolytic preparation (glucanex) in the hydrolysis of two gluco-polysaccharides substrates: curdlan (β(1→3)) and laminarin (β(1→3):β(1→6) ratio of 3:1).

2. MATERIALS AND METHODS

2.1. Chemicals

Both substrates, laminarin from *Laminaria digitata* and curdlan from *Alcaligenes faecalis* were provided by Sigma (St. Louis, MO, USA). Glucanex® 200G was obtained from Novozymes Corp (Copenhagen, Denmark). Other reagents used in enzymatic assays were of analytical grade and purchased from Sigma.

2.2. Methodology for measuring the enzyme activity of Glucanex® 200G

Two different substrates (curdlan and laminarin) were dissolved in 0.02M citric/phosphate buffer at varius pHs. The enzyme activity was tested by placing 0.4 mL of the substrate solution, at the required pH for the analysis, into a 30 mL tube (in duplicate) with a teflon cap. The tubes were then put into a controlled thermostatic water bath with continuous mild agitation. After reaching the bath temperature, 0.1 mL of a fresh enzyme solution (0.005 M citric/phosphate buffer for each pH assay) of Glucanex® 200G (the concentration varied) was added, and thus 2.5 mg/L of substrate was obtained in the final reaction solution. The reaction was ended when the analytical time (varied) finished by adding 0.5 mL of 3,5-dinitrosalicylic acid (DNS). The enzyme activity was measured by determining the sugars released by the reaction with DNS using glucose as the substrate.[23]

2.3. Preliminary assays for establishing the initial conditions

In order to determine the proper conditions for evaluating the joint effect of temperature and pH on the enzymatic activity, initial experiments were carried out to establish: a) an appropriate range for the temperature and pH; b) a suitable ratio between the substrate and enzyme (Glucanex® 200G); and c) an analytical time in which the product formation rate would continue to show a linear profile.

2.3.1. The temperature and pH range

The appropriate pH and temperature ranges were obtained by studying the two variables separately (with a constant pH of 4.5 for the temperature assays, and at 45ºC for the pH assays) with curdlan as the substrate, an analytical time of 20 min and an enzyme-substrate ratio of 3:10 (750 µg/L). The maximum enzyme activity was obtained at 45 ºC and pH 4.5 (Figure 1a). The final range selection for pH (3.5 to 6) and temperature (32 to 60 ºC) were chosen around the individual optima where the product conversion reaches 50% (see experimental points included into the selected range box, Figure 1a). The experimental data were expressed as the percentage of the maximum concentration of the product formed (RS).

2.3.2. The substrate and enzyme ratio

The suitable substrate/enzyme ratio was selected by carrying out a kinetic assay, measuring the enzyme activity with different enzyme concentrations (from 0 to 1000 µg/L) at pH 4.5 and 45ºC with a constant curdlan concentration of 2.5 mg/L in the final solution. The experimental results are shown in Figure 1b together with the profiles obtained by fitting the data to a first-order kinetic model:

$$A_{Ez} = K\left(1 - e^{-\alpha t}\right) \qquad [1]$$

where A_{Ez} as the enzymatic activity of glucanex (mg/L of RS released), t is the time of hydrolysis (min), K is the asymptotic product formation (mg/L) obtained and α is the specific RS production rate (min^{-1}). In the case of Glucanex, due to the absence of saturation, the value of K it is equal to the maximum possible product conversion (2.5 mg/L) for all enzymatic concentrations tested except for the case when no enzyme is present. The parameter α increases as the enzyme concentration increases.

Finally, Figure 1c shows the nonlinear relation between the specific rate α, and the enzyme concentration along with the fit to a similar mathematical expression [1], allowing us to analyse its derivative and to obtain the optimum value for the enzyme-substrate ratio. The ideal concentration was found to be approximately 250 μg of glucanex/L (a ratio of 1:10) and this value was maintained for all subsequent experiments.

2.3.3. The optimum analytical time

The results obtained for the initial times (<1 h) are plotted in Figure 1d. A linear correlation between the product formed and reaction time was observed for all enzyme concentrations over the initial 30 minutes. An analytical time of 15 min was chosen in order to ensure the linearity of product formation (mg/L) throughout the experiment. This time choice also avoids enzyme denaturation, shortens the time of the assay, and produces enough reducing sugars for accurate quantification.

2.4. Combined effect of pH and temperature on glucanex activity

The combined effect on glucanex activity (GA) was measured at several pHs (from 3.5 to 6 in steps of 0.5) and at different incubation temperatures (from 32 to 60°C with different interval steps for each substrate) with curdlan and laminarin as substrates. The enzyme activity (with

0.02M citric/phosphate buffer) was tested following the procedure described above using the conditions previously selected: an analytical time of 15 min and enzyme/substrate ratio of 1:10 (250 μg/L) in the final solution. The experimental data were expressed as a percentage of the maximum concentration obtained for each substrate.

2.5. Fitting procedure and common statistical values

Fitting procedures and parametric estimates from the experimental results were performed by minimizing the sum of quadratic differences between the observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro '*Solver*' of the *Microsoft Excel XP* spreadsheet. The confidence intervals from the parametric estimates (Student's *t* test) and the goodness of fit and consistency of the mathematical models (Fisher's *F* test) were determined using *DataFit 9.0.59* (Oakdale Engineering, Oakdale, PA, USA).

2.6. Criteria used to assess the selection of the best model

2.6.1. Criteria based on model selection criteria (MSC)

In the present work, the AICc, BIC, RIC, Cp, R^2_{adj}, FPE and MSC criteria (Table 1) were directly obtained using an *Excel* spreadsheet. The leave one out cross-validation (LOO-CV) procedure and Monte Carlo cross-validation (MCCV) were obtained with an *Excel* spreadsheet using the *Excel* add-in *Solverstat* macro. This selected group is a combination of different criteria that can discriminate between the models based on their goodness of fit, complexity, overfitting and generalizability.

2.6.2. Additional statistical criteria

Additional criteria based on the following features were used to evaluate the mathematical models: a) the residual distribution; b) the number of non-significant (NS) parameters ($\alpha = 0.05$); c) the number of parameters with biological or physical meaning.

3. RESULTS AND DISCUSSION

3.1. Mathematical models describing the combined effect of temperature and pH on GA

We reviewed and studied the appropriateness of nine models from the literature for predicting GA under different pH and temperature conditions. Those equations have different origins and mathematical structure and can be classified as: a) regular models with empirical forms (polynomials) whose parameters do not have any physical meaning; b) models useful and widely in other fields of knowledge (i.e., microbial growth) for similar purpose; c) structured models developed to study the combined effect of temperature and pH on enzymatic reactions.

3.1.1. Regular models

Model 1: The simplest approach for describing the joint effect of temperature and pH is defined by a quadratic polynomial with a multiplicative term that combines the action of the two independent variables on the response (enzymatic activity). This resource has been applied, for example, to study amylase,[1] chitinase[33] and 1,3-glucanase[34] activity. When *r* is the enzymatic activity, the mathematical function is as follows:

$$r = b_0 + b_1 T + b_2 pH + b_{12} TpH + b_{11} T^2 + b_{22} pH^2 \qquad [2]$$

Model 2: The previous equation [2] defines a parabolic surface that can acceptably approach the response in a nearby environment to its maximum value. Thus, the following equation [3] could represent a useful resource for determining the temperature and pH values that maximize the activity. However, the response is generally asymmetric, and thus better fits are obtained by adding two more terms to [2]:

$$r = b_0 + b_1 T + b_2 pH + b_{12} TpH + b_{11} T^2 + b_{22} pH^2 + b_{112} T^2 pH + b_{122} TpH^2 \qquad [3]$$

3.1.2. Models used in other fields of knowledge

Model 3: If the objective is to describe a profile with an asymmetric dome form, a function with bias towards the right side (*i.e.*, with an abrupt drop when $X \to 0$ and gentle drop when $X \to \infty$) is:

$$r = aX^{n} \exp\left(-bX\right) \qquad [4]$$

When $n = 1$, the equation represents a classic model of the population dynamics that describes with basic principles the effect of intraspecific competition on reproductive success.[35] The arbitrary resource of allowing n values that are different from 1 makes the profile of the mathematical function more versatile.

Equation [4] was used by Murado et al.[36] to describe the production of amylases in solid-state cultures by *Aspergillus oryzae* as a function of the saturation of the support in the liquid phase. The same authors found that the results obtained by Lindenfelser and Ciegler,[37] relative to the effect of the humidity percentage in ochratoxin A production with *Aspergillus ochraceus* using solid-state fermentation on wheat grains, were satisfactorily fitted to equation [4].

The bivariate model applied to the activity is the multiplication of two equations [4]:

$$r = aT^{n_1} pH^{n_2} \exp\left(-b_3 T - b_4 pH\right) \qquad [5]$$

However, since equation [4] defines a curve with an initial null ordinate, to use equation [5] in our context we need to modify the origin by introducing two parameters (T_0 and pH_0) that represent the T and pH values that make the activity null:

$$r = a(T - T_0)^{n_1} (pH - pH_0)^{n_2} \exp[-b_3 (T - T_0) - b_4 (pH - pH_0)] \quad [6]$$

Model 4: The effect of temperature on the rate of nucleotide decomposition (r) in cold-stored carp muscle was described by Ohta and Hirabara[38] with the empirical relation: $r^{1/2} = 0.065T + 0.518$, which[8] is generalized as:

$$r^{1/2} = c(T - T_0) \quad [7]$$

It was applied to bacterial growth in a range of temperatures (in K) that covers a range from the minimum temperature (T_0) when the growth rate is null to the maximum temperature of growth.

Later on, the equation was reformed[7] to expand its descriptive capacity to any temperature, and took on the following mathematical form:

$$r^{1/2} = c(T - T_{\min})\{1 - \exp[a_1 (T - T_{\max})]\} \quad [8]$$

where $T_{\min}$ (with the same meaning as T_0 in equation [7]) and $T_{\max}$ represent the limits of the temperature range beyond which the growth rate is null. Indeed, the exponential term becomes nil when $T_{\max} >> T$ (so that equation [8] can be simplified to equation [7]) and increases when T is close to $T_{\max}$, so that r decreases from a certain T value and tends to zero when $T = T_{\max}$.

In a subsequent modification, Pronk et al.[10] proposed the equation:

$$r = c(T - T_{\min})^2 \{1 - \exp[a_1 (T - T_{\max})]\} \quad [9]$$

This equation differs from [6] because the decrease in *r* from a maximum is due to an exponential function instead of its square. In both cases, equations [8] and [9] produce more versatile profiles than [4], with the possibility of obtaining biases to the left or right.

The inclusion of the pH in this model can be done by multiplying [9] by a polynomial equation formulated with this variable:

$$r = c\left(T - T_{\min}\right)^2 \left\{1 - \exp\left[a_1\left(T - T_{\max}\right)\right]\right\}\left(c_0 + c_1 pH + c_2 pH^2\right) \qquad [10]$$

Model 5: Another option would be to accept that the relationship between the pH and the enzymatic activity leads to a function with the same structure as that used for the temperature. Thus, the combined response could be described by multiplying the two effects:

$$r = c\left(T - T_{\min}\right)^2 \left\{1 - \exp\left[a_1\left(T - T_{\max}\right)\right]\right\}\left(pH - pH_{\min}\right)^2 \left\{1 - \exp\left[a_2\left(pH - pH_{\max}\right)\right]\right\} \qquad [11]$$

Model 6: Initially, the Rosso equation was used to describe the joint effect of temperature and pH on microbial growth,[39] but it can also be used in other fields, such as enzyme kinetics. It establishes the enzymatic activity (*r*) as a dependent variable:

$$f\left(T\right) = \frac{\left(T - T_{min}\right)^2\left(T - T_{max}\right)}{\left(T_{opt} - T_{min}\right)\left[\left(T_{opt} - T_{min}\right)\left(T - T_{opt}\right) - \left(T_{opt} - T_{max}\right)\left(T_{opt} + T_{min} - 2T\right)\right]}$$

$$f\left(pH\right) = \frac{\left(pH - pH_{min}\right)\left(pH - pH_{max}\right)}{\left[\left(pH - pH_{min}\right)\left(pH - pH_{max}\right) - \left(pH - pH_{opt}\right)^2\right]}$$

$$r = r_m f(T) f(pH) \quad [12]$$

where r is the enzymatic activity, r_m is the maximum enzymatic activity, T is the temperature (°C), T_{min} is the temperature below which no activity occurs, T_{max} is the temperature above which no enzymatic activity occurs, T_{opt} is the temperature at which the enzyme activity is optimal, pH_{min} is the pH below which no catalytic activity occurs, pH_{max} is the pH above which no activity occurs, and pH_{opt} is the pH at which the enzyme activity is optimal.

Model 7: The accumulated function of the Weibull distribution is a very versatile resource when a symmetric sigmoid or parabolic profiles do not need to be simulated.[40] It has been successfully used in diverse experimental fields such as the study of biotoxins[41] and the antioxidant capacity of different compounds.[42] Its mathematical expression, in the case of defining the combined effect of temperature and pH on GA, can be written as:

$$r = k\left(\frac{\alpha T^{\alpha-1}}{q^{\alpha}}\right)\exp\left[-\left(\frac{T}{q}\right)^{\alpha}\right]\left(\frac{\beta pH^{\beta-1}}{p^{\beta}}\right)\exp\left[-\left(\frac{pH}{p}\right)^{\beta}\right] \quad [13]$$

where r is the enzymatic activity, k, α, q, β and p are empirically determined parameters, and T is the temperature (ºC).

3.1.3. Structured models

Model 8: The model proposed by Sharpe et al.[4] is based on a combination of three Arrhenius equations:

$$r = \frac{k_r \exp\left(\frac{-E_r}{RT}\right)}{1 + k_a \exp\left(\frac{-E_a}{RT}\right) + k_b \exp\left(\frac{-E_b}{RT}\right)} \quad [14]$$

where T is the temperature (K), R is the ideal gas constant (8.314 J mol^{-1} K^{-1}), and the meaning of the parameters is defined by the Arrhenius model for each reaction considered: 1) substrate transformation (represented by subindex r), and 2) the reversible enzyme deactivations at high (subindex a) and low (subindex b) temperatures. Thus, k_i is the pre-exponential terms and E_i the activation energies (J mol^{-1}).

The reformulation used by Schoolfield et al.[6] is:

$$r = \frac{r_r \frac{T}{T_r} \exp\left[\frac{H_r}{R}\left(\frac{1}{T_r} - \frac{1}{T}\right)\right]}{1 + \exp\left[\frac{H_a}{R}\left(\frac{1}{T_a} - \frac{1}{T}\right)\right] + \exp\left[\frac{H_b}{R}\left(\frac{1}{T_b} - \frac{1}{T}\right)\right]} \quad [15]$$

involving the enzymatic activity r_r to a reference temperature T_r (319 K), the substitution of activation energies by the enthalpies H_i and the introduction of temperatures T_a and T_b that determine, for excess and defect, respectively, the 50% drop in enzymatic activity. As in equation [9], the profile can be biased to the left or right.

The effect of pH could also be included by multiplying equation [15] and a quadratic polynomial as follows:

$$r=\frac{r_r\frac{T}{T_r}\exp\left[\frac{H_r}{R}\left(\frac{1}{T_r}-\frac{1}{T}\right)\right]}{1+\exp\left[\frac{H_a}{R}\left(\frac{1}{T_a}-\frac{1}{T}\right)\right]+\exp\left[\frac{H_b}{R}\left(\frac{1}{T_b}-\frac{1}{T}\right)\right]}\left(c_0+c_1pH+c_2pH^2\right) \quad [16]$$

Model 9: The only theoretical approach developed for fitting the joint effect of temperature and pH on enzymatic activity was proposed by Tijskens et al.[3] in order to study this effect in phytase, peroxidase and lipase catalysis.[43] This equation combines the Arrhenius model that explains the temperature effect with an acid-base dissociation reaction for the pH effect:

$$r=\frac{r_mk_{sr}\exp\left[\frac{E_s}{R}\left(\frac{1}{T_r}-\frac{1}{T}\right)\right]\exp\left[-k_{dr}t\exp\left[\frac{E_d}{R}\left(\frac{1}{T_r}-\frac{1}{T}\right)\right]\right]}{1+\frac{H^+}{K_{EH}}+\frac{K_w}{K_{EOH}}\frac{1}{H^+}} \quad [17]$$

where r is the enzymatic activity, r_m is the maximum enzymatic activity, which was maintained constant at a value of 100, t is the reaction time (10 min), T is the temperature (K), T_r is the reference temperature (313 K), 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}), R is the ideal gas constant (8.314 J mol^{-1} K^{-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.

The combined effects of pH and temperature on GA using laminarin and curdlan as substrates are displayed in Figure 2 and Figure A (Supplemental Material), respectively. In both cases, the experimental domains ranged from 32 to 60ºC and from 3.5 to 6.0 for pH. In these figures the experimental data were fitted to the equations specified on the top of the graphs. The parametric estimates and corresponding confidence intervals of the proposed equations are summarized in Table 2. In all cases for both substrates, the fitting of experimental data to equations (F_{ratio}>45, *p*<0.005) was statistically satisfactory and consistent (data not shown).

3.2. Model selection by statistical criteria

Since there are many models able to fit the combined effects of T and pH reasonably well for the data presented, a selection process was carried out to determine the model that best predicts the joint effect of the two variables in the interval studied. In order to assist us with selecting the best model, we used different statistical criteria to evaluate the multivariable fit and explanatory appropriateness of the equations.

3.2.1. Model selection criteria (MSC)

The usefulness of MSC to compare a group of possible models is well-documented.[44] A model should be complex enough to extract the regularities in data, but simple enough not to overfit it and thereby reduce predictiveness. MSC adjust the goodness of fit in order to penalize model complexity, overfitting and lack of generalizability. Currently, there are a variety of MSC available,[26,45] but there is no one criterion that can lead to a perfect choice.[46] A summary of the MSC used to evaluate the results obtained for the nine models with curdlan and laminarin as substrates is shown in Table 1.

Table 3 shows the model rank (Rk) obtained for each MSC and the final ranking (Rk_F) based on the ranking sum of each MSC (∑Rk) for the two substrates. With curdlan, equation [3] was the best model with respect to the sum of all MSC, followed by equations [12], [13], [11] and [17]. In the case of laminarin, equation [16] was the best model with respect to the sum of all MSC, followed by equations [17], [12], [11] and [10]. When the sum of the model rank (Rk) for the two substrates is applied, equation [12] followed by [17] and [16] are the models most likely to be correct.

3.2.2. Additional statistical criteria

The residuals should be randomly scattered around zero to avoid autocorrelation.[47] These residuals should not be grouped and should not increase or decrease as a function of the independent variable. In general terms all the models used showed a relatively good distribution of the residuals, and autocorrelation was not observed with the Durbin-Watson test (data not shown).

The confidence intervals at a level of 95% for each parameter are reported in Table 2. The parametric estimates in many cases led to large confidence intervals, and therefore these parameters were considered not significant. For example, in equation [6] only one parameter (b_3) was significant. In equations [10] and [11] the most important coefficients with physical meaning (T_{max}, T_{min}, pH_{max}) were significant. Equation [16] has good fitting levels in both cases (the best fit was when laminarin was used); however; just three out of the nine parameters were significant.

Only in equations [12] and [13] were all the parameters statistically significant. In equation [16], the parameters K_{dr} and K_{sr} showed confidence levels below 95% in both cases (laminarin and curdlan). As explained by Tijskens et al.[3], fitting should be carried out in two steps: first, obtain the parameters K_{dr}, E_d, K_{sr} and E_s; and second, adjust the other two parameters (K_{EH} and K_{EOH}) separately. When these indications are followed all the parameters are significant ($\alpha = 0.05$). Conversely, when the fitting procedure is applied in one single step, as we reported here, the parameters K_{dr} and K_{sr} are not statistically significant.

Finally, many parameters from equations [2], [3], [6] and [13] do not have a biological or simple physical meaning, and therefore these equations are not very appropriate for describing enzymatic activity. Equations [10] and [11] have a small number of interpretable parameters. Only in equations [12] and [17] do all the parameters have physical meaning. In equation [17], additional values for pH_{opt}, pH_{min} and pH_{max} can be calculated from the parameters obtained. However, equation [12] seems to be more convenient (from an industrial point of view) because its parameters that describe the limits and optimal conditions of pH and T as well as the maximum amount of product released are easily interpreted. Many authors have also emphasized that the applied models should have a clear meaning and use the minimum number of parameters that can be successfully employed for biological optimizations and descriptions.[39,48]

3.3. Model selection and application

The combination of the above mentioned criteria indicated that equations [12] and [17] are suitable for predicting the join effect of temperature and pH on GA with both substrates. In the case of curdlan, equation [12] seems to be more accurate than [17]; however, with laminarin, equation [17] was the best model for predicting the experimental data. These models can be used to determine a set of parameters with geometric and physical meaning that describe completely the joint effect of pH and T on the enzyme activity.

3.3.1. Application when curdlan is used as substrate

Figure 3 shows the interactive effects of pH and temperature on GA using curdlan as a substrate. Experimental data were fitted to equation [12], which showed that a joint maximum is achieved from 46 to 53ºC and in the pH range 4.5 to 5.0.

Practical and operative descriptions of the limits and optimal glucanex activity can be established using the numerical values of the biologically meaningful parameters from equation [12] (Table

2). Thus, the joint optimal pH (pH_{opt}) and temperature (T_{opt}) for GA was 4.64±0.22 and 50.48±1.38°C, respectively, using curdlan as substrate. Both values were in agreement with the results obtained by[49] using 1,3-glucanase from *Trichoderma harziaum,* but studying one factor at a time. Other interesting parameters obtained from equation [16] were the maximum temperature (T_{max}) and pH (pH_{max}) and minimum temperature (T_{min}) and pH (pH_{min}) for enzymatic activity. The values for glucanex with curdlan were 65.55±2.29°C, 6.83±0.51, 22.96±4.63°C and 2.88±0.42 respectively.

3.3.2. Application when laminarin is used as substrate

The experimental data and the simulated profiles fitted to equation [17] are shown in Figure B (Supplementary Material). The optimal activity was found at 47.58±0.70°C for T and 4.64±0.11 for pH. Using a conventional study of one factor at a time with laminarin, other authors established the optimal pH and T conditions for an exo-β-1,3-glucanase from *Trichoderma asperelhum* as 5.1 and 55ºC, respectively [35].

The large number of runs in the residual plot indicates that there is no clustering in the distribution in certain zones, which suggests that this distribution is random and that the equation estimated all datasets perfectly (avoiding under or overestimations) (see Figure B, Supplemental Material).

4. CONCLUSIONS

The results of the comparison of several mathematical models for describing the experimental profiles of the combined effect of pH and temperature on glucanex activity highlighted the fitting and description capacities of the Rosso [12] and Tijskens [17] equations. The two models were used separately to obtain a set of parameters, based on first principles or with clear geometric and physical meaning, which described the GA characteristics completely.

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FIGURE CAPTIONS

Figure 1: Determination of the optimal conditions for enzyme assay in order to evaluate the combined effect of temperature and pH on GA: a) selection of pH and T intervals; b) kinetics data obtained at different enzyme concentrations (0 ■, 15 □, 30 ●, 75 ○, 150 ▲, 250 △, 500 ◆, 1000 ◇ µg/L) and adjusted to the equation (1); c) values of specific rate of RS produced (α) for each glucanex concentration; d) selection of the analytical time in the linear section of the initial rates.

Figure 2: Response surfaces of the combined effect of temperature and pH on GA (%) with laminarin as substrate. Fit of the experimental results (●) according to the equations defined in the text.

Figure 3: Combined effect of temperature and pH on GA (%) with curdlan as substrate. **A:** 2D representation of pH and T effects. Fit of the experimental results (●) according to the equation (12) (continuous line). **B:** correlation between expected and observed data and plots of residuals (%) in relation with pH, T and GA. **C:** 3D representation of pH and T effects on GA. Fit of the experimental results (●) according to the equation (12) (response surface).

Supplemental Figure A: Response surfaces of the combined effect of temperature and pH on GA (%) with curdlan as substrate. Fit of the experimental results (●) according to the equations defined.

Supplemental Figure B: Combined effect of temperature and pH on GA (%) with laminarin as substrate. **A:** 2D representation of pH and T effects. Fit of the experimental results (●) according to the equation (17) (continuous line). **B:** correlation between expected and observed data and plots of residuals (%) in relation with pH, T and GA. **C:** 3D representation of pH and T effects on GA. Fit of the experimental results (●) according to the equation (17) (response surface).

TABLE CAPTIONS

Table 1: Different model selection criteria (MSC) used to compare the nine models reviewed from the bibliography to predict the joint effect of pH and T. *n*: number of independent measurements considered in the fit. *k*: number of fitted parameters. *RSS*: residual sum of squares. *ESS*: explained sum of squares.

Table 2: Parametric estimates and confidence intervals obtained from the equations used in the evaluation of the joint effect of pH and temperature on the glucanex activity with curdlan (A) and laminarin (B) as substrate. CI: confidence intervals were evaluated by t-Student test (α=0.05). NS: non significant. ** Further interesting values calculated from the parameters of equation (17).

Table 3: Model ranking (Rk) obtained for each MSC and the final ranking (Rk_F) based on the total ranking average ($\sum$Rk) for the two substrates.

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FIGURES

Figure 1

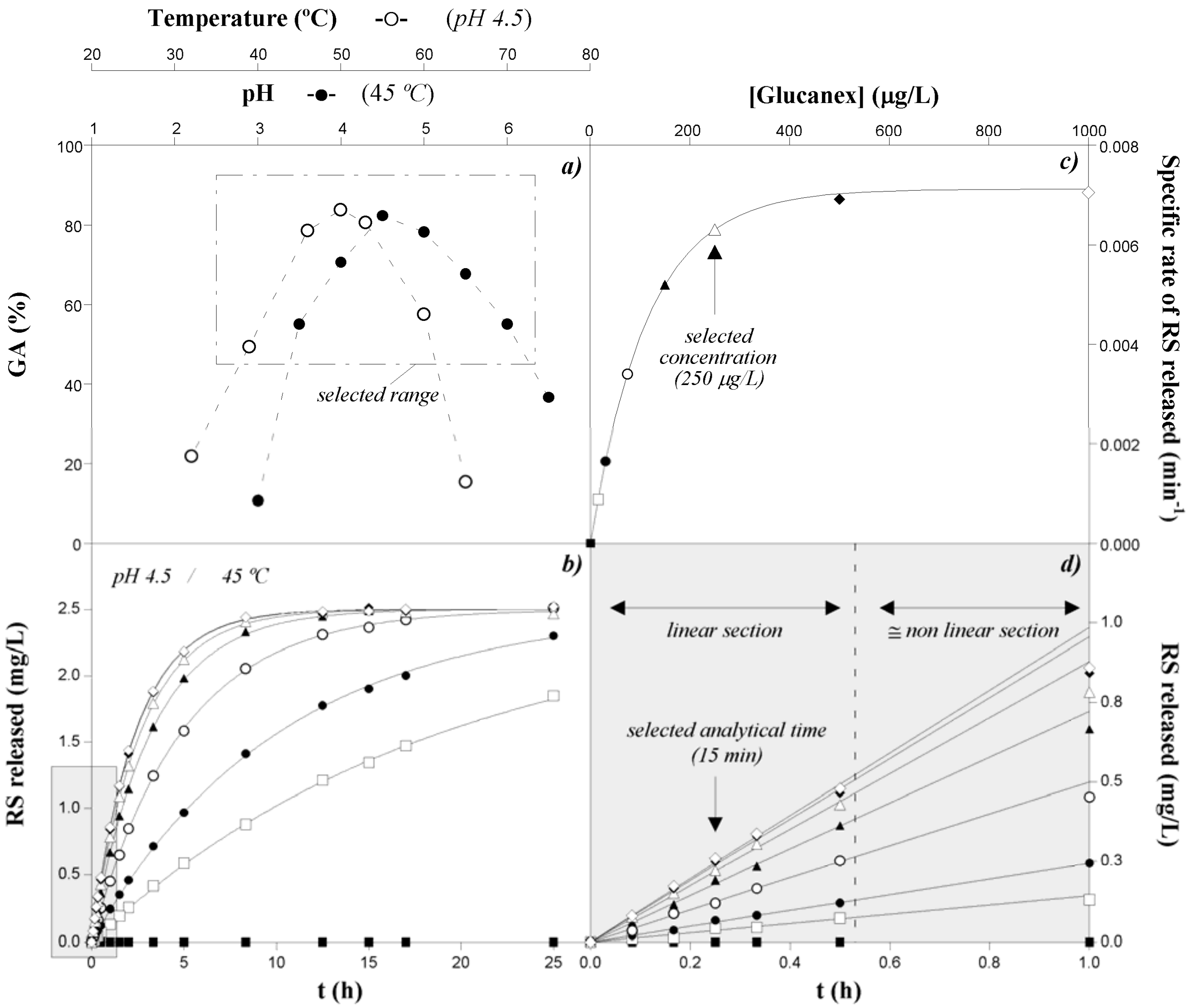
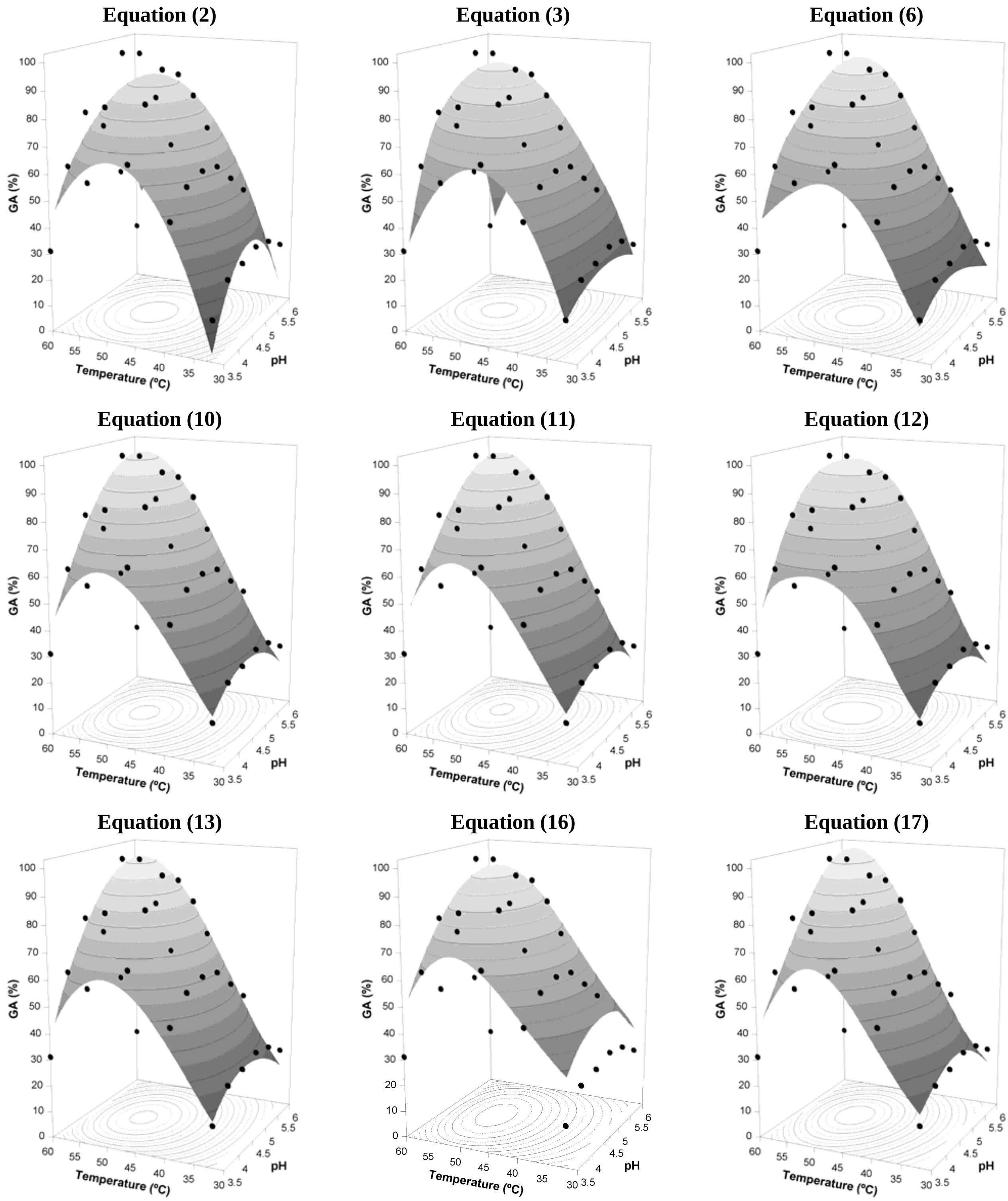


Figure 2



Equation (2)
Equation (3)
Equation (6)
Equation (10)
Equation (11)
Equation (12)
Equation (13)
Equation (16)
Equation (17)
GA (%)
0
10
20
30
40
50
60
70
80
90
100
Temperature (ºC)
60
55
50
45
40
35
30
pH
3.5
4
4.5
5
5.5
6

Figure 3

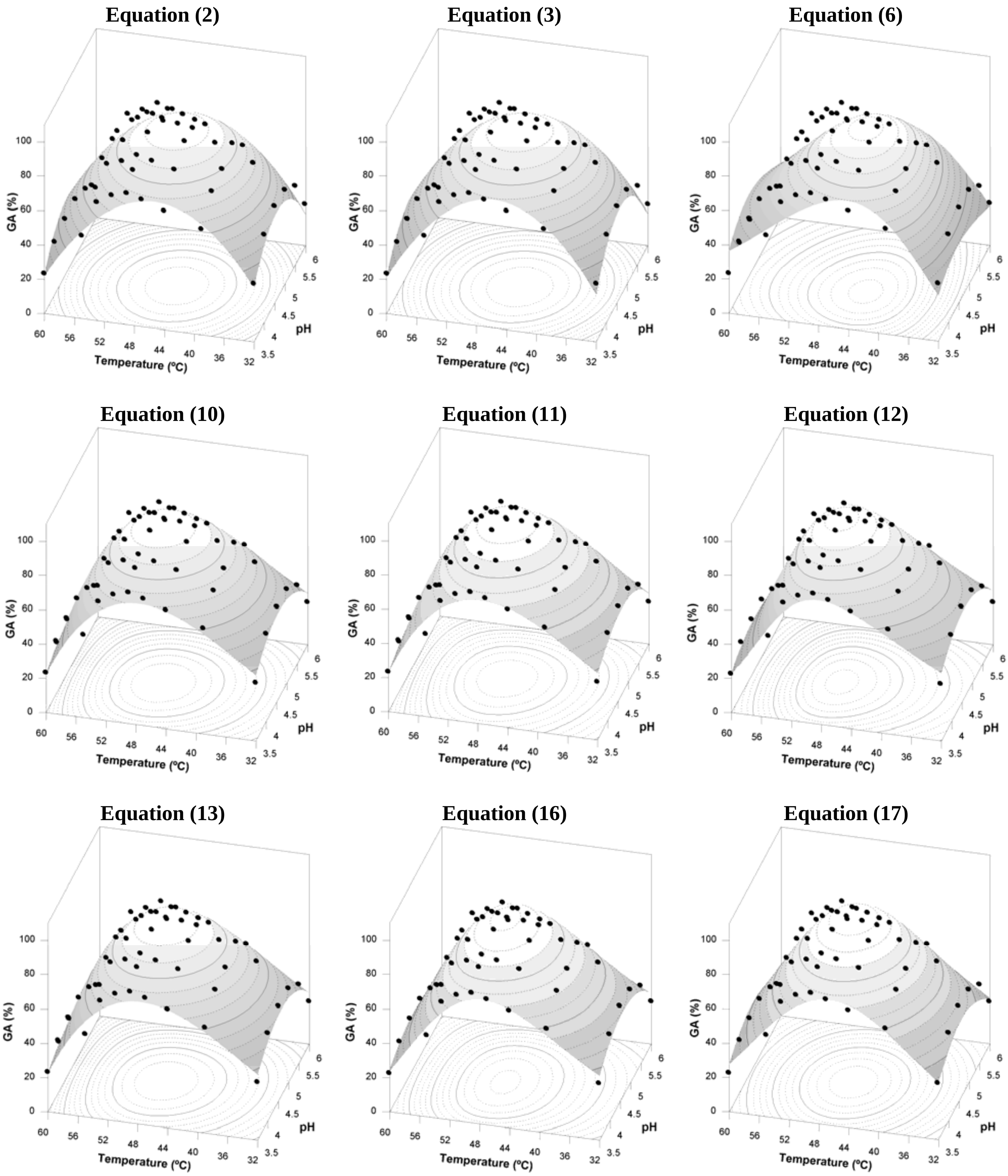
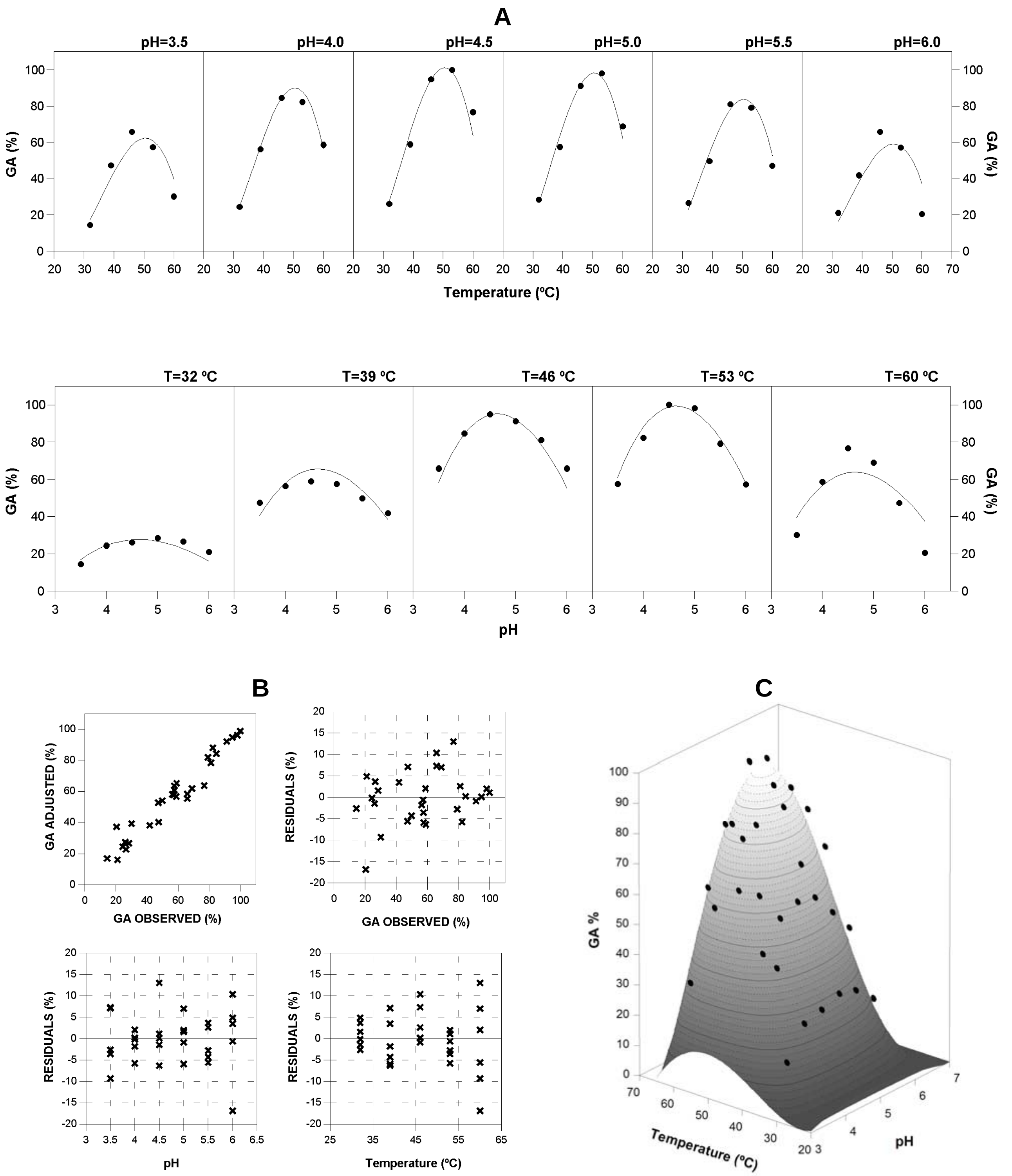
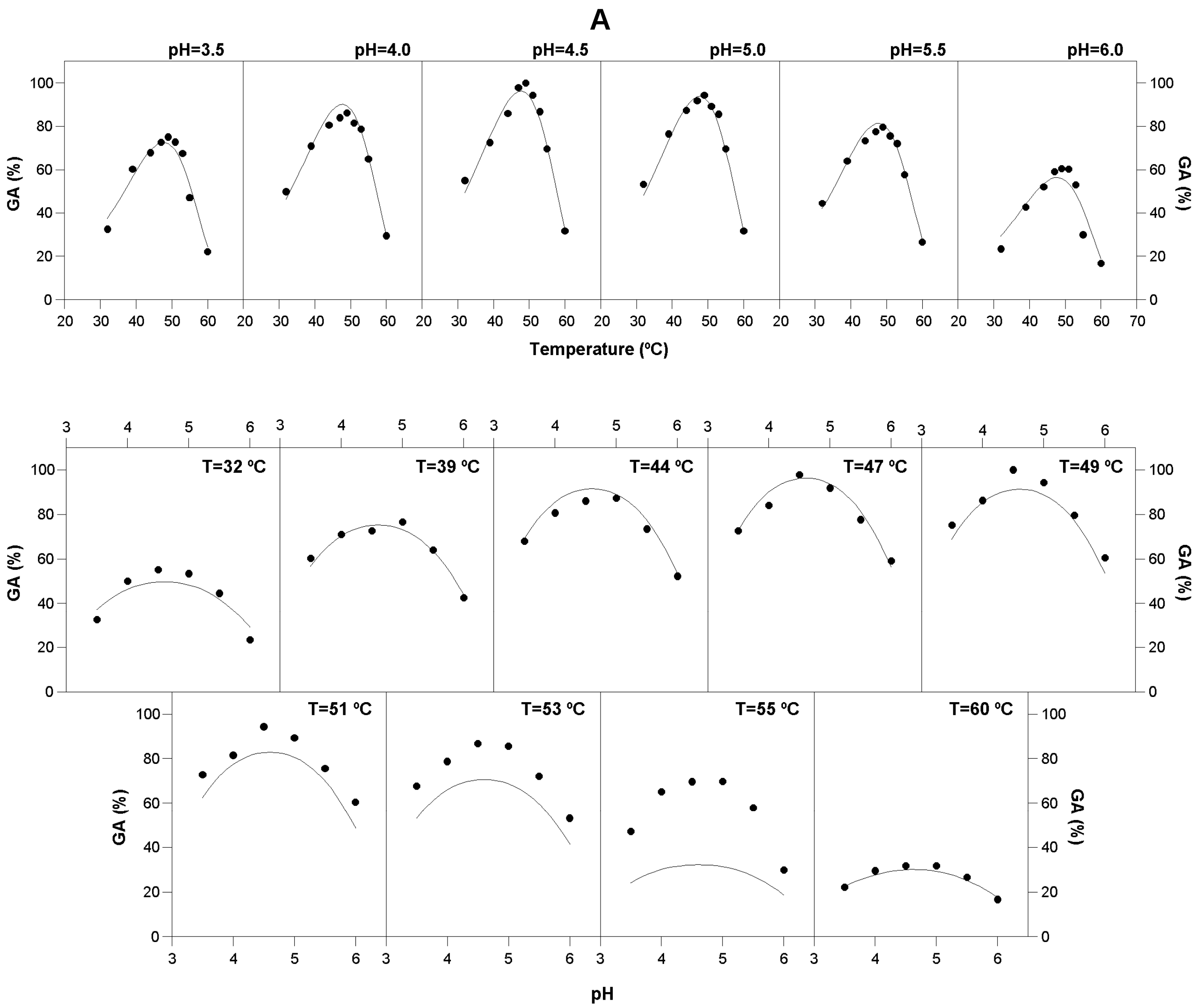

Equation (2)
Equation (3)
Equation (6)
Equation (10)
Equation (11)
Equation (12)
Equation (13)
Equation (16)
Equation (17)
GA (%)
0
20
40
60
80
100
Temperature (ºC)
60
56
52
48
44
40
36
32
pH
3.5
4
4.5
5
5.5
6

Figure 4



A
pH=3.5
pH=4.0
pH=4.5
pH=5.0
pH=5.5
pH=6.0
GA (%)
Temperature (ºC)
T=32 ºC
T=39 ºC
T=46 ºC
T=53 ºC
T=60 ºC
pH
B
GA ADJUSTED (%)
GA OBSERVED (%)
RESIDUALS (%)
Temperature (ºC)
C
GA %

Figure 5



A
pH=3.5
pH=4.0
pH=4.5
pH=5.0
pH=5.5
pH=6.0
GA (%)
Temperature (ºC)
T=32 ºC
T=39 ºC
T=44 ºC
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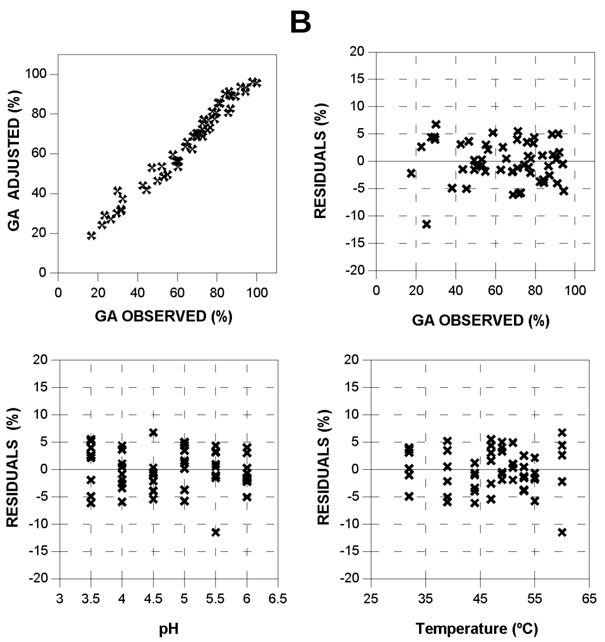
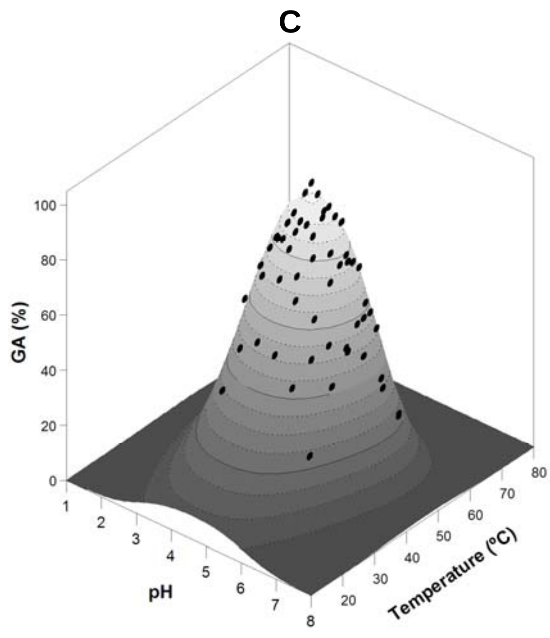
T=60 ºC
pH



B
GA ADJUSTED (%)
GA OBSERVED (%)
RESIDUALS (%)
GA OBSERVED (%)
RESIDUALS (%)
pH
RESIDUALS (%)
Temperature (ºC)



C
GA (%)
pH
Temperature (ºC)

TABLES

Table 1: Different model selection criteria (MSC) used to compare the nine models reviewed from the bibliography to predict the joint effect of pH and T. n: number of independent measurements considered in the fit. k: number of fitted parameters. RSS: residual sum of squares. ESS: explained sum of squares.

Criterion	Key	Claim	Formula
Akaike Information Criterion Corrected[23,24]	AICc	complexity (efficient)	$AIC_C = n\ln\left(\frac{RSS}{n}\right)+\left(\frac{2(k+1)}{n-k-2}\right)$
Bayesan Information Criterion[25]	BIC	complexity (consistent)	$BIC = n\ln(RSS)+\ln(n)k$
Akaike's Final Prediction Error[24]	FPE	complexity	$FPE = n\frac{RSS(n+k)}{(n-k)}$
Mallows' Cp[23,24]	Cp	goodness of fit / overfitting	$C_P = n\left[RSS/(ESS/n-1)\right]-n+2k$
Adjusted Coefficient of Determination[24]	R^2_{adj}	goodness of fit / complexity	$R^2_{adj} = \frac{(n-1)R^2-k}{n-1-k}$
Residual Information Criterion[24]	RIC	goodness of fit / overfitting	$RIC=(n-k)\ln(RSS)+k\left[\ln(n)-1\right]+\frac{4}{n-k-2}$
Model Selection Criterion[26]	MSC	goodness of fit	$MSC = \ln\left(\frac{ESS}{RSS}\right)-\frac{2k}{n}$
Leave One Out Cross-Validation[26,27,28,29]	LOO-CV	generalizability	--
Monte Carlo Cross-Validation[27,28,30]	MCCV	generalizability / overfitting	--

Table 2: Parametric estimates and confidence intervals obtained from the equations used in the evaluation of the joint effect of pH and temperature on GA with curdlan (A) and laminarin (B) as substrate. CI: confidence intervals were evaluated by t-Student test (α=0.05). NS: non significant.

A) PARAMETERS OBTAINED WITH CURDLAN AS SUBSTRATE									
Equations	b_{00}	b_{12}	b_1	b_{11}	b_2	b_{22}	b_{221}	b_{112}	--
(2)	-856±164	-0.186 (NS)	181±53	-18.37±5.22	21.42±4.10	-0.21±0.04	--	--	--
(3)	116.2 (NS)	9.01±4.42	-240±170	25.90±16.64	0.146 (NS)	-0.21±0.15	-0.0006 (NS)	-0.96±0.35	
	a	T_0	n_1	pH_0	n_2	b_3	b_4	--	--
(6)	6x10^{-6} (NS)	17.17 (NS)	6.54 (NS)	17.17 (NS)	6.31 (NS)	0.210±0.207	2.06 (NS)	--	--
	T_{max}	T_{min}	c	a_1	c_0	c_1	c_2	--	--
(10)	65.12±2.25	21.97±4.98	22.20 (NS)	0.0003 (NS)	-5.92 (NS)	3.05 (NS)	-0.32 (NS)		--
	T_{max}	T_{min}	pH_{max}	pH_{min}	c	a_1	a_2	--	--
(11)	65.11±2.45	21.98±5.42	6.52±0.36	1.26 (NS)	40.68 (NS)	0.0001 (NS)	0.083 (NS)	--	--
	T_{max}	T_{min}	pH_{max}	pH_{min}	r_m	T_{opt}	pH_{opt}	--	--
(12)	65.55±2.29	22.96±4.63	6.83±0.51	2.88±0.42	101.76±6.55	50.48±1.38	4.64±0.22	--	--
	k	α	q	β	p	--	--	--	--
(13)	8445±758	5.32±0.40	52.35±0.70	4.33±0.45	5.04±0.09	--	--	--	--
	c_0	c_1	c_2	r_r	H_r	T_a	H_a	T_b	H_b
(16)	-5.71 (NS)	2.950 (NS)	-0.313 (NS)	1.189 (NS)	-17.37 (NS)	334.0±46.6	687.7 (NS)	311.9±41	-177 (NS)
	E_s	E_d	pH_{max}**	pH_{min}**	K_{EH}	K_{EOH}	pH_{opt}**	k_{sr}	k_{dr}
(17)	65.5±42.6	104.28±92.59	7,96**	3,38**	0.0004±0.0002	1.1x10-8±10^{-10}	4,70**	7.5x10^{10} (NS)	3.83x10^{15}(NS)
B) PARAMETERS OBTAINED WITH LAMINARIN AS SUBSTRATE									
Equations	b_{00}	b_{12}	b_1	b_{11}	b_2	b_{22}	b_{221}	b_{112}	--
(2)	-747.8±94.4	0.032 (NS)	153.1±28.2	-16.77±2.69	21.58±2.42	-0.24±0.02	--	--	--
(3)	-1029±447	-2.59 (NS)	245.3±164.4	-23.5±16.3	30.72±14.11	-0.31±0.13	0.01 (NS)	0.14 (NS)	
	a	T_0	n_1	pH_0	n_2	b_3	b_4	--	--
(6)	0.0001 (NS)	15.22 (NS)	6.26 (NS)	1.13 (NS)	6.08 (NS)	0.21 (NS)	1.82 (NS)	--	--
	T_{max}	T_{min}	c	a_1	c_0	c_1	c_2	--	--
(10)	62.17±0.52	12.50±5.51	0.38 (NS)	0.03 (NS)	-2.29 (NS)	1.28 (NS)	-0.14 (NS)	--	--
	T_{max}	T_{min}	pH_{max}	pH_{min}	c	a_1	a_2	--	--
(11)	62.11±0.52	12.51±5.60	6.60±0.18	0.75 (NS)	0.026 (NS)	0.047 (NS)	0.18 (NS)	--	--
	T_{max}	T_{min}	pH_{max}	pH_{min}	r_m	T_{opt}	pH_{opt}	--	--
(12)	62.18±0.54	13.10±4.58	6.69±0.22	2.26±0.45	94.82±2.44	47.58±0.70	4.64±0.11	--	--
	k	α	q	β	p	--	--	--	--
(13)	9019±280	4.87±0.24	48.81±0.42	3.83±0.26	5.00±0.06	--	--	--	--
	c_0	c_1	c_2	r_r	H_r	T_a	H_a	T_b	H_b
(16)	-318.6 (NS)	178.8 (NS)	-19.43 (NS)	1.14 (NS)	-52.33 (NS)	327.4±25.6	226.4 (NS)	329.2±27.6	-98.35±46
	E_s	E_d	pH_{max}**	pH_{min}**	K_{EH}	K_{EOH}	pH_{opt}**	k_{sr}	k_{dr}
(17)	59.09±14.3	109.1±24.9	7,91**	3,11**	8x10^{-4}±2x10^{-4}	1x10^{-8}±2x10^{-9}	4,60**	7.8x10^{9} (NS)	3.1x10^{16} (NS)

** Further interesting values calculated from the parameters of equation (17).

Table 3: Model ranking (Rk) obtained for each MSC and the final ranking (Rk_F) based on the total ranking average (∑Rk) for the two substrates.

CRITERIA	AICc		BIC		RIC		Cp		R^2_{adj}		FPE		MSC		LOO-CV (MEP)		MCCV (MEP)		AVERAGE	
Equations	**Value**	**Rk**	**Value**	**Rk**	**Value**	**Rk**	**Value**	**Rk**	**Value**	**Rk**	**Value**	**Rk**	**Value**	**Rk**	**Value**	**Rk**	**Value**	**Rk**	**∑Rk**	**Rk_F**
A) MODEL RANK USING CURDLAN AS SUBSTRATE																				
(2)	123.30	(9)	245.11	(8)	194.35	(9)	65.1	(9)	0.8798	(9)	80551.8	(9)	1.95	(9)	114.24	(9)	124.57	(6)	77	(9)
(3)	96.70	(1)	225.04	(1)	164.49	(1)	19.9	(1)	0.9448	(1)	37878.6	(1)	2.71	(1)	37.87	(2)	47.35	(3)	12	(1)
(6)	115.63	(8)	240.72	(7)	183.30	(6)	48.1	(8)	0.9005	(8)	66629.4	(7)	2.14	(7)	43.06	(3)	43.02	(2)	56	(7)
(10)	113.96	(6)	239.04	(6)	182.01	(5)	44.6	(5)	0.9213	(4)	63014.2	(5)	2.20	(5)	58.94	(7)	164.41	(8)	51	(6)
(11)	108.80	(4)	233.88	(4)	178.05	(4)	35.1	(4)	0.9074	(7)	53050.8	(4)	2.37	(4)	54.07	(5)	135.91	(7)	43	(4)
(12)	107.76	(2)	232.84	(3)	177.26	(3)	33.3	(3)	0.9241	(3)	51243.9	(3)	2.40	(3)	36.43	(1)	40.69	(1)	22	(2)
(13)	108.31	(3)	226.83	(2)	187.03	(8)	30.6	(2)	0.9288	(2)	45786.7	(2)	2.51	(2)	56.99	(6)	60.87	(5)	32	(3)
(16)	113.85	(5)	245.44	(9)	172.20	(2)	47.8	(7)	0.9122	(6)	71771.1	(8)	2.08	(8)	95.81	(8)	234.96	(9)	62	(8)
(17)	114.89	(7)	238.70	(5)	186.22	(7)	47.1	(6)	0.9160	(5)	65061.9	(6)	2.16	(6)	53.87	(4)	54.87	(4)	50	(5)
B) MODEL RANK USING LAMINARIN AS SUBSTRATE																				
(2)	189.74	(8)	428.77	(7)	377.88	(8)	163.4	(8)	0.9191	(7)	121683.6	(7)	2.41	(7)	41.42	(7)	42.61	(7)	66	(7)
(3)	187.79	(7)	434.70	(8)	367.12	(6)	159.7	(7)	0.9186	(8)	126311.3	(8)	2.38	(8)	47.25	(8)	50.27	(8)	68	(8)
(6)	237.01	(9)	479.99	(9)	414.48	(9)	452.3	(9)	0.8018	(9)	302935.9	(9)	1.50	(9)	74.33	(9)	74.05	(9)	81	(9)
(10)	152.21	(5)	395.18	(5)	340.66	(5)	62.4	(5)	0.9610	(4)	62992.5	(5)	3.07	(5)	19.86	(3)	16.78	(3)	40	(5)
(11)	150.23	(4)	393.20	(4)	338.94	(4)	58.7	(4)	0.9603	(5)	60727.3	(4)	3.11	(4)	20.24	(4)	21.02	(4)	37	(4)
(12)	148.64	(3)	391.61	(3)	337.55	(3)	55.8	(3)	0.9614	(3)	58962.6	(3)	3.14	(3)	20.45	(5)	21.56	(5)	31	(3)
(13)	176.93	(6)	412.03	(6)	370.81	(7)	118.1	(6)	0.9374	(6)	92548.7	(6)	2.69	(6)	32.57	(6)	33.39	(6)	55	(6)
(16)	129.43	(1)	380.27	(1)	313.96	(1)	31.0	(1)	0.9719	(1)	44470.9	(1)	3.42	(1)	13.80	(2)	11.84	(1)	10	(1)
(17)	141.31	(2)	380.35	(2)	334.83	(2)	41.8	(2)	0.9670	(2)	49632.8	(2)	3.31	(2)	13.62	(1)	13.50	(2)	17	(2)