# ARTIFICIAL NEURAL NETWORKS TO MODEL THE PRODUCTION OF BLOOD PROTEIN HYDROLYSATES FOR PLANT FERTILIZATION

**Modeling the hydrolysis of blood protein by artificial neural network**

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## ABSTRACT

BACKGROUND. Amino acid-based fertilisers increase the bioavailability of nitrogen in plants and help withstand stress conditions. Besides, porcine blood protein hydrolysates are able to supply iron, which is involved in chlorophyll synthesis and improves the availability of nutrients in soil. A high degree of hydrolysis (DH) is desirable when producing a protein hydrolysate intended for fertilization, since it assures a high supply of free amino acids. Given the complexity of enzyme reactions, empirical approaches such as artificial neural networks (ANN) are preferred for modelization.

RESULTS. Porcine blood meal was hydrolysed for 3 hours with subtilisin. The time evolution of the degree of hydrolysis was successfully modelled by means of a feedforward ANN comprising 10 neurons in the hidden layer and trained by the Levenberg-Marquardt algorithm. The ANN model described adequately the influence of pH, temperature, enzyme concentration and reaction time upon DH, and was employed

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to estimate the optimal operation conditions (pH 6.67, 56.9ºC, enzyme to substrate ratio of 10 g $kg^{-1}$ and 3 h of reaction) leading to the maximum DH (35.12%).

CONCLUSIONS. ANN modeling was a useful tool to model enzymatic reactions and was successfully employed to optimise the degree of hydrolysis.

**Keywords:** Blood protein; fertilization; enzymatic hydrolysis; degree of hydrolysis; artificial neural networks; modelization

## INTRODUCTION

Protein concentrates are a source to provide nitrogen for fertilization. The enzymatic hydrolysis of these materials leads to a final product with enhanced functional properties (solubility, root and leaf absorption), able to supply small peptides and free amino acids to the plant[1]. It is proved that plants need to increase their content of free amino acids to overcome some adverse situations such as hydric stress, salinity, pests, heat-strokes, or burns caused by herbicides or other agents. This is done at the expense of decreasing the anabolism of the structural proteins, which is prejudicial to the plant growth. Therefore, the supplementation of amino acid- based biostimulants enhances the photosynthesis process and helps to withstand stress conditions[2,3]. Compared to traditional fertilizers, amino acid enriched fertilizers avoid the chemical transformation of nitric and ammonium nitrogen into amino acids inside the plants, as it provides them directly.

Among these protein sources, blood meal provides a high protein content (above 85%) as well as supplementation of hemic iron [4–7]. Iron is essential in plant physiology for chlorophyll synthesis and certain enzyme functions. Besides, the presence of iron in soil improves the stability and availability of nutrients owing to its chelating effect [8]. Chan et al. [9] pointed out the poor solubilization of blood meal as the main constraint for its use as liquid fertiliser, and proposed a microwave-aided digestion of the dried powder with hydrogen peroxide. This method enhanced nitrogen solutibilization at the expense of protein denaturation and the presence of toxic substances in the final product. This could be alleviated by the enzymatic hydrolysis of blood meal. In a previous work, we

reported that enzymatic hydrolysis of blood meal with subtilisin favoured protein solubilization [6] and increased the content of free amino acids in the final hydrolysate.

Enzymatic hydrolysis presents a number of advantages compared to chemical catalysis. Chemical hydrolysis produces toxic substances (salts) which should be removed prior to fertilization. Besides, amino acids released from chemical hydrolysis are present under the D and L isomers, whereas enzymatic hydrolysis only leads to L-amino acids. The latter are the only form recognised by the enzymatic locus and therefore can participate in the metabolic routes in the plant[10].

The degree of hydrolysis is a key parameter when producing a protein hydrolysate intended for fertilization. High degrees of hydrolysis ensure adequate levels of free amino acids and small peptides, as well as favouring the solubilization of the final product. Phenomenological approaches to modeling the hydrolysis of blood protein are normally based on simple Michaelis-Menten mechanisms[1,11,12]. Given the complexity of an enzymatic reaction, influenced by a range of processes such as enzyme thermal deactivation or substrate inhibition, empirical methods based on direct analysis of experimental data are preferred[13,14]. These methods employ response surface methodology and artificial neuronal networks as main tools to generate predictive models. The former technique has been widely employed for the modelization of biochemical systems since it requires a small number of experimental data. In the field of protein hydrolysis, it has been successfully used to estimate kinetic constants[13,15] or to optimise operation parameters for a given purpose [6]. The main limitation of response surface methodology lies in the fact that many biochemical processes (e.g. substrate activation, thermal enzyme deactivation) cannot be modeled adequately by a second

order polynomial[13]. To this regard, artificial neural networks are a potent alternative providing better estimating and predicting capabilities[14,16].

An artificial neural network (ANN) is composed of a large number of interconnected processing elements (neurons) working in parallel to solve specific problems. Basically, the neural network receives the input variables which are previously transformed into a single sign by means of weight coefficients. The weighted function is then fed to the processing element (neuron), where it is converted into an output by means of an activation function. Finally, output values are usually arranged inside a given range (e.g. from -1 to 1) by means of a transfer function. In contrast with traditional computational methods, neural networks do not follow sequential rules or algorithms, but learn and adapt their responses by examples. This is achieved by training algorithms, which continuously update weight coefficients until obtaining an actual value of the output variable close to the target [17]. In the field of enzymatic hydrolysis, ANNs have been successfully employed to predict reaction rates in the absence of phenomenological models [14,16,18,19], as well as to estimate kinetic constants when a reaction mechanism was available [20,21].

The purpose of this paper was to test that ANN models were able to adequately predict the time evolution of the degree of hydrolysis of blood meal with subtilisin at different initial conditions of pH, temperature and enzyme-substrate ratio. To this end, a set of neural networks will be generated by employing three training algorithms (Levenberg-Marquardt, resilient and gradient descent with momentum) and a variable number of neurons in the hidden layer (from 0 to 10). Besides modelling the reaction kinetics, the predictive ANN could be subsequently been employed to obtain the optimal conditions

leading to maximum degree of hydrolysis, which is desirable to provide a high content of free amino acids in the final product.

## EXPERIMENTAL

### Raw material

Porcine blood meal was purchased from APC Europe (Barcelona, Spain). It contained 890 ± 10 g $kg^{-1}$ protein, with less than 100 ± 9 g $kg^{-1}$ moisture. The amino acid composition of the blood meal reveals an important proportion in glutamic acid, phenylalanine, leucine and lysine, all of them around 100 ± 15 g $kg^{-1}$.

### Hidrolysis procedure.

The enzyme employed was Subtilisin (EC 3.4.21.62), purchased from Novozymes (Bagsvaerd, Denmark). This enzyme hydrolyses peptide bonds in a long extent and wide range of specifity. It is stable within a wide range of pH between 5.0 and 11.5, with maximum activity at temperatures between 50 and 60ºC.

Each hydrolysis experiment employed 10 g $L^{-1}$ of substrate. The reaction was carried out for 3 h in a jacketed reactor of 200 mL. pH was controlled throughout the hydrolysis by means of an automatic titrator (718 Stat Titrino, Metrohm, Herisau, Switzerland), equipped with temperature and pH probes. The degree of hydrolysis (DH), defined as the fraction of peptide bonds cleaved during the reaction, was calculated from the amount of base needed to keep pH constant, according to Eq. [1] [1]:

$$DH = \frac{V \cdot N}{m_P \cdot \alpha \cdot h_{tot}} \qquad [1]$$

where V and N are the volume(mL) and normality (eq $L^{-1}$) of titration agent (NaOH 0.5 N) consumed during the reaction, $m_P$ is the mass of protein (g) fed to the reactor, α is the degree of dissociation of the amino group for a given operation temperature and $h_{tot}$ is the average amount of peptide bonds present in blood protein, which was assumed to be 8.62 meq $g^{-1}$ of protein [1]. After three hours, the reaction mixture was heated at 100ºC during 15 min in order to stop enzymatic reaction.

## Experimental design

Three operation parameters, pH, temperature and enzyme-substrate ratio were varied according to a 4 x 3 x 3 factorial design [22]. The levels assayed were 6.0, 6.5, 7.0 and 7.5 for the pH and 50, 55 and 60 ºC for the reaction temperature. Enzyme-substrate ratio was defined as the quotient between the mass of enzyme and the amount of protein fed into the reactor; it was assayed at three levels, 50, 75, and 100 g $kg^{-1}$ of substrate. For each of the 36 hydrolysis experiments, the automatic titrator monitored base consumption (and therefore, the degree of hydrolysis) throughout 3 hours of reaction.

## Artificial neural network

A feedforward artificial neural network (ANN) was implemented by means of Matlab 7.0 Neural Network Toolbox. The arquitecture of the artificial neural network assayed consisted in an input layer, a hidden layer and an output layer, as shown in Fig. 1. Four input variables ($X_i$), temperature, enzyme-substrate ratio, pH and time, were fed into an input layer with i = 4 neurons, where they were processed into weighted functions ($s_k$) as follows:

$$s_k = \sum_{i=1}^{4} w_{ki} \cdot X_i + b_k \qquad [2]$$

where $w_{ik}$ are the weight factors and $b_k$ is the bias for the neuron k.

These signals were then received by the hidden layer, which comprised a variable number of neurons between 1 and 10. Each neuron applied a transfer function Φ(s) to the weighted signal $s_k$, given by the equation 3:

$$\Phi(s_k) = \frac{1}{1+e^{-s_k}} \qquad [3]$$

This function returns a value ranging between 0 and 1, and is easily differentiable. Finally, the output lawyer consists in one neuron which receives a single weighted signal from the hidden layer. This input is then processed by means of the linear transfer function (purelin command), which returns the same value received from the hidden layer.

In order to train the neural network, the input data were divided into three subsets. The first subset, which comprised 70% of the experimental data, was employed to fit the output responses to the experimental data. This was achieved by testing three different training algorithms, Levenberg-Marquardt (lm), resilient backpropagation (rp) and gradient descent with momentum (gdm), implemented in the software Matlab 7.0. These algorithms update weight and bias values systematically so the mean squared error (MSE) is minimum, defined by the equation 4:

$$MSE = \frac{\sum_{i=1}^{N}\left(e_i - p_i\right)^2}{N} \qquad [4]$$

A total number of 30 runs was performed for a variable number of neurons (1-10) in the hidden layer and three training algorithm (lm, rp and gdm), with a number of iterations per run limited to 10000. In backpropagation methods, overfitting occurs when a better fit of the training data is achieved at the expense of larger generalization errors. In order

to avoid overfitting of the training dataset, a validation subset containing 15% of the experimental data was processed. The error obtained from this subset was monitored during the training process and used as criterion for early stopping. Indeed, when this value increased for 10 iterations, the training process was stopped and the algorithm returned the weights and biases corresponding to the minimal validation error. Finally, the remaining data (15%) were processed to compute the test error. This error is used to evaluate the predictability of the neural network model, since it is computed independently of the training process. Furthermore, it provides information on the quality of the division of the data set.

## RESULTS AND DISCUSSION

### Selection of the training algorithm.

For a given number of neurons in the hidden layer, the selection of the training algorithm was done according to the average value of mean square error (MSE) over 30 trials. Each trial started with different combinations of weight and biases, and stopped when the validation error increased for 10 iterations. Average training, validation and test errors were in all cases very similar, presenting differences below 2%. Therefore, test error was chosen to assess the predictability of each backpropagation algorithm, since it is computed independently of the training process.

Fig. 2 plots the average test error against the number of neurons in the hidden layer for the three training algorithms studied. It can be observed that the Levenberg-Marquardt algorithm (lm) showed the best performance, followed by resilient backpropagation (rp). Although the resilient backpropagation algorithm presented the lowest test error at 1 neuron in the hidden layer (0.0160), it was surpassed by the Levenberg-Marquardt

algorithm at 3 neurons. This latter reduced continuously the value of test error from 0.0297 at one neuron to 0.0013 at ten neurons. Contrarily, the test error for the gradient descent with momentum (gdm) started at 0.0512 for one neuron and then showed large fluctuations with the number of neurons, presenting a test error of 0.1914 at 10 neurons in the hidden layer.

The predictability of the artificial network was also evaluated by the coefficient of determination r-squared between calculated (i.e. outputs of the ANN) against experimental data. The goodness of the linear fit is assessed by this coefficient, which ideally would take the values of 1. Figure 3 plots the average values (over 30 trials) of r-squared against the number of neurons in the hidden layer for the three training algorithms. As expected, the highest values of r-squared were achieved by the Levenberg-Marquardt algorithm (lm) followed by the resilient backpropagation (rp). In both cases r-squared increased rapidly up to 2 neurons and then continued at lower rate, attaining a maximum value of 0.9964 (lm) and 0.9870 (rp) at 10 neurons. In contrast, the values of the coefficients of determination for the gradient descend algorithm fluctuated with the number of neurons, ranging from the maximum at 4 neurons (0.8462) to the minimum at 10 neurons (0.1791), far from the values of r-squared obtained with the other two algorithms.

It can be concluded that the Levenberg-Marquardt training was the most appropriate for modeling the kinetics of the hydrolysis, since it improved significantly the values of MSE and r-squared with increasing number of neurons in the hidden layer, reaching the lowest values of MSE (and therefore highest r-squared) at 10 neurons in the hidden layer. This algorithm computes numerically the Jacobian matrix of the system, which permits to update weight and bias iteratively until minimising the error function (in our

case MSE). Similarly, the Levenberg-Marquardt training algorithm was chosen by Nikzad et al.[23]to model the enzymatic hydrolysis of cellulose with an ANN comprising 7 neurons in the hidden layer. This model was found to be more accurate than two phenomenological models chosen from literature. Other authors such as Buciński et al.[24] or Abakarov et al.[21] employed satisfactorily conjugate gradient descent algorithms to model enzymatic hydrolysis.

## ANN model of the degree of hydrolysis

According to the values of test MSE and regression slope, the ANN architecture which best reproduced the experimental data comprised ten neurons in the hidden layer. As shown in figures 2 and 3, both the values of test error and r-squared hardly varied from 9 to 10 neurons. Increasing the number of neurons in the hidden layer above 10 would not improve significantly the test error of the model, at the expense of larger computation times.

The normalised output from such neural network was given by Eq. [5]:

$$Y = \sum_{k=1}^{10} \omega_k \cdot \Phi\left( \sum_{i=1}^{4} w_{ki} \cdot X_i + b_k \right) + \beta$$

where X (T, eS, pH, t) denotes the vector of input variables; $w_{ki}$ and $b_k$ are the weight factors and bias of the input layer, respectively; ω and β are the weights and bias of the hidden layer and Φ is the transfer function of the hidden layer. Table 1 summarises the parameters (weights and bias) for the ANN model with ten neurons in the hidden layer. This values were taken for the trial presenting the highest value of coefficient of determination $r^2$= 0.9978.

Figures 4a, 4b and 4c illustrate the influence of different levels of enzyme-substrate ratio, temperature and pH on the hydrolysis curves, respectively. Experimental data are

represented as point markers, while solid lines represent the predicted DH obtained by application of Eq. [5] with the parameters listed in Table 1. It can be observed that the ANN model assayed fits experimental data adequately, which is confirmed by the high value of the coefficient of determination.

The time evolution of the degree of hydrolysis followed the general pattern described for enzymatic reactions [25,26]. Indeed, the hydrolysis curves presented a high reaction rate at the beginning and then decreased progressively throughout the reaction. This is attributable to the exhaustion of available peptide bonds, together with other phenomena which modulate the proteolytic activity such as thermal enzyme inactivation or product inhibition [26].

Fig. 4a shows the influence of the enzyme-substrate ratio on the degree of hydrolysis at 55ºC and pH 6.5. It can be observed that increasing concentrations of enzyme improved the reaction rate, since the amounts of enzyme assayed were far below the saturation of the peptide bonds. Fig. 4b shows the influence of temperature on the hydrolysis curves, setting enzyme-substrate ratio and pH at 7.5% and 6.5, respectively. The kinetics of the reaction was favoured at T=55ºC, attaining the maximum DH (26.77%) at 180 min of hydrolysis. On the contrary, the curve at 60ºC presented similar initial rate than that of 55ºC but soon flattened, indicating loss of enzyme activity due to thermal deactivation. Indeed, second order thermal deactivation has been reported for the hydrolysis of hemoglobin with subtilisin [27]. As for pH, Fig. 4c plots the curves hydrolysis at 55ºC and enzyme-substrate ratio of 75 g $kg^{-1}$ at the levels of pH assayed. Overall, the degree of hydrolysis was affected positively by the decrease in pH, with an optimum at pH=6.5. The behavior of experimental DH with pH was successfully reproduced by the ANN model. The highest deviations between experimental and calculated values were

recorded at the initial times of reaction, especially for the curve at pH 7. The influence of pH on the degree of hydrolysis is the consequence of the trade-off between the denaturation of the substrate structure and the proteolytic activity of the subtilisin. As the pH becomes acid, the denaturation of the quaternary structure of hemoglobin (major protein present in blood meal) increases the exposure of peptide bonds to enzyme attack, resulting in higher degrees of hydrolysis[12]. Contrarily, the proteolytic activity of subtilisin is favoured by alkaline conditions, with an optimal pH between 7 and 8[28]. Despite the high accessibility of peptide bonds at pH 6, the enzyme activity under these conditions was far from its optimal range.

## Optimization of the degree of hydrolysis

The ANN model expressed by Eq. 5 was used to generate contour maps, where the degree of hydrolysis was plotted against a combination of two input variables. In our case, Fig. 5 plots the DH as a function of the pH and the temperature, while the enzyme-substrate ratio and the reaction time were set at 100 g $kg^{-1}$ and 3 h, respectively. This contour map allows obtaining the optimal reaction conditions for maximum degree of hydrolysis under the experimental conditions of temperature, enzyme-substrate and pH tested. As stated above, it is desirable that DH be maximal, since it ensures a high supply of free amino acids for fertilization.

The contour plot confirms the influence of pH and reaction temperature upon DH explained above. Effectively, the optimum value of DH (35.11%) was attained at 56.9ºC and pH=6.67. Under acidic conditions and levels of temperature below 55ºC, the degree of hydrolysis increased continuously with decreasing pH, attaining a local maximum (DH=31.82%) at pH=6 and 51.43ºC. Contrarily, DH decreased with increasing reaction temperatures and alkalinity, reaching a minimal DH of 15.15% at 58ºC and the upper

bound of pH. Finally, the contour shows a local maximum of DH (29.62%) at neutral pH (7.22) and T=53.03ºC. These conditions favour the solubilization of the blood protein[29], while the activity of subtilisin is optimal.

The application of ANN can offer a complete vision of the phenomena involved in the hydrolysis and solubilization of blood hydrolysis. In a previous work[6], the degree of hydrolysis was modelled to a second order polynomial by Response Surface Methodology. In this case, the fit of the experimental data to the model was poorer, with a coefficient of determination $r^2$=0.9410. Finally, the quadratic model only allowed an optimum value (DH = 28.90 % at pH=6.25 and T= 54.3ºC) while the ANN model explored the DH function in more detail, detecting several experimental conditions (pH, T) where the degree of hydrolysis was maximum. These conditions ensure an average content of free amino acids in the final hydrolysate between 0.37 – 0.39 g· $kg^{-1}$ of liquid fertilizer, mainly cysteine, tyrosine and lysine. This final hydrolysate may be concentrated in free amino acids prior to its application as a plant fertilizer.

## CONCLUSIONS

ANN modelling was successfully used to model the enzymatic hydrolysis of blood meal. This approach was preferred to traditional phenomenological models given the complexity of the reaction, where several phenomena (e.g. partial substrate solubilization, enzyme adsorption, enzyme deactivation) are involved simultaneously.

To this end, the degree of hydrolysis was monitored for 3 h, under different conditions of enzyme-substrate ratio (50, 75 and 100 g $kg^{-1}$ of substrate), temperature (50ºC, 55ºC, 60ºC) and pH (6, 6.5,7, 7.5), arranged according to a factorial design. These experimental data were related to the degree of hydrolysis by an ANN comprising an

input layer, a hidden layer with 10 neurons and an output layer. The Levenberg-Marquardt algorithm was successfully employed as training procedure, so the ANN model presented a mean square error of 0.0013 and a coefficient of determination of 0.9978. This model was optimised with the aim to obtaining the operation conditions which maximised the degree of hydrolysis. The proposed ANN model allowed the optimisation of the operation parameters (i.e. pH, temperature, enzyme-substrate ratio) leading to a maximum DH. The optimum value of DH (35.12%) was attained at pH 6.67, 56.9ºC, an enzyme-substrate ratio of 100 g $kg^{-1}$ and 3 h of hydrolysis.

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**Table 1. Parameters of the ANN model with k = 10 neurons in the hidden layer**

| k | Input layer | | | | | Hidden layer | |
|---|---|---|---|---|---|---|---|
| | $w_{k1}$ | $w_{k2}$ | $w_{k3}$ | $w_{k4}$ | $b_k$ | $\omega_k$ | $\beta$ |
| 1 | 0.1625 | 0.0524 | 0.4222 | 1.4041 | -15.8176 | 2.4717 | 33.1885 |
| 2 | -0.2446 | 0.0237 | -0.2986 | 1.1825 | -18.6174 | 2.2845 | |
| 3 | -1.1320 | -0.0625 | 0.8887 | 0.1085 | 3.1686 | -0.1431 | |
| 4 | -31.4637 | -4.9266 | 13.6690 | -1.5070 | -0.3356 | 15.9032 | |
| 5 | -2.3641 | 0.7590 | -2.4768 | 0.2393 | 14.6346 | -0.6395 | |
| 6 | -2.6101 | 0.7837 | -2.6973 | 0.2695 | -13.7942 | -0.7995 | |
| 7 | -4.7021 | -0.7437 | 5.8165 | -0.3558 | -1.2170 | 1.5031 | |
| 8 | 0.0325 | -0.0019 | -0.0651 | -1.0669 | -80.8024 | -2.9621 | |
| 9 | -13.1571 | -6.2745 | -10.6569 | 0.5048 | -0.3718 | -16.4398 | |
| 10 | 5.3910 | 0.4309 | -7.0554 | 0.3408 | 1.3076 | 8.2216 | |

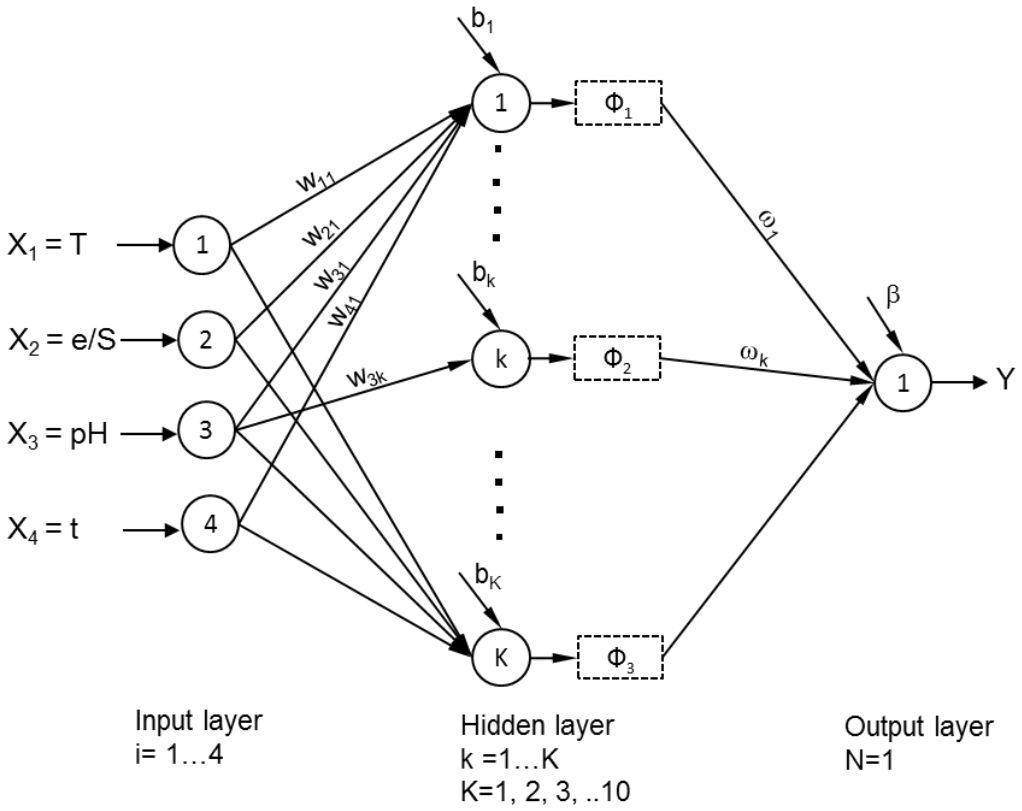


**Figure 1**. Representation of the architecture and model parameters of the artificial neural network.

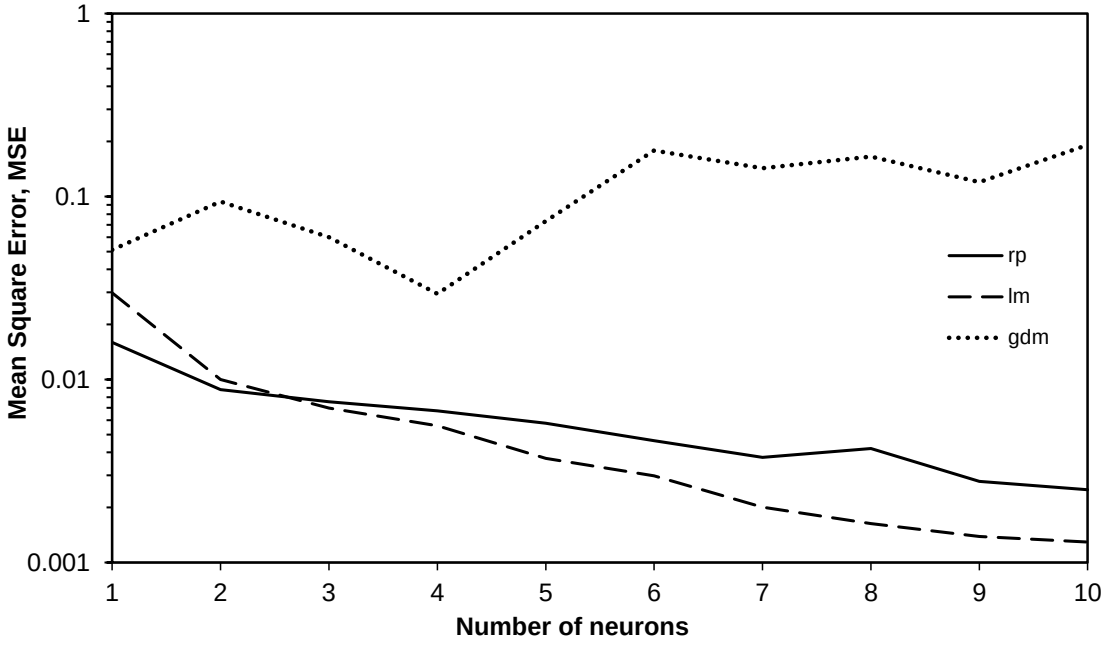


**Figure 2**. Mean Square Error of the test subset against the number of neurons in the hidden layer for the three training algorithms assayed.

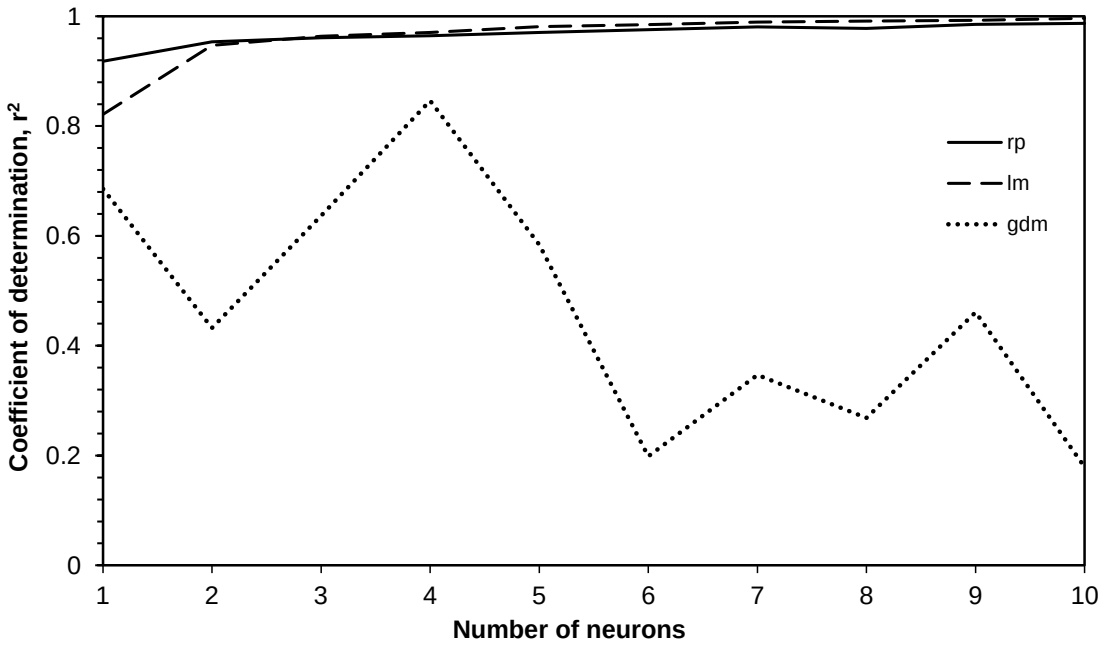


**Figure 3**. Coefficient of determination $r^2$ against the number of neurons in the hidden layer for the three training algorithms assayed.

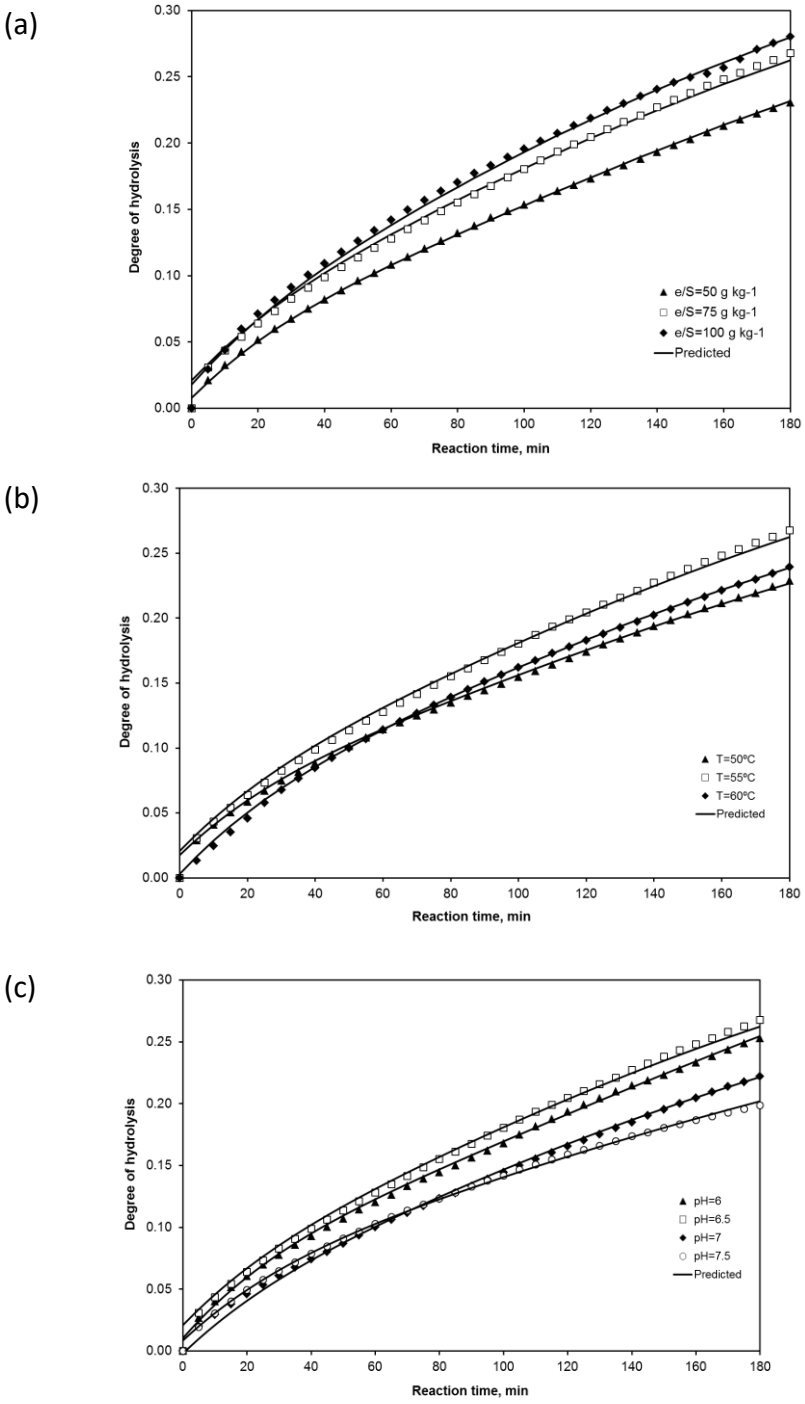


**Fig. 4.** Hydrolisis curves for blood protein with subtilisin. (a) Influence of enzyme-substrate ratio at pH=6.5 and T=55ºC, (b) influence of temperature at pH =6.5 and e/S = 7.5% and (c) influence of pH at T=55ºC and e/S=7.5%.

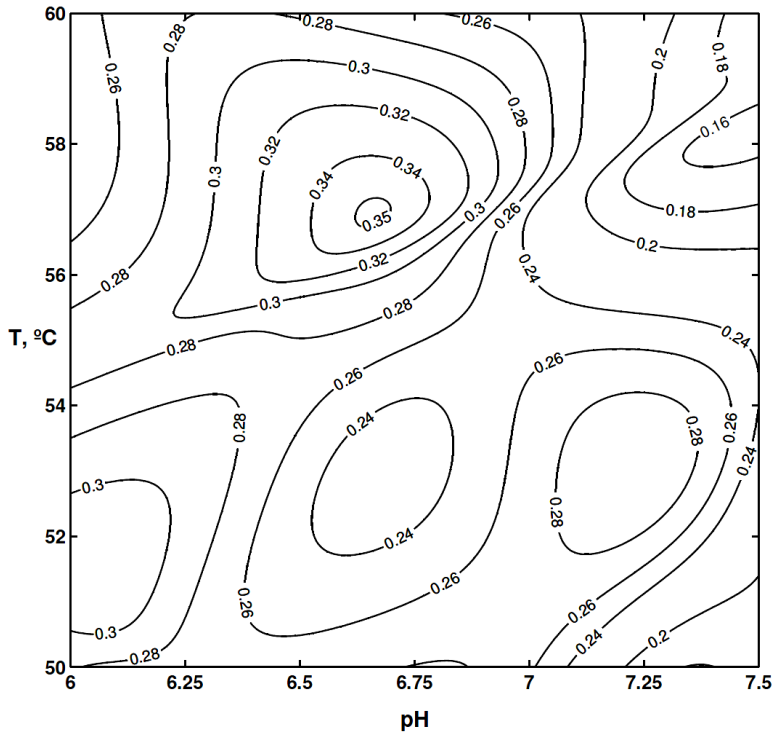


**Figure 5**. Contour plot for the degree of hydrolysis against pH and reaction temperature. Enzyme-substrate ratio and time of hydrolysis were set at 10% and 3 h, respectively.