# Bi-objective optimisation of the enzymatic hydrolysis of porcine blood protein

Raúl Pérez-Gálvez, M. Carmen Almécija, F. Javier Espejo, Emilia M. Guadix, Antonio Guadix*

Department of Chemical Engineering, University of Granada, 18071 Granada, Spain

## Abstract

Protein from porcine blood meal was hydrolysed with Alcalase to obtain a final revalorised product suitable, for example, to take part in the composition of an organic fertiliser. Three experimental factors of the reaction (pH, temperature and enzyme-substrate ratio) were optimised by means of a statistically designed experiment and response surface methodology. The goal of the optimisation problem was to maximise both the degree of hydrolysis and solubilisation of the substrate, obtaining a maximum degree of hydrolysis (28.89%) with pH 6.24, 54.2ºC and a enzyme-substrate ratio of 10%. Regarding the content of suspended solids, its minimum value (30.29% related to the initial weight of blood meal) was attained at pH 7.5, 59.8ºC and an enzyme-substrate ratio of 10%. The controversial effects of pH and temperature on the substrate solubilisation and the final degree of hydrolysis, suggested employing a multiobjective

* Corresponding author. Tel.: +34 958242925; fax: +34 958248992.

E-mail address: aguadix@ugr.es

optimisation technique. A Pareto Front was generated in order to find a set of intermediate solutions which satisfied both objectives in an adequate degree.

*Keywords*: Blood meal; Enzyme bioreactors; modelling; bi-objective optimisation; proteolysis; response surface methodology.

## 1. Introduction

Blood meal, the blood of animals processed into a dehydrated powder form, is a major by-product from slaughterhouses. Earlier approaches to provide a better utilisation for the blood streams originating from abattoirs date from the end of the seventies, driven by the improvements in the hygiene of blood collection systems [1,2]. The up-grading of these by-products solves the problem of their disposal as blood is the major pollutant from slaughterhouse waters, with a $BOD_5$ between 250000 and 375000 mg/L [3].

The raw blood is separated after centrifugation into a plasma fraction and a red cell fraction. The former exhibits good emulsifying and heat coagulating properties, and it is therefore a protein material of interest for food applications [4,5]. The red cell fraction, despite its high protein concentration and quality (it accounts for 70 – 75% of the total blood protein), has so far found a limited use in foodstuffs intended to human consumption, due to its intense brown colour and therefore the bad appearance of the products formulated with blood [2,6]. To this regard, various attempts have been made to decolourise the haemoglobin, which accounts for 90-91% of the red cell fraction on a dry basis [7,8], including enzymatic hydrolysis [6,9], where the heme iron was separated from the globine, yielding a final hydrolysate with good foaming and

emulsifying properties [10], as well as better solubility and minimal risk of harmful residues compared to other decolourisation methods [11].

Besides its original application as decolourisation technique, the enzymatic hydrolysis of the red cell fraction has increasingly drawn the interest of researches for two reasons:

- The isolation of bioactive peptides from the globine protein. For instance, the peptides LVV-hemorfin-7, VV-hemorfin-4 or VV-hemorfin-7 [12-14], which exhibit opiaceous activity, were obtained after partial hydrolysis of haemoglobin at acid pH.

- Recovery of heme iron. This form is of potential interest as dietary supplement for the treatment of iron deficiency anemia, based on its higher absorption rate, compared with non hemic iron [15]. Vaghefi *et al*. [16] conducted several in vitro tests on rats, employing as source of iron bovine haemoglobin hydrolysates. The biochemical assays proved that the intestinal absorption of heme iron was increased by a higher degree of hydrolysis. Furthermore, porcine haemoglobin hydrolysates have proved to have antioxidant activity [17], owing to the reducing power and chelation properties of the ferrous ion.

Together with other protein concentrates such as meat or feather meal, blood meal is an available source to provide nitrogen for fertilisation. The enzymatic hydrolysis of these materials leads to a final product with enhanced functional properties (solubility, root and leaf absorption), supplying a varied composition of peptides and free amino acids [18].

Furthermore, blood meal provides higher protein content as well as supplementation of hemic iron. This has proved to improve the stability and availability of nutrients in soil owing to its chelating effect [19,20], resulting in larger agricultural outputs. To this regard, Douglas [21] reported an increase of 8.3% in the crop yield of a soil fertilised with abattoir wastes compared to that obtained by traditional fertilisation with ammonium nitrates.

Chan *et al*. [22] considered the low solubility of blood meal as the main drawback for its use as liquid fertiliser, since the suspended particles can partially block the pipelines and reduce the flow. A combined treatment of microwaves and peroxide oxidation was employed to digest the blood meal. Although this procedure achieved good results in terms of nitrogen solubilisation, it was accompanied of high protein denaturation.

Enzyme hydrolysis is a potential alternative to solubilise blood meal protein because of the shorter length of the peptide chains in the hydrolysate compared to the original protein. In addition, it has been suggested that the supplementation of protein hydrolsates enhances the photosynthesis process and helps to withstand stress conditions such as hydric stress or salinity [23, 24]. This approach, compared to traditional fertilisation based on nitric and ammonium nitrogen, does not involve the chemical transformation of these compounds into amino acids inside the plants, which implies a high energy cost.

Both the control of the degree of hydrolysis and the solubilisation of the final product are of key importance when the blood meal is intended to take part in the formulation of a liquid fertiliser. It is noticeable the lack of previous research works on the enzymatic hydrolysis of blood related substrates. Among the few references available, Márquez

and Vázquez [25] reported that the optimal parameters for the hydrolysis of bovine haemoglobin with Alcalase 0.6 L were pH 7 and a 55ºC.

The aim of this paper is to optimise the operational conditions of the enzymatic hydrolysis of blood meal to obtain a final product able to be used as organic fertiliser. Thus, two objectives are pursued: a maximal degree of hydrolysis, as it assures an adequate content of peptides and free amino acids in the final product, and a good solubilisation of the substrate in the course of the hydrolysis reaction, as it facilitates its absorption by soil or its direct foliar application as spray fertiliser.

## 2. Materials and methods

### 2.1. Substrate and enzyme

The substrate employed in this study was blood meal (Protesan, APC, Barcelona, Spain), obtained from porcine blood by coagulation and drying, containing 89% in protein, 1.5 % in fat and 1.2 % in ash. The amino acid composition of the blood meal reveals an important proportion in glutamic acid, phenylalanine, leucine and lysine, all of them around 10%. The data from the complete aminogram were used to estimate the total number of peptide bonds in the protein $h_{tot}$ = 8.62 mmol/g, useful to calculate and monitor the degree of hydrolysis during the reaction .

The hydrolysis was undertaken with the enzyme Alcalase 2.4 L (EC 3.4.21.62) from Novozymes (Bagsvaerd, Denmark). This enzyme acts as a serine endroprotease, which hydrolyses peptide bonds in a long extent and wide range of specifity. Alcalase is stable within a wide range of pH, between 5.0 and 11.5, showing a maximum activity at temperatures between 50 and 60ºC.

## 2.2. Experimental rig

The hydrolysis reaction was carried out in a jacketed reactor with a capacity of 200 mL. A magnetic stirrer was employed to ensure a perfect mix in the reactor, while temperature was kept constant by connecting the reactor jacket to a thermostatic bath (F423, Haake, Karlsruhe, Germany). A 718 Stat Titrino (Metrohm, Herisau, Switzerland) was employed to keep pH constant during the reaction. This automatic titrator was equipped with temperature and pH probes and a dosing burette connected to a 1 L reservoir containing 0.5 M NaOH.

## 2.3. Input and output variables

Each hydrolysis experiment was carried out for 3 h with a substrate concentration of 10 g/L. Aiming to optimise the hydrolysis operation, the influence of 3 experimental factors as input variables was studied: pH, temperature (T) and enzyme-substrate ratio (E/S).

As output variables, 2 responses were measured for: degree of hydrolysis (DH) and the content of suspended solids (SS). The degree of hydrolysis, defined as the fraction of peptide bonds cleaved during the reaction, was calculated by considering the amount of base needed to keep pH constant, according to the pH-stat method [2, 26]. In order to determine the content of suspended solids (SS), the hydrolysate was vacuum filtered by means of a Büchner funnel coupled with a cellulose filter paper Whatman grade 40 with a pore size of 8 μm. Once filtered, the retained matter was dried at 110ºC during 30 minutes to remove the moisture before being weighted. The suspended solids were reported as the ratio between the mass of solids retained by the filter and the initial mass of blood meal added to the reactor in each experiment.

## 2.4. Experimental design

The effects of the 3 independent input variables on the degree of hydrolysis and the suspended solids were investigated using a 4 x 3 x 3 factorial design and response surface methodology [27]. The levels assayed were 6.0, 6.5, 7.0 and 7.5 for the pH; 50, 55 and 60 ºC for the temperature; and 0.050, 0.075 and 0.100 for the ratio enzyme-substrate.

The Statgraphics software (version 5.1) was used to generate the experimental designs, the statistical analysis and the regression model. The response functions were related to the input variables by a second degree polynomial as follows:

$$Y_j = b_0 + \sum_{i=1}^{3} b_i \cdot X_i + \sum_{i=1}^{3} b_{ii} \cdot X_i^2 + \sum_{i<j}^{j=3} b_{ij} \cdot X_{ij} \qquad (1)$$

where the coefficients $b_i$ and $b_{ii}$ are related to the linear and quadratic effects, respectively, of each input factor on the response and the cross-product coefficients $b_{ij}$ represent the interactions between two input variables.

In our case, each output variable could be related to the input factors by a model containing one constant $b_0$, 3 linear terms (those associated to pH, T and E/S), 3 quadratic ($pH^2$, $T^2$ and $(E/S)^2$) and 3 interactions related to the cross-products pH·T, pH·(E/S) and T·(E/S).

The significance of each term (and therefore its associated effect) on the response variables was judged statistically by means of the analysis of variance (ANOVA). The statistical significance of each effect was evaluated by means of the F-test. By this procedure, the F-value calculated for each effect is compared with a Fisher's

distribution of 1 and 26 degrees of freedom (those corresponding to each single effect and the total error). The deviation from the listed and calculated values is evaluated by means of an associated probability or p-value. Setting a confidence level 1- $\alpha$ = 95%, those effects having an F-test value or associated probability (p-value) lower than 0.05 will be significant on the response.

The regression models are useful to generate contour maps, where the degree of hydrolysis or the content of suspended solids are plotted against a combination of two input factors (in our case, pH and temperature). In addition, the regression model permits to optimise the response variables according to a given criterion, i.e. to obtain the set of input variables which leads to an optimum (maximum or minimum) value for a given response variable. The optimum solution should be obtained by a combination of the experimental factors inside their ranges of application. In this work, two single optimisation problems were defined, maximisation of the degree of hydrolysis attained after 3 hours and minimisation of the content of suspended solids in the final product.

### 2.5. Multiobjective optimisation

A problem of multiobjective optimisation arises when several objectives (possibly conflicting) must be satisfied, in our case, obtaining a maximum degree of hydrolysis and a minimum content of suspended solids in the final hydrolysate. A multiobjective optimisation problem can be defined as the problem of finding a vector of decision variables which satisfies constraints and optimises a vector function whose elements represent the objectives [28]. The solution vector should give values of all the objective functions acceptable to the decision maker. If any of the components of the objective function are competing, there is no unique solution to this problem. Then the concept of

Pareto front must be used in order to identify an adequate solution. A Pareto front consists of a set of non inferior solutions, which are defined as those in which an improvement in one objective requires a degradation of another [29].

The techniques to generate the Pareto Front are wide and varied. In our case the weighted-sum method [30] was chosen, which consists in obtaining an objective function (F), expressed as linear combination of the individual objectives (DH and SS), by means of a weight factor (α), which quantifies the relative importance given to the accomplishment of each individual objective.

$$F(pH, T, E/S) = \alpha \cdot (-DH) + (1-\alpha) \cdot SS \ , \qquad 0 \le \alpha \le 1 \qquad (2)$$

where each decision variable lays within its experimental range: 6.0 ≤pH ≤ 7.5; 50 ºC ≤ T ≤ 60 ºC; 0.050 ≤ E/S ≤ 0.100.

Note that the contribution of DH is negative in order to pose the problem as a minimisation of the objective function. Also, this general problem comes down to one single objective optimisation problem when α takes one of its boundary values. Between these bounds, a set of intermediate solutions are obtained, with more or less weight for each one of the individual objectives.

The selection of a single optimal solution inside the Pareto Front will depend on the technical and economical viability of the process for the production of the fertiliser, as well as the nature of the Pareto Front itself (whereas it attains a plateau with slight variation of the response variables or it hits one of the constraints set for the input variables).

## 3. Results and discussion

### 3.1. Regression model and response surfaces

As evidenced by the experimental results, Alcalase was successfully employed to hydrolyse blood meal within the ranges of the input variables. Table 1 shows the experimental design and the measured values of the response variables. Three experimental factors (pH, T, and E/S) were varied according to a 4 x 3 x 3 multilevel design, involving 36 experimental runs.

The actual values for both the degree of hydrolysis were fitted to the complete quadratic model described by equation (1). The polynomial coefficients for the surface response models were calculated by multiple regression, obtaining the equations (3) and (4), which relate both responses to the input factors (pH, T, E/S):

$$DH = -4.24 + 7.09E\text{-}1 \cdot pH + 7.46E\text{-}2 \cdot T + 4.80 \cdot (E/S) - 4.87E\text{-}2 \cdot pH^2 - 1.47E\text{-}3 \cdot pH \cdot T - 2.11E\text{-}1 \cdot pH \cdot (E/S) - 5.78E\text{-}4 \cdot T^2 - 2.77E\text{-}2 \cdot T \cdot (E/S) - 4.63 \cdot (E/S)^2 \quad (3)$$

$$SS = 9.94 - 1.71 \cdot pH - 1.03E\text{-}1 \cdot T + 4.77 \cdot (E/S) + 1.06E\text{-}1 \cdot pH^2 + 1.85E\text{-}3 \cdot pH \cdot T - 7.93E\text{-}1 \cdot pH \cdot (E/S) + 7.68E\text{-}4 \cdot T^2 - 2.42E\text{-}2 \cdot T \cdot (E/S) + 3.23 \cdot (E/S)^2 \quad (4)$$

Figures 1a and 1b compare the predicted values versus the observed data for both response variables, evidencing a reliable correlation with determination coefficients $R^2 = 0.912$ for the degree of hydrolysis and $R^2 = 0.941$ for the content of solids. Most of the experimental points presented a deviation between their actual and predicted value lower than ± 5% (represented by the dotted lines above and below the diagonal).

Statistical testing of the model was performed by the Fisher's statistical test for analysis of variance (ANOVA), which was performed separately to both response variables, as shown in Tables 2 and 3.

According to the Table 2, the influence of both the pH and the temperature on the degree of hydrolysis is non linear, as their quadratic effects are highly significant, with associated probabilities $p = 0.000$ and $p = 0.006$, respectively. On the contrary, it can be noticed that DH is linearly dependent of enzyme-substrate ratio, while the quadratic ($p = 0.557$) and interaction effects ($p = 0.304$ for pH·E/S, $p = 0.325$ for T·E/S) are not significant. DH increases linearly with the enzyme-substrate ratio until attaining an optimum at the highest level of enzyme concentration assayed (E/S = 0.100), which constitutes an upper bound for the optimisation of the hydrolysis operation.

The value of the coefficient of determination $R^2$ indicates that the proposed model can explain 94.1% of the variability of the degree of hydrolysis. Likewise, the 0.881 value of the adjusted $R^2$ is sufficiently good.

The goodness of the polynomial fit was also assessed by means of the residuals, which presented an average value (Mean Absolute Error) of 0.009 and a standard deviation (Standard Error of Estimate) of 0.014.

The Table 3 shows the analysis of variance for the content in suspended solids (SS). From the F-test it was concluded that SS was highly dependent on the linear effects of pH, T and E/S, with associated probabilities $p < 0.001$. However, the influence of quadratic effect was found to be significant only for pH ($p=0.000$), and the p-values for the remaining effects indicated a lack of interaction between the input factors ($p > 0.05$). Similarly to the degree of hydrolysis, the proposed quadratic model explained the

variability of the data to a large extent ($R^2$ = 0.941), as well as its adjusted $R^2$ = 0.920. With regard to the residuals, they presented a mean value of 0.030 and a standard deviation of 0.041.

The Figures 2a and 2b show the contour plots for both response variables. In both cases, the enzyme-substrate ratio was set at its maximum level (0.100) while pH and T laid on the Y and X axis, respectively. Several contour lines are marked with the corresponding value for the response variable (DH or SS). The curvature and separation between consecutive lines indicates the rapidity of the ascent (or descent) for the output variable.

As shown in Figure 2a, the degree of hydrolysis corresponded to a curved surface, owing to the significance of the quadratic effects and the optimum degree of hydrolysis (DH = 0.289) was found inside the experimental ranges of both input variables, at pH = 6.25 and T = 54.3 ºC.

With regard to the content of suspended solids SS, it decreased as pH and T were higher, attaining a minimum value (SS =0.303 w/w) at pH = 7.50, T = 59.8ºC and again the maximal ratio enzyme-substrate (0.100).

The results obtained show that the behaviour of the output variables with two of the input variables is conflicting, since the optimal values are located at different values of pH and temperature. Considering the biochemical nature of the hydrolysis reaction, as the pH becomes acid, the denaturation of the structure of the haemoglobin (major compound of the blood meal) facilitates the availability of the peptide bonds to the enzyme attack, leading to higher degrees of hydrolysis. The optimal pH = 6.25 results as a trade-off between this phenomenon and the loss of activity of the Alcalase, which usually presents optimal performances in the pH = 7-9 range. With respect to the

suspended solids, the opening of the globular structure promotes the exposure of hydrophobic groups [3] which are normally hidden in native state, increasing the insolubility of the hydrolysate. This fact justifies why the minimum suspended solids are obtained at the upper bound for pH = 7.50. In summary, better degrees of hydrolysis can be achieved working at pH values below 7, at the expense of producing more insoluble hydrolysates.

In the case of relationship between temperature and degree of hydrolysis, the optimal value of T = 54.3 ºC is a consequence of the trade-off between the kinetics of the hydrolysis reaction and the simultaneous deactivation of the enzyme. Higher reaction temperatures increase the initial reaction rate by unfolding the peptide chains and thus favouring the access to the peptide bonds. On the other hand, higher temperatures provoke protein denaturation with loss of its proteolitic activity [31]. This explains why the maximal solubility of the blood meal hydrolysate was observed at T = 59.8 ºC, close to the experimental upper bound T = 60 ºC, while the final degree of hydrolysis attained at this temperature was moderate, without exceeding 0.20 at a maximal enzyme concentration.

With regard to the enzyme-substrate ratio, the addition of enzyme increases the reaction rate as the hydrolysis were conducted with low enzyme concentrations, far from those of saturation of the substrate. Its general effect on the protein solubilisation is also positive, as the average molecular weight of the polypeptides contained in the hydrolysis reaction is increasingly lower.

### 3.2. Bi-objective optimisation

The conflict between the two responses described above suggested employing a multiobjective optimisation technique. A Pareto front was generated (Fig. 3) in order to find a set of solutions which satisfied in an adequate degree both objectives by following the weighted-sum method.

The optimisation of the objective function for each bound of the weight interval corresponds to the optimisation of a single objective, that is, the minimisation of the content of suspended solids ($\alpha = 0$, left-top end of the curve ), or the maximisation of the degree of hydrolysis ($\alpha = 1$, right-bottom end of the curve) . Between these values a set of noninferior solutions is represented, where each point corresponds to a particular value of $\alpha$, i.e. the relative weight given to each objective. Each solution or efficient point (SS, -DH) is determined by a decision vector or combination of factors inside the ranges of the independent variables (pH, T, E/S). It can be noticed that an increase of the degree of hydrolysis implies an augmentation of content of suspended solids in the final hydrolysate. This trend is more marked at values of DH higher than 28%. Above this value, the degree of hydrolysis can only be increased up to 28.89%, which only represents a percentage improvement of 3%, at the expense of an augmentation of the content of suspended solids higher than 25%.

Then, the Pareto front can be translated to the decision space by obtaining the optimal combination of experimental factors for each weight factor $\alpha$, as shown in Fig. 4.

As the weight factor $\alpha$ decreases from $\alpha = 1$ to $\alpha = 0$, moving from the left to the right along the curve depicted in Fig. 4, the objective of minimum suspended solids is

favoured over that of maximum degree of hydrolysis. It is important to note that for $\alpha \leq$ 0.601, the optimal valued of pH hits the upper bound of 7.5. In this critical value DH = 0.201 and SS = 0.307, for the triplet pH = 7.5, T = 57.42 ºC, E/S =0.100. Below this critical value for the weight factor, an increase of the temperature will not reduce significantly the content of suspended solids (attaining a minimum value of 0.303), while the degree of hydrolysis will be lower than 0.20. The optimum combination of pH and T should be determined for a weight factor higher than 0.601, according to the technical and economical viability of the process (e.g. operational costs for the removal of the suspended matter, minimal desired concentration of free amino acids, etc).

## 4. Conclusions

The blood meal was successfully hydrolysated with Alcalase 2.4 L in the range of temperatures from 50 to 60ºC, a pH between 6 and 7.5 and a ratio enzyme-substrate of 5-10%.

With the help of the response surface methodology, the influence of the input variables on the degree of hydrolysis and suspended matter content was studied and operation conditions were optimised, obtaining a maximum degree of hydrolysis (28.89%) with pH 6.24, 54.2ºC and a ratio enzyme-substrate of 10%. Regarding the content of suspended solids, its minimum value (30.29% related to the initial weight of blood meal) was attained at pH 7.5, 59.8ºC and a enzyme-substrate ratio of 10%.

There is a competition between both objectives as the influence of pH and temperature upon the response variables is opposite. This can be attributed to the denaturation of the quaternary structure of the haemoglobin in the course of the enzymatic hydrolysis,

appearing hydrophobic groups, previously hidden inside the macromolecule, which decrease the solubility of the substrate. The opposite behaviour of the experimental factors towards the accomplishment of the optimisation objectives obliges to find a compromise solution by using multiobjective optimisation techniques. The weighted-summed method was chosen for this purpose, generating a set of optimal solutions (Pareto Front) which assures an adequate degree of hydrolysis with a limited content of suspended matter in the final hydrolysate.

## Acknowledgements

This work was funded by the project CTQ2008-02978 from the Spanish Ministerio de Ciencia e Innovación.

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## Figure and Table captions

- Figure 1. Comparison between experimental and calculated values of the response variables: (a) Degree of hydrolysis, (b) Suspended solids. The straight line calculated by linear regression can be compared to the diagonal. Dotted lines represent a 5 % deviation.

- Figure 2. Contour plots for the response variables: (a) Degree of hydrolysis, (b) Suspended solids. The influence of temperature and pH is shown, while enzyme-substrate ratio was set at 0.100.

- Figure 3. Set of non-inferior solutions (Pareto front) for the multiobjective optimisation problem.

- Figure 4. Set of efficient solutions for the multiobjective optimisation problem. Optimum temperature and pH are shown, while optimum enzyme-substrate ratio was 0.100 in all cases.

- Table 1. Experimental design and measured values for the response variables

- Table 2. Analysis of variance for the degree of hydrolysis.

**Table 1. Experimental design and measured values for the response variables**

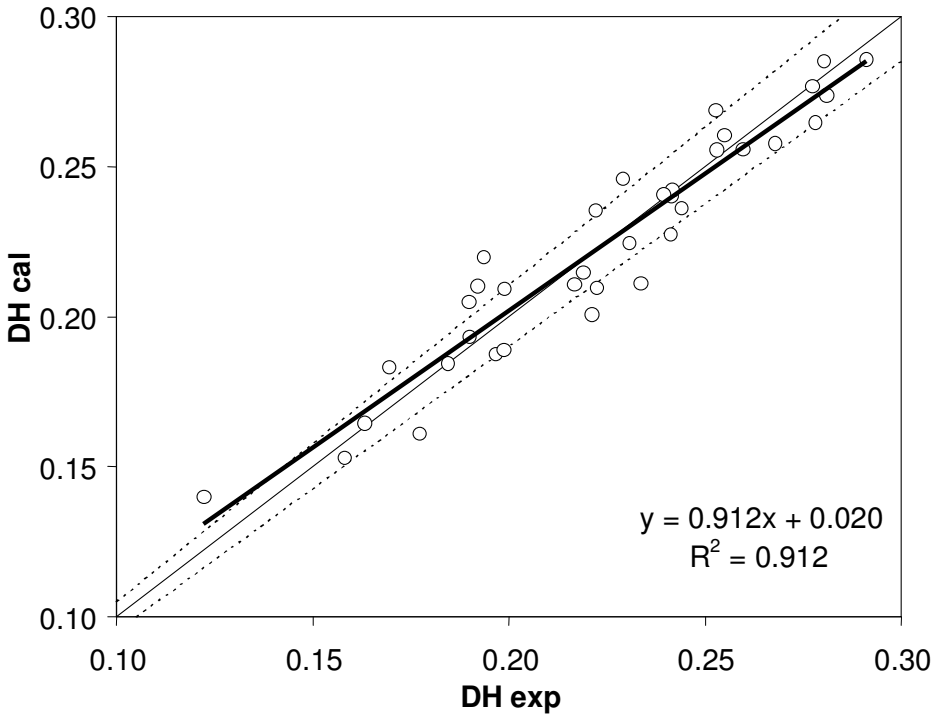
| Exp | pH | T (ºC) | E/S | DH | SS |
|---|---|---|---|---|---|
| 1 | 6.0 | 50 | 0.050 | 0.221 | 0.739 |
| 2 | 6.0 | 50 | 0.075 | 0.241 | 0.753 |
| 3 | 6.0 | 50 | 0.100 | 0.281 | 0.746 |
| 4 | 6.0 | 55 | 0.050 | 0.194 | 0.750 |
| 5 | 6.0 | 55 | 0.075 | 0.253 | 0.721 |
| 6 | 6.0 | 55 | 0.100 | 0.291 | 0.665 |
| 7 | 6.0 | 60 | 0.050 | 0.222 | 0.697 |
| 8 | 6.0 | 60 | 0.075 | 0.242 | 0.685 |
| 9 | 6.0 | 60 | 0.100 | 0.253 | 0.700 |
| 10 | 6.5 | 50 | 0.050 | 0.199 | 0.702 |
| 11 | 6.5 | 50 | 0.075 | 0.229 | 0.659 |
| 12 | 6.5 | 50 | 0.100 | 0.277 | 0.612 |
| 13 | 6.5 | 55 | 0.050 | 0.231 | 0.607 |
| 14 | 6.5 | 55 | 0.075 | 0.268 | 0.581 |
| 15 | 6.5 | 55 | 0.100 | 0.280 | 0.553 |
| 16 | 6.5 | 60 | 0.050 | 0.217 | 0.554 |
| 17 | 6.5 | 60 | 0.075 | 0.239 | 0.531 |
| 18 | 6.5 | 60 | 0.100 | 0.278 | 0.455 |
| 19 | 7.0 | 50 | 0.050 | 0.190 | 0.496 |
| 20 | 7.0 | 50 | 0.075 | 0.241 | 0.459 |
| 21 | 7.0 | 50 | 0.100 | 0.260 | 0.420 |
| 22 | 7.0 | 55 | 0.050 | 0.190 | 0.501 |
| 23 | 7.0 | 55 | 0.075 | 0.222 | 0.421 |
| 24 | 7.0 | 55 | 0.100 | 0.255 | 0.373 |
| 25 | 7.0 | 60 | 0.050 | 0.196 | 0.402 |
| 26 | 7.0 | 60 | 0.075 | 0.219 | 0.369 |
| 27 | 7.0 | 60 | 0.100 | 0.244 | 0.332 |
| 28 | 7.5 | 50 | 0.050 | 0.158 | 0.477 |
| 29 | 7.5 | 50 | 0.075 | 0.184 | 0.442 |
| 30 | 7.5 | 50 | 0.100 | 0.192 | 0.397 |
| 31 | 7.5 | 55 | 0.050 | 0.177 | 0.366 |
| 32 | 7.5 | 55 | 0.075 | 0.199 | 0.339 |
| 33 | 7.5 | 55 | 0.100 | 0.234 | 0.305 |
| 34 | 7.5 | 60 | 0.050 | 0.122 | 0.480 |
| 35 | 7.5 | 60 | 0.075 | 0.163 | 0.360 |
| 36 | 7.5 | 60 | 0.100 | 0.169 | 0.359 |

**Table 2. Regression coefficients and p-values for the response variables**

| Term | DH | | SS | |
|---|---|---|---|---|
| | **Coefficient** | **p-value** | **Coefficient** | **p-value** |
| Constant | -4.244E+00 | - | 9.943E+00 | - |
| pH | 7.090E-01 | 0.000 | -1.705E+00 | 0.000 |
| T | 7.463E-02 | 0.119 | -1.032E-01 | 0.000 |
| E/S | 4.804E+00 | 0.000 | 4.769E+00 | 0.000 |
| $pH^2$ | -4.867E-02 | 0.000 | 1.063E-01 | 0.001 |
| pH·T | -1.465E-03 | 0.158 | 1.847E-03 | 0.541 |
| pH·E/S | -2.109E-01 | 0.304 | -7.929E-01 | 0.196 |
| $T^2$ | -5.779E-04 | 0.006 | 7.678E-04 | 0.195 |
| T·E/S | -2.771E-02 | 0.325 | -2.419E-02 | 0.769 |
| $(E/S)^2$ | -4.632E+00 | 0.557 | 3.228E+00 | 0.889 |

**Figure 1. Comparison between experimental and calculated values of the response variables: (a) Degree of hydrolysis, (b) Suspended solids.**

(a)




(b)

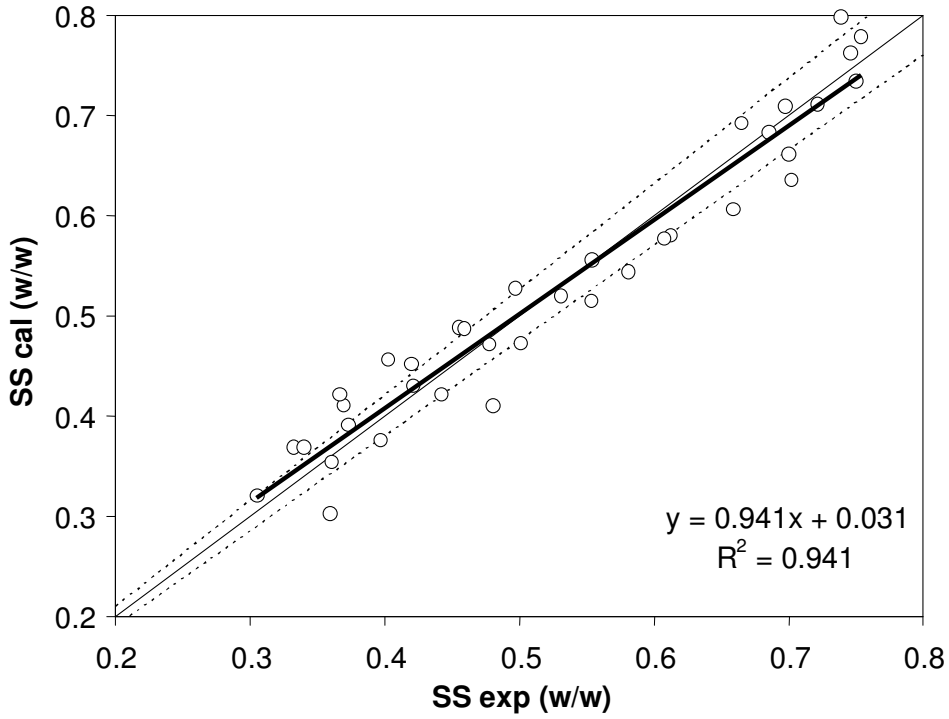
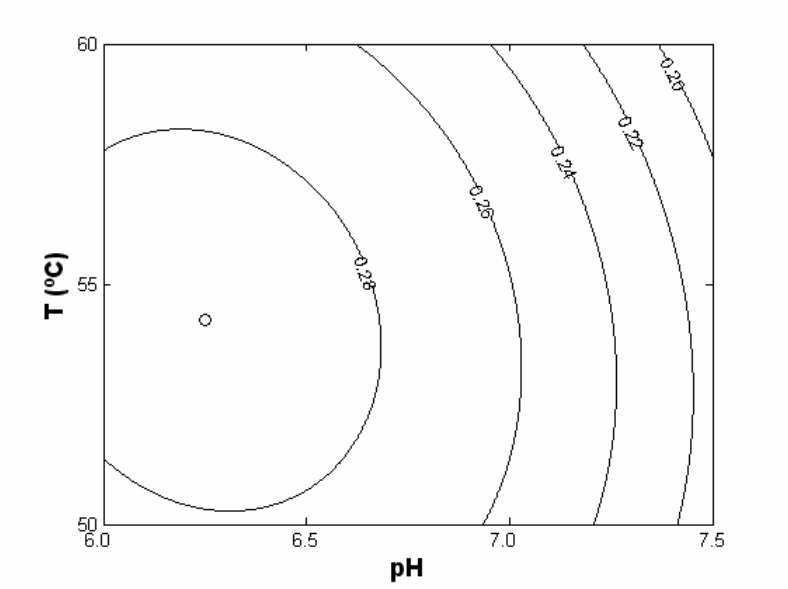


**Figure 2. Contour plots for the response variables: (a) Degree of hydrolysis, (b) Suspended solids. (E/S = 0.100)**

(a)




(b)

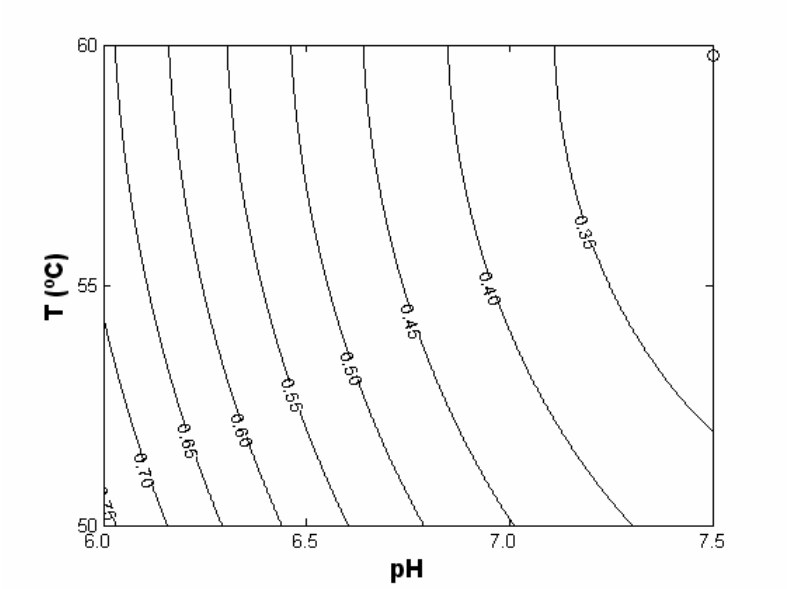


**Figure 3. Pareto front for the multiobjective optimisation problem**

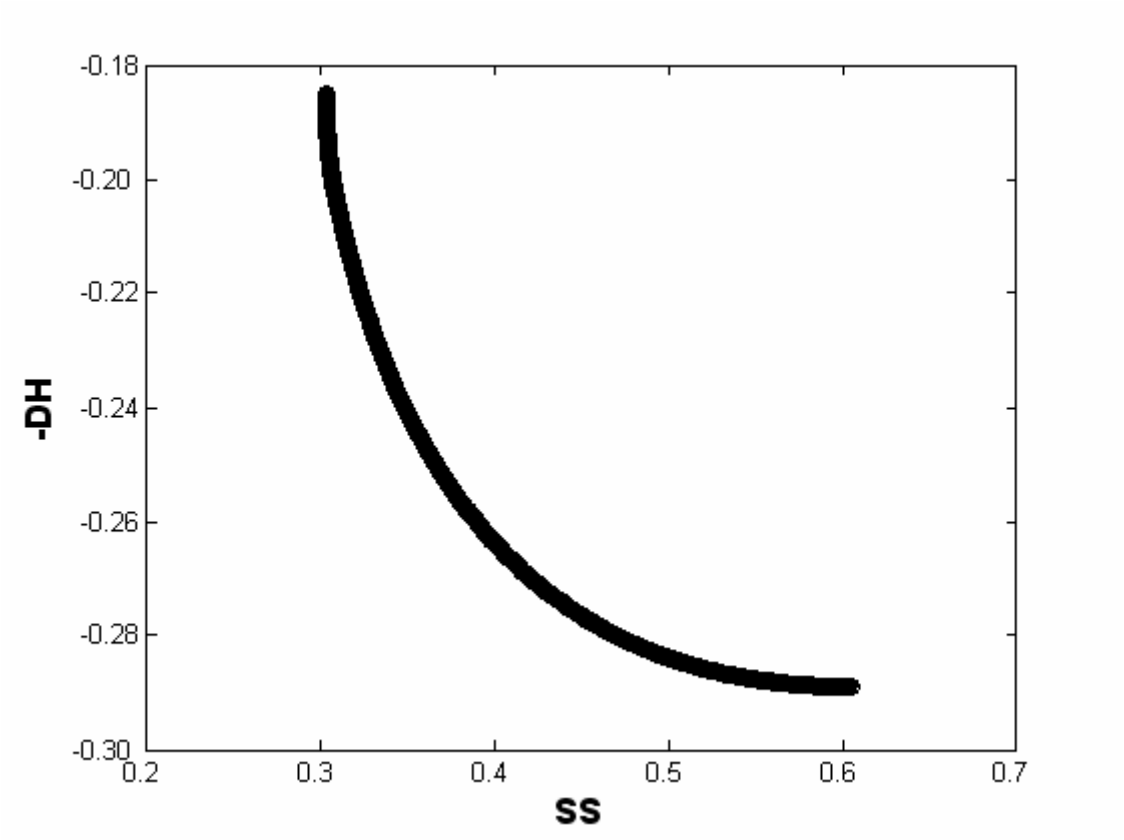


**Figure 4. Set of efficient solutions for the multiobjective optimisation problem (E/S=0.100)**