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

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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, 54.2 °C and 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, 59.8 °C and 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 optimisation technique. A Pareto Front was generated in order to find a set of intermediate solutions which satisfied both objectives in an adequate degree.

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 250,000 and 375,000 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

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regard, Douglas et al. [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 that 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 55 °C.

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 specificity. Alcalase is stable within a wide range of pH, between 5.0 and 11.5, showing a maximum activity at temperatures between 50 and 60 °C.

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 110 °C during 30 min 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 $4 \times 3 \times 3$ 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 °C 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:

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

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 is plotted against a combination of two input factors (in our case, pH and temperature). In addition, the regression model permits