Bi-objective optimization of tuna protein hydrolysis to produce aquaculture feed ingredients.

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ABSTRACT

Fish meal is commonly employed as protein source in aquaculture diets. The enrichment of this ingredient with fish protein hydrolysates (FPH) and free amino acids has proved to improve larval development and feed assimilation. In this work, we produced tuna head hydrolysates using a sequential enzymatic treatment employing Alcalase and Flavourzyme. Statistical modelization coupled with bi-objective optimization were employed to optimize the operating parameters (i.e. pH, temperature and duration of the Flavourzyme treatment) for producing a FPH with a desired molecular weight profile. More specifically, this work focused on the content of small peptides between 700 – 2500 Da (F_{2500}) and that of free amino acids (F_{250}), supported by their benefits as aquaculture feed ingredients.

The optimal reaction conditions for maximimizing the release of free amino acids F_{250} (i.e, pH 7.2, 43-49ºC, Flavourzyme treatment above 160 min) were detrimental for the content of F_{2500}. A bi-objective optimization approach was then proposed, able to find a set of intermediary solutions (Pareto Front) presenting maximal F_{2500} for a range of free amino acids level between 2 - 30%. This allows the selection of the operating parameters for producing a FPH with a desired weight profile, based on the specific needs of the farmed species.

Keywords: Tuna head hydrolysates, Alcalase, Flavourzyme, molecular weight distribution, bi-objective optimization, aquaculture

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1. INTRODUCTION

Last FAO assessment on catches from tuna and tuna-like species estimates a worldwide figure around 6 million tons for the year 2010. According to this study, the main trading species are tropical tunas such as skipjack (*Katsuwonus pelamis*), yellowin (*Thunnus albacares*) and bigeye (*Thunnus obesus*), which account for more than 93% of worldwide production.

Tuna processing industry transforms raw material into fillets intended for human consumption. In the case of Tunisia, tuna canning industry has increased over the past years achieving a yearly average production of canned tuna around 6850 tons for the period 2004-2008 (GIPP, 2017). Tuna loins and fillets are the main parts extracted from tuna in processing industry. These final products represent only 17% of body weight, while the rest (i.e. viscera, heads, fins) is discarded as waste (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012). Given the high amount of by-products resulting from tuna processing facilities, as well as the environmental impact caused by their disposal and elimination, the valorization of the protein and lipid fraction from these materials is receiving increasing attention. As proximate composition, tuna heads present on average 15% proteins, 13.5% lipids and 12% mineral matter (Nguyen et al., 2011). Apart from these macroconstituents, they contain vitamins and minerals of high nutritive value

Enzymatic hydrolysis allows high protein recovery from tuna heads, yielding fish protein hydrolysates with improved functional and biological activities (Han, Byun, Park, & Kim, 2015; Klomklao & Benjakul, 2016; Nguyen et al., 2011; Saidi, Belleville, Deratani, & Ben Amar, 2016; Wang, Leng, Chen, & Wang, 2015). Furthermore, some authors have employed enzymatic hydrolysis to simultaneously recover protein and oil from fish by-products (de Oliveira et al., 2017).

Fish protein hydrolysates are potential ingredients for the formulation of aquaculture diets, replacing fish meal as protein source. First- breeding diets in aquaculture require a high supply of proteins, which are commonly incorporated as fish meal from herring, anchovy, sardine or lean species. To this regard, the partial replacement of fish meal by fish protein hydrolysates presents a number of advantages for the development of larvae and juvenile individuals. In the case of larvae, protein assimilation is deficient due to the incomplete development of the digestive system and the low activity of trypsin and other proteases (K. Hamre et al., 2013;

Zambonino Infante & Cahu, 2010). This drawback can be alleviated by the incorporation of fish protein hydrolysates into the feeding diets. To this regard, Kotzamanis et al. (2007) recommend the supplementation of aquaculture diets with peptides in the range between 500 and 2500 Da. In the case of juvenile individuals, the incorporation of low molecular weight peptides and free amino acids improves the palatability of the powdered diet, increasing the dietary intake of protein. This has been reported for aquaculture species such as shrimp (Nunes, Sá, Andriola-Neto, & Lemos, 2006) or Senegalese sole (Barroso, Rodiles, Vizcaino, Martínez, & Alarcón, 2013). Besides their nutritional value, fish protein hydrolysates may exert a number of biological activities. In the case of aquaculture, much research has been devoted to seek for natural stimulators which enhance the immune response of farmed fish against pathogens. The vaccination of mature individuals is difficult and the incorporation of antibiotics to the aquaculture diets is not recommended since they increase bacterial resistance. To this regard, some studies concluded that some specific peptides could enhance the immune system of fish in farm rearing conditions. For example, Tang et al. (2008), incorporated increasing levels of fish protein hydrolysates (i.e. 5%, 10% and 15% on weight basis) in the powder diets of meagre *(Pseudosciaena crocea).* The authors reported an increase in the lysozyme activity as well as the levels of immunoglobulins in blood, both parameters related to stronger immunitary resistance.

The aim of this paper is to optimize the operation conditions of the enzymatic hydrolysis of tuna head by-products to obtain a final product able to be used as dietary supplement in aquaculture diets. According to previous literature, this product should present a balanced distribution of short chain peptides and free amino acids, depending on morphological stage and specific needs of the fish reared species. To this end, a set of protein hydrolysates were produced by a combined enzymatic treatment employing Alcalase and Flavourzyme within their ranges of maximal activity. The influence of three operation parameters (i.e. pH, reaction temperature and duration of the Flavourzyme treatment) on the final degree of hydrolysis and the molecular weight distribution was modelled by Response Surface Methodology. Finally, a bi-objective optimization technique was employed to find the optimal operating conditions for obtaining a tuna head hydrolysate with a specific molecular weight profile. This approach is a first approximation to the production of taylor-made hydrolysates able to fulfill the specific needs of aquaculture species.

2. MATERIALS AND METHODS

2.1. Raw material and biochemical analysis

Skipjack tuna (*Katsuwonus pelamis*) heads were provided by the canning factory El Sultan, located in Sfax (Tunisia). These by-products were grinded and stored at -20ºC prior to analysis. The proximate composition of the raw material was determined according to the official methods recognized by the Association of the Official Analytical Chemists (A.O.A.C., 2012). Both the moisture and mineral content were determined gravimetrically, heating the samples at 105°C and 550°C, respectively. Nitrogen content was determined experimentally by the Kjeldahl method, and then converted into crude protein by employing a factor of 6.25 grams of protein per gram of nitrogen (Adler Nissen, 1986). Finally, lipid content was extracted and quantified according to the Soxhlet semi-continuous method (Rocío Morales-Medina et al., 2016). The average proximal composition of tuna heads was 38.4% of protein, 9.6% of lipid, 11.5% of ashes and 40.5 % of water.

2.2. Enzymes and hydrolysis procedure

Two commercial food grade proteases were selected to produce the protein hydrolysates, purchased from Novozymes (Bagsvaerd, Denmark) as Alcalase 2.4.L and Flavourzyme 1000L. Alcalase (subtilisin, EC 3.4.21.62) is a protease extracted from *Bacillus licheniformis* and purchased from Novozymes as Alcalase 2.4 L. It acts as wide spectrum endoprotease with maximal proteolitic activity within the range of pH 7-10 and the range of temperature 50ºC – 60ºC (Adler Nissen, 1986). The second catalyst was Flavourzyme, a mixture of aminopeptidases, exoproteases and endoproteases extracted from *Aspergillus oryazae*. According to Merz et al. (2015), this cocktail presents optimal proteolitic activity within the interval of pH 7-10 and temperature 40-60ºC.

Hydrolysis experiments were carried out in a jacketed stirred tank reactor of 250 mL. The raw material was homogenized with distilled water to obtain a 15% (w/w) protein. A volume of 200 mL of this suspension was transferred to the batch reactor, where the conditions of pH and temperature were adjusted prior to the hydrolysis. The enzymatic treatment lasted 5 hours, comprising two sequential stages of different duration. The first stage started with the addition of alcalase at enzyme to substrate ratio 3% (w/w). After a certain period of time,

Flavourzyme was added at enzyme to substrate ratio of 3% (w/w). At this point the reaction was allowed until completing 5 hours of enzymatic treatment.

The degree of hydrolysis (DH), defined as the percentage ratio of the number of peptide bonds cleaved to the total number of peptide bonds available in the substrate, was monitored throughout the reaction. According to the pH stat method, DH was related to the amount of base consumed to keep the pH constant during the reaction (Rutherfurd, 2010). To this end, the batch reactor was attached to an automatic titrator 718 Stat Titrino (Metrohm, Switzerland), employing 1N NaOH as titration agent. The degree of hydrolysis (DH, %) was calculated by the equation [1]:

$$DH = \frac{V_b \cdot N_b}{\alpha \cdot m_P \cdot h_{TOT}} \cdot 100 \quad [1]$$

where V_b (mL) is the amount of base of normality N_b (eq/L) consumed during the reaction, m_P (g) is the mass of protein fed to the reactor, h_{TOT} was assumed to be 8.6 milliequivalents of peptide bonds per gram of protein and α is the average degree of dissociation of the α-NH_2 amino groups, which can be related to reaction pH and temperature by Eq. [2]:

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \quad [2]$$

The average pK value of the α-NH_2 amino groups was estimated according to Steindhardt and Beychok (1964).

$$pK = 7.8 + \frac{298-T}{298 \cdot T} \quad [3]$$

After completing 5 hours, the reaction was stopped by heating the solution at 100 °C for 15 min, assuring the thermal deactivation of the enzymes. The resulting hydrolysate was cooled down to room temperature and freeze-dried in a Labconco freeze drying system (Kansas City, MO, USA). The powdered product was employed for the subsequent analysis.

2.3. Size exclusion chromatography (SEC)

Molecular weight distribution of the hydrolysates was determined by size exclusion chromatography on a Superdex peptide 100/300 GL column (GE, Health care, Uppsala, Sweden) mounted in an Akta Purifier UPC100 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Dried hydrolysate samples were dissolved in ultrapure water at protein concentration of 5 mg/mL. To perform the analysis, 100 μL of the above solution was injected to the system and eluted at 0.5 mL/min with a mobile phase of ultra-pure water and acetonitrile (70:30) containing 0.1% of trifluoroacetic acid. Absorbance of the eluted samples was monitored at 280 nm. A total of eight standard were employed for calibrating the column: Gly (75 Da), Ala (89 Da), Phe-Gly-Gly (279 Da), $(Gly)_6$ (360 Da), vitamin B12 (1355 Da), insulin (5733 Da), aprotinin (6511 Da) and ribonuclease (13700 Da).

2.4. Amino acids analysis

The free amino acids released by the enzymatic treatment were recovered by SEC separation and analyzed according to Liu et al. (1995). A tuna head hydrolysate was produced at the optimal conditions of pH, temperature and duration of the flavourzyme treatment for maximum content of free amino acids. The hydrolysate was freeze dried and the resulting powder was dissolved in ultrapure water at protein concentration of 5 mg/mL. An aliquot of 500 μL was injected in the Superdex peptide 100/300 GL column for SEC separation. Free amino acids were defined from the SEC profile as the fraction with molecular weight below 250 Da, noted as F_{250}. This fraction was recovered by means of a fraction collector FRAC-920 (General Electric Healthcare, Chicago, USA). This procedure was repeated several times in order to collect enough amount of sample for the amino acid analysis. The samples collected were pooled and freeze dried prior to amino acid determination by Liu et al. (1995).

To this end, the samples were filtered and mixed with borate buffer. The resulting solution was derivatised at 55 °C for 10 min with 20 μl of AccQ Fluor reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). The derivatives were subsequently separated by reversed-phase chromatography, employing Waters Alliance 2695 system mounted with AccQTag column (Waters Corporation, Milford, Massachusetts, USA). Sample were eluted in a mobile phase composed of AccQ·Tag Eluent A, acetonitrile and water at 37 °C and the derivatives were detected after separation by Waters 474 Scanning fluorescence detector.

2.5. Design of experiments and statistical analysis

The effects of pH, temperature and duration of the Flavourzyme treatment were related to both the final degree of hydrolysis (DH %) and the molecular weight distribution of the resulting hydrolysate. The enzymatic treatment starting with the addition of alcalase, which acted until completing 5 hours of hydrolysis. Flavourzyme was added at a second stage whose duration varied from 19 to 221 min. The first stage, where only the endoprotease (i.e. alcalase) was acting as enzyme, was noted as t_{endo}. The duration of the second stage, where both Flavourzyme and Alcalase were acting in the reaction vessel, was noted as t_{exo}. Since the total duration of the enzymatic treatment was fixed at 5 hours (i.e. 300 min), both input variables are related as shown in equation [4]:

$$t_{endo} + t_{exo} = 300 \quad [4]$$

The input variables described above were assayed at different levels according to a central composite design (CCD) of 16 experimental runs, including 2 replicates of the central point. The complete CCD design matrix, as well as the observed values for the response variables, are shown in Table 1. The levels of pH (7.2 – 8.8) and temperature (41.6 ºC – 58.4 ºC) were chosen considering the intervals of activity reported for Alcalase and Flavourzyme. The third input factor was the duration of the Flavourzyme treatment (t_{exo}), which was varied from 19 to 221 min. Given the linear dependence expressed in equation [4], t_{endo} and t_{exo} cannot be present in the same regression model.

The final degree of hydrolysis and the molecular weight distribution of the resulting hydrolysates were related to the input factors defined above. The degree of hydrolysis DH was calculated by equation [1] and expressed as percentage. Two SEC fractions were chosen to study the influence of the enzymatic treatment upon the molecular weight distribution of the hydrolysales: the fraction below 250 Da, and a second fraction between 700 and 2500 Da. Each fraction was quantified as percentage area and noted as F_{250} and F_{2500}, respectively.

Statgraphic Centurion XV (statistical graphic corps, Rockville, MD, USA) was used for the regression model and statistical analysis. According to the response surface methodology, the responses were related to the input factors by second order polynomials. The regression models were expressed by the general quadratic equation [5]:

$$\left\{DH, F_{250}, F_{2500}\right\} = b_0 + b_1 \cdot pH + b_2 \cdot T + b_3 \cdot t_{exo} + b_{11} \cdot pH^2 + b_{12} \cdot pH \cdot T + b_{13} \cdot pH \cdot t_{exo} + b_{22} \cdot T^2 + b_{23} \cdot T \cdot t_{exo} + b_{33} \cdot t_{exo}^2 \quad [5]$$

The intercept b_0 and the coefficients b_1 to b_{33} were estimated by multiple regression. The significance of each term on the response variables was then judged statistically by means of the analysis of variance (ANOVA). This approach computes an associated probability (p-value) for each effect (i.e. linear, quadratic or interaction term in the polynomial) at a confidence level of 95%. Those effects whose p-values are below 0.05 are not statistically significant and were subsequently eliminated from the regression model. Among the different techniques available to reduce regression models, the backward selection (Kroese & Chan, 2014) was chosen in this work. This approach starts with the complete model, and eliminates progressively the terms with the p-value ($p > 0.05$). The goodness of the quadratic model was assessed by the coefficient of determination R^2, as well as the mean absolute error (i.e. average value of residuals) and the standard error of estimate (standard deviation of the residuals).

2.6. Single optimization of the response variables

The regression equations for both response variables (DH, F_{250} and F_{2500}) allowed the optimization of the experimental conditions of the hydrolysis (i.e. pH, T and t_{exo}) to maximize either the degree of hydrolysis DH or the area percentages F_{250} and F_{2500}.

The generalized reduced gradient algorithm (GRG), implemented in the Solver Tool of the MS Excel software, was chosen for the optimization. This solver method is based on the algorithm developed by Lasdon et al. (1978). By this approach, a generalized reduced gradient is computed by a combination of the gradient of the objective function (i.e. vector of partial derivatives of the response variable with respect the experimental factors) and a pseudo-gradient derived from the equality constraints. Based on the reduced gradient, the algorithm determines the search direction for the optimum solution. Since this algorithm is local, the optimal solutions are highly dependent on the starting conditions (pH, T, t_{exo}), and may not find the global optimum. To overcome this drawback, the Solver tool includes a multistart method which runs the GRG algorithm from a grid of starting points) and then selects the best solution among the set of local optimums.

Based on previous literature, the objectives of the single optimization problems would be the maximization of DH, F_{2500} and F_{250}. The maximization of DH has a positive impact on some technological properties of the resulting hydrolysate, such as solubilization or water/fat holding capacity (Gbogouri et al., 2004; Balti et al., 2010; García-Moreno et al., 2017). These properties are desirable for the incorporation of fish hydrolysates as ingredients of feed diets. As for the molecular weight distribution, some studies relate the partial replacement of fish meal by hydrolysates in the range 500 – 2500 Da to higher feed utilization in larvae (Kotzamanis et al., 2007; Kristin Hamre et al., 2013). Most of the studies on amino acid supplementation highlight their role as feeding stimulants in juvenile individuals, increasing feeding utilization and weight gain (Barroso et al., 2013; Morais, 2017). In larval stages, free amino acids and other small compounds may modulate the activity of some specific enzymes and hormones related to larval immunity and nutrient assimilation (Li et al., 2009; Cai et al., 2015).

2.7. Bi-objective optimization problem

A Multiobjective Optimization Problem (MOP) arises when several objectives (possibly conflicting) must be satisfied. In MOP there is no unique optimal solution (i.e. combination of the decision variables) that simultaneously optimizes all the objective functions. In this work, the simulateous maximization of both F_{2500} and F_{250} cannot be achieved by a single combination of pH, T and t_{exo}. Indeed, it is expectable that the maximization of content of amino acids F_{250} would be reached by employing optimal conditions for maximal Flavourzyme activity. Nevertheless, an intensive exoprotease treatment would reduce the content of large and medium peptides (i.e. would be detrimental to the maximization of F_{2500}).

In this context, where two or more objectives are pursued and the improvement of one objective may be detrimental for the other, multiobjective optimization techniques are necessary. Contrarily to single optimization, the result of a MOP is not a single solution but a set of intermediary solutions (i.e. Pareto optimal solutions) which satisfy to a certain degree both objectives.

The Pareto Front is defined as the set of non-inferior (or efficient, non-dominated) solutions which satisfies all the constraints of the MOP. Non-inferior solutions are those that cannot improve one objective function without degrading at least one of the others (Halsall-Whitney & Thibault, 2006; Mavrotas, 2009).

The most widely used methods to approximate the Pareto domain are the weighted sum method and the ε-constraint method. Both techniques can provide a representative subset of the Pareto Front, which in most cases is adequate for the decision-maker. The former consists in obtaining a single objective function, expressed as linear combination of the individual objectives of the MOP. The combined objective function is obtained by means of a set of weight factors, which quantify the relative importance attached to every individual objective (Kim & de Weck, 2004; Marler & Arora, 2009). The selection of the weight factors, as well as the adequate scaling of the single objective functions, are the main issues concerning the application of the weighted sum method. Indeed, there are several combinations of weights leading to the same non-inferior solution, resulting in an inefficient use of the computation time (Mavrotas, 2009). The ε-constraint method consists in optimizing one single objective while the others are employed as constraint equations (Chaturvedi & Bandyopadhyay, 2014; R. Morales-Medina, Pérez-Gálvez, Guadix, & Guadix, 2017). In our case, the optimization problem was stated as finding the operating conditions (i.e. pH, T. t_{exo}) within their experimental range which maximizes the fraction F_{2500} for a fixed value of F_{250} (noted as ε), as follows:

$$
\begin{aligned}
&\textit{Maximize } F_{2500}(pH, T, t_{exo}) \\
&\textit{subjected to}: \\
&\quad 7.2 \le pH \le 8.8 \\
&\quad 41.6 \le T \le 58.4 \\
&\quad 19 \le t_{exo} \le 221 \\
&\quad F_{250}(pH, T, t_{exo}) = \varepsilon
\end{aligned}
\qquad [6]
$$

The main advantage of this method is that the decision-maker can control the number of non-dominated solutions which depict the Pareto approximation (Mavrotas, 2009). In our case, we can adjust the number of grid points of the objective function F_{250} (i.e. the step value of ε) and thus increase the subset of optimal solutions.

3. RESULTS AND DISCUSSION

3.1. Modelization of the degree of hydrolysis

The final values of DH are summarized in Table 1 as a function of the experimental conditions. It can be observed that the observed values of final DH varied from 11.79% to 32.18%. The final degree of hydrolysis was modeled as a function of the operation conditions (i.e. pH, reaction temperature and duration of the exoprotease treatment) by the equation [7].

$$DH = -717.6120 + 120.9560 \cdot pH + 7.8787 \cdot T + 0.1635 \cdot t_{exo} - 7.2015 \cdot pH^2 - 0.0685 \cdot T^2 + 0.0004 \cdot t_{exo}^2 \quad [7]$$

This predictive model was obtained by non linear regression and reduced by backward elimination, where the terms with associated p-value lower than 5% were removed. The goodness of fit of the reduced model was confirmed by the coefficient of determination (R^2= 93.3%, R^2 = 88.8% adjusted to the degrees of freedom).

The predicted model of DH allowed the generation of a surface plot (Fig. 1a) where the final degree of hydrolysis, calculated by equation [7], was plotted against the conditions of pH and temperature in the reaction vessel. The response surface depicted in Fig. 2a presents a typical rising ridge condition, where the maximum is located at the upper value of one of the experimental factors. It was observed that DH was favoured by increasing levels of pH and reaction temperature, reaching a maximum (31.7%) at pH 8.4, 57.4ºC and 183 min of exoprotease treatment.

Alcalase is a broad sprectrum serine protease, which cleaves preferably peptide bonds involving aromatic and methionine residues. It presents maximal proteolitic activity at pH between 8-9 and temperatures 50-60ºC (Adler Nissen, 1986; Valencia, Pinto, & Almonacid, 2014). Given its broad selectivity and high endoprotease activity within our experimental range, it is expectable that extent of the reaction would be mostly determined by the intensity of the Alcalase treatment. As for the Flavourzyme complex, it is sold as enzymatic cocktail extracted from *Aspergillus oryzae*, mostly comprising amino and dipeptidases. These enzymes catalyze the cleavage of terminal or penultimate peptidic bonds, releasing amino acids and dipeptides, respectively. According to Merz et al. (2015), the dipeptidase and

aminopeptidase fractions contained in the Flavourzyme complex presented optimal activity at neutral pH (around 6 – 7) and relatively high temperatures (55-65ºC).

Under the experimental conditions for maximum DH (pH 8.4, 57.4ºC and t_{exo} 183 min), Alcalase presents optimal proteolytic activity, while Flavourzyme aminopeptidase activity is limited. Thus, it is expected that the Alcalase treatment was responsible for most of the solubilization and hydrolysis of the tuna proteins. Nevertheless, the combination of Alcalase and Flavourzyme has proved to achieve higher degrees of hydrolysis than using Alcalase alone (Nchienzia, Morawicki, & Gadang, 2010). A previous digestion of raw proteins by Alcalase increases the availability of N-terminal sites available for the exopeptidase attack. This is in agreement with our results, where maximum DH was reached when Flavourzyme was added after 117 min of Alcalase treatment.

The combined treatment with Alcalase and Flavourzyme has been reported by several authors to obtain hydrolysates from by-products of animal origin. Nchienzia et al. (2010) processed poultry by-products employing as catalysts Alcalase and Flavourzyme (1:2 v/v), either simultaneously or added sequentially (Alcalase and then Flavourzyme). The authours found that the sequential treatment, where Alcalase was allowed to digest the hydrolysate prior to the addition of Flavourzyme, led to slightly higher DH than the simultenous addition of both enzymes. Harnedy et al. (2018) employed a mixture of Alcalase 2.4 L and Flavourzyme 500 L to produce hydrolysates from salmon skin gelatin and trimmings, which were investigated for their antidiabetic potential. Compared to the sole addition of Alcalase 2.4 L, the incorporation of Flavourzyme 500 L to the enzyme mixture increased the final degree of hydrolysis of Atlantic salmon (*Salmo salar*) skin gelatin and trimmings by 56% and 28%, respectively. The same combined treatment was chosen to produce protein hydrolysates from blue whiting (*Micromesistius poutassou*), attaining an extensive hydrolysis of the muscle (DH 29% at 4 hours) (Harnedy et al., 2018b).

3.2. Modelization of the molecular weight distribution

The molecular weight distribution of the hydrolysates was determined by size exclusion chromatography as explained in the Materials and Method section. As an example, the Fig. 2 shows the SEC profiles of the experimental hydrolysates no. 13, 10 and 15, all of them produced at pH 8, 50ºC and increasing durations of Flavourzyme treatment (i.e. 19, 120 and

221 min, respectively). For discussion purposes, we divided the SEC profiles into four distinct regions, noted as F_{5000}, F_{2500}, F_{700} and F_{250}. Regardless the hydrolysis conditions, all the SEC profiles presented a dominant broad region between 2500 and 5000 Da (F_{5000}), associated to partially hydrolysed proteins. A second peak, between 700 – 2500 Da, corresponds to a range of peptides with chain length between 4 – 15 residues. The third region between 250 – 700 Da, corresponds to short peptides between 2 and 3 residues. Finally, a single peak was observed at retention times above 43 min (i.e. molecular weight below 250 Da, F_{250}). This peak is associated to free amino acids released during the hydrolysis. It was observed that increasing durations of the Flavourzyme treatment had a significant impact on the content (expressed as percentage areas) of free amino acids in the final hydrolysate, evidencing the aminopeptidase activity of the Flavourzyme complex. As mentioned above, we focused on the fractions F_{2500} and F_{250} to assess the influence of the operational conditions upon the molecular weight distribution of the resulting hydrolysates. Both fractions are of interest as ingredients for aquaculture diets, based on scientific literature supporting their positive effect on larval growth and feed palatability (Nunes et al., 2006; Li et al., 2009).

Both weight fractions F2500 and F250 were modeled as a function of the operation conditions (i.e. pH, reaction temperature and duration of the exoprotease treatment) by the complete quadratic model stated by equation [5]. Both empirical models were reduced by backward elimination, where those terms presenting an associated probability value less than 5% were removed. The resulting reduced models for F_{2500} and F_{250} are expressed equations [8] and [9], respectively.

$$F_{2500} = 128.603 - 11.8449 \cdot pH - 1.9119 \cdot T - 0.2819 \cdot t_{exo} + 0.1055 \cdot pH \cdot t_{exo} + 0.0345 \cdot T^2 - 0.0128 \cdot T \cdot t_{exo} + 0.0002 \cdot t_{exo}^2 \quad [8]$$

$$F_{250} = 8.7249 - 32.6041 \cdot pH + 3.9332 \cdot T + 0.8255 \cdot t_{exo} + 0.8051 \cdot pH \cdot T - 0.0752 \cdot pH \cdot t_{exo} - 0.1019 \cdot T^2 - 0.0008 \cdot t_{exo}^2 \quad [9]$$

The goodness of fit of both reduced model was confirmed by their coefficients of determination R^2, which were 89.8% and 89.6% for F_{2500} and F_{250}, respectively

The Figures 1b and 1c represent the surface plots of F_{2500} and F_{250}, calculated by equations [8] and [9], against the pH and the reaction temperature. The duration of the Flavourzyme

treatment (t_{exo}) was set at its optimum value for maximizing each percentage area (i.e. 19 min and 172 min for maximum F_{2500} and F_{250}, respectively). The surface plot for F_{2500} presents a hillside shape, where the maximum was located at one extreme vertex of the design region. According to the optimization procedure, the experimental conditions for maximum F_{2500} (42.5 % of total area) were pH 7.2, 58.4ºC and the lowest level of Flavourzyme treatment (i.e. 19 min). These conditions are outside the range of optimal proteolitic activity for Alcalase, which reduces the availability of terminal residues for Flavourzyme attack (Nchienzia et al., 2010). Moreover, the duration of the Flavourzyme treatment, which is responsible for the release of short chain peptides and amino acids, is short (19 min). Several studies on larval rearing diets confirm that the partial substitution of dietary protein (mainly under the form of fish meal) by fish protein hydrolysates has a positive impact on both weight gain and survival rate (Zheng et al., 2013; Ovissipour et al., 2014; Cai et al., 2015; Khosravi et al., 2017). Specifically, Kotzamanis et al. (2007) recommend the enrichment of dietary protein with short peptides within the range 500 – 2500 Da for optimal larval development.

The surface plot of the content of free amino acids (F_{250}, Fig. 1c) shows that the release of free amino acids is favoured by low levels of pH and moderate temperatures around 50ºC. The maximum for F_{250} (30.7% of total area) was reached at pH 7.2, 48.3ºC and 172 min of Flavourzyme treatment. These values of temperature and duration of the exoprotease treatment assure maximal amino acid content in the range 30.7 – 25.9% for the pH interval 7.2 – 8.8. Alcalase presents optimal proteolytic activity at pH between 8-9 and temperatures 50-60ºC (Adler Nissen, 1986; Valencia et al., 2014). Under the optimal conditions estimated above, the substrate was digested for 128 min by Alcalase before the addition of Flavourzyme. The release of free amino acids was then controlled by the aminopeptidase activity of Flavourzyme, which correlates inversely with pH within the interval 7.2 – 8.8. Most of studies dealing with amino acid supplementation of aquaculture diets highlight their role as feeding stimulants, increasing feeding utilization and therefore weight gain, especially in juvenile individuals (Nunes et al., 2006; Li et al., 2009; Barroso et al., 2013; Morais, 2017). Besides being feeding attractants, some amino acids are important regulators of key metabolic pathways involved in larval immunity, resistance, growth and nutrient assimilation (Li et al., 2009). To this regard, Cai et al. (2015) concluded that dietary amino acids and small peptides had a significant effect on digestion and absorption of protein in yellow croaker (*Larimichthys crocea*) larvae, by modulating the activity of some specific enzymes and hormones.

3.3. Composition of free amino acids

The Table 2 shows the amino acid profile of the fraction F_{250} recovered from the hydrolysate obtained at optimal conditions (pH 7.2, 48.3ºC and 172 min of Flavourzyme treatment) for maximal content of free amino acids. Under these conditions the percentage area below 250 Da was F_{250}=31.1 ± 0.6% w/w (mean ± standard deviation of triplicate measurements), close to the predicted maximum (30.7% w/w) computed by optimization.

Dietary amino acids play a significant role in supporting growth and physiological development of aquaculture species. The amount essential amino acids supplied by diet is especially important, since they cannot be synthesized by fish metabolism. The amino acid composition of fish protein hydrolysates is highly influenced by the source of protein employed for the hydrolysis. For instance, aspartic and glutamic acids are usually found in large concentrations in hydrolysates from fish muscle (Chalamaiah et al. 2012). In our case, the tune head hydrolysate presented high concentration of arginine and glycine, as shown in Table 2. This is in line with the amino acid composition reported for tuna by-products hydrolysates produced by Flavourzyme, which exhibited high concentrations of arginine, lysine and histidine (Nilsang, Lertsiri, Suphantharika, & Assavanig, 2005). These are essential amino acids for fish species, which are required in the formulation of feeding diets. Compared to other protein sources, the nutritional quality of our tuna head hydrolysate, in terms of essential amino acids (46.5 % molar), is higher than most of the hydrolysates found in literature. As far as the author´s knowledge, only the hydrolysate produced from round scad (*Decapterus maruadsi*) using Flavourzyme (Thiansilakul, Benjakul, & Shahidi, 2007) had a slightly higher content of essential amino acids (48% molar) . The content of hydrophobic amino acids is also relevant since it could determine a range of functional and biological properties in the hydrolysate. For instance, Cai et al. (2015) provided evidence that some small molecular weight compounds such as free amino acids may be essential for the normal absorption of short peptides in the intestine of yellow croaker (*Larimichthys crocea*) larvae. More specifically, Chi et al. (2015) related the presence of hydrophobic amino acids to the antioxidant activity displayed by skipjack tuna (*Katsuwonus pelamis*) muscle hydrolysates. In our case, the molar percentage of hydrophobic amino acids (60.2 % molar) is in the upper limit of the range (30-60%) usually reported for fish hydrolysates (Chalamaiah et al. 2012).

3.4. Bi-objective optimization of the molecular weight distribution

The results obtained after single optimization of the fractions F_{2500} and F_{250} are conflicting, since the optima are located under different operating conditions. For instance, under the optimal conditions for maximum F_{2500} (42.4%), the amount of free amino acids in the resulting hydrolysate were only 2.28%. Contrarily, optimal hydrolysis conditions for maximal F_{250} (30.7%) lead to 15.2% of the fraction F_{2500}. As a general trend, the reaction conditions leading to increasing levels of free amino acids were detrimental to the content of fraction F_{2500} in the final hydrolysate. This suggested employing a bi-objective optimization approach, able to find intermediary solutions satisfying to a certain degree both objectives (i.e. maximization of F_{2500} and F_{250}). There is an increasing interest in scientific literature in designing valorization strategies through multi-criteria optimization techniques. For instance, Antelo et al. (2015) proposed a multiobjective optimization technique (the epsilon-constraint method) to select the best processing route to convert discarded fish species into valuable products. Moreover, multiobjective optimization methods have already been employed to produce fish protein hydrolysates from horse mackerel with optimized antioxidant properties (Morales-Medina et al., 2017). Similarly, García-Moreno et al. (2014) optimized simultaneously the yield and oxidation stability of fish oil extracted mechanically from sardine discards.

Considering the bi-objective optimization problem stated in [6], the ε-constraint optimization procedure can be formulated mathematically as follows:

$$\begin{aligned} &\textit{Maximize } F_{2500} = 128.603 - 11.8449 \cdot pH - 1.9119 \cdot T - 0.2819 \cdot t_{exo} + 0.1055 \cdot pH \cdot t_{exo} \\ &\qquad + 0.0345 \cdot T^2 - 0.0128 \cdot T \cdot t_{exo} + 0.0002 \cdot t_{exo}^2 \\ &\textit{subjected to}: \\ &\quad 7.2 \le pH \le 8.8 \\ &\quad 41.6 \le T \le 58.4 \\ &\quad 19 \le t_{exo} \le 221 \\ &\quad F_{250} = 8.7249 - 32.6041 \cdot pH + 3.9332 \cdot T + 0.8255 \cdot t_{exo} + 0.8051 \cdot pH \cdot T \\ &\qquad - 0.0752 \cdot pH \cdot t_{exo} - 0.1019 \cdot T^2 - 0.0008 \cdot t_{exo}^2 = \varepsilon \end{aligned} \qquad [10]$$

Considering the nature of F_{2500} and F_{250} within the experimental range of pH, T and t_{exo}, the Pareto Front is a continuous curve over its entire domain. Table 3 presents a set of 17 solutions of the optimization problem [10], which represents a discrete approximation of the

Pareto Front. Each row in Table 3 contains the optimal operating conditions (i.e. pH, T, t_{exo}) for maximizing the content of F_{2500} at a fixed value of free amino acids between 2.28 % and 30.7%. The lower level of free amino acids (2.28%) in Table 3 was obtained under the optimal conditions (pH 7.4, 58.4ºC and 19 min of exoprotease treatment) for maximal content of the fraction F_{2500} (42.4%). Above this value, a set of intermediary solutions was computed by allowing the content of F_{250} to increase up to its single optimum 30.7% (pH 7.2, 47.3ºC and 169 min). Within this interval, the Pareto Front can be broken down into three main subsets:

- Subset I. The content of free amino acids increased from 2.28% up to 22% by keeping temperature at 58.4ºC and allowing pH to increase from 7.4 up to its upper bound 8.8. All the optimal solutions in this range implied short durations of the Flavourzyme treatment, increasing slightly from 19 to 30 min. Under these experimental conditions, an increase of pH favoured the endoprotease activity of Alcalase. This action increased the availability of terminal sites for Flavourzyme attack, and thus the release of free amino acids.
- Subset II. An augmentation of F_{250} from 22% to 27.6% was achieved by keeping pH at its upper experimental bound (pH 8.8) and allowing the variable t_{exo} to increase from 30 to 94 min. As for reaction temperature, it slightly decreased from 58.4ºC to 55.5ºC.
- Subset III. The triplet (pH 8.8, 55.5ºC, 94 min) is a critical point above which an augmentation of the content free amino acids determined a sharp decrease of pH and temperature down to their lower bounds (7.2 and 42.6ºC, respectively). From this point on, the value of F_{250} could be improved by rising both the reaction temperature and the duration of the Flavourzyme treatment. The maximum value of free amino acids allowable by hydrolysis conditions was 30.7% (pH 7.2, 48.3ºC, 169 min).

In practice, the selection of a single optimal solution inside the Pareto Front depends on several factors such as the morphological state (i.e. larvae or juvenile) of the fish farmed, the rearing conditions (e.g. larval density, rearing system), specific dietary requirements of the reared species or technical or economic viability of the process, among others.

4. CONCLUSIONS

A combined enzymatic treatment employing two commercial proteases, Alcalase and Flavourzyme, was proposed to obtain tuna head hydrolysates intended for aquaculture

ingredients. Statistical modeling coupled with multiobjective optimization was successfully employed to relate hydrolysis operating parameters (i.e. pH, reaction temperature and duration of the Flavourzyme treatment) to the degree of hydrolysis (DH) and the molecular weight profile of the resulting hydrolysate. More specifically, we focused on two fractions, the peptides between 700 – 2500 Da (F_{2500}) and the free amino acids (fraction below 250 Da, F_{250}), due to their interest as ingredients in aquaculture diets.

The proposed statistical models fitted satisfactorily the observed responses (i.e., DH, F_{2500}, F_{250}) to the operating conditions (r^2 = 93.3%, 89.8% and 89.6%, respectively). The predictive model for DH concluded that this variable was favoured by increasing levels of pH and reaction temperature, reaching a maximum (31.7%) at pH 8.4, 57.4ºC and 183 min of exoprotease treatment. Under these conditions, Alcalase presents optimal proteolytic activity, being responsible for most of the solubilization and hydrolysis of the substrate. As for the Flavourzyme treatment, it had a major impact on the molecular weight profile of the hydrolysates, especially on the content of free amino acids. The optimal reaction conditions for maximimizing the release of free amino acids (i.e, pH 7.2, 43-49ºC, duration of the Flavourzyme treatment above 160 min) were detrimental to the content of small chain peptides F_{2500}. A bi-objective optimization approach was then proposed, able to find a set of intermediary solutions (Pareto Front) satisfying to a certain degree both objectives (i.e. maximization of F_{2500} and F_{250}). This approach allows predicting the optimal operating parameters to produce a hydrolysate with a desired molecular weight profile. The selection of a single optimal solution inside the Pareto Front depends to a large extent on the specific dietary requirements of farmed species. Although dietary protein levels are determined for most of the commercial species, further research on amino acid requirements and composition is necessary.

The novelty of this work lies in the application of multiobjective optimization for the design of protein dietary supplements for aquaculture, with a focus on their molecular weight profile. This is the first approximation to the production of taylor-made hydrolysates able to fulfill specific dietary requirements of farmed species.

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FIGURE AND TABLE CAPTIONS

- **Figure 1**. Surface plots of (a) final degree of hydrolysis, (b) percentage area of peptides in the range 700-2500 Da (F_{2500}) and (c) percentage area below 250 Da (F_{250}) as a function of pH and reaction temperature at fixed times of Flavourzyme treatment.

- **Figure 2**. Size Exclusion Chromatography profiles of the hydrolysates produced at pH 8, 50ºC and increasing durations of the Flavourzyme treatment (t_{exo}).

- **Table 1**. Final degree of hydrolysis (DH, %) and molecular weight distribution (percentage area, %) of the tuna head hydrolysates as a function of the reaction parameters.

- **Table 2**. Composition of free amino acids (molar percentage) of the hydrolysate obtained under the conditions for maximal F_{250} (pH 7.2, 49ºC and 172 min of Flavourzyme treatment).

- **Table 3**. Set of optimal solutions for the multiobjective optimization problem: maximization of F_{2500} at a fixed content of free amino acids F_{250}.

Table 1. Final degree of hydrolysis (DH, %) and molecular weight distribution (percentage area, %) of the tuna head hydrolysates as a function of the reaction parameters.

Exp. No.	Operating conditions				Final DH	Molecular weight distribution Percentage area, %				
	pH	T, ºC	t_{endo}, min	t_{exo}, min	%	>5000 Da	5000 – 2500 Da	2500 -700 Da	700 – 250 Da	< 250 Da
1	8.5	45	240	60	15.17	8.68	10.53	23.63	40.18	17.3
2	8.5	45	120	180	20.71	8.77	10.43	23.76	41.87	15.4
3	7.5	45	240	60	7.49	3.54	6.41	19.40	41.27	29.7
4	8	41.6	180	120	11.80	6.84	8.93	22.89	41.15	20.19
5	8.5	55	120	180	25.83	5.04	7.76	20.07	44.72	22.7
6	7.5	55	240	60	18.32	9.18	10.59	29.29	35.51	15.43
7	7.2	50	180	120	15.58	4.61	7.13	18.04	42.33	28.2
8	8.5	55	240	60	25.46	9.68	7.38	22.93	36.91	23.10
9	8.8	50	180	120	26.73	4.50	7.59	19.10	42.34	26.8
10	8	50	79	221	28.33	4.10	7.31	20.35	45.15	22.0
11	7.5	45	120	180	14.09	4.46	7.40	19.26	42.85	24.8
12	7.5	55	120	180	27.51	4.25	11.74	10.11	43.96	25.69
13	8	50	281	19	24.90	4.65	9.62	22.55	41.24	17.4
14	8	58.4	180	120	29.91	6.35	10.50	19.94	41.50	21.71
15	8	50	180	120	24.76	4.36	7.66	19.97	42.80	25.6
16	8	50	180	120	24.76	3.52	6.39	17.78	41.89	29.0

Table 2. Composition of free amino acids (molar percentage) of the hydrolysate obtained under the conditions for maximal F_{250} (pH 7.2, 49ºC and 172 min of Flavourzyme treatment).

Amino acid	Molar percentage
HIS	5.19 ± 0.06
ILE	5.12 ± 0.05
LEU	8.70 ± 0.06
LYS	3.08 ± 0.06
MET	3.78 ± 0.07
PHE	9.34 ± 0.03
TYR	6.10 ± 0.06
PRO	0.69 ± 0.03
THR	4.22 ± 0.23
ARG	18.17 ± 0.32
VAL	7.04 ± 0.11
ASP + ASN	0.03 ± 0.00
GLY	15.86 ± 0.18
ALA	7.40 ± 0.06
SER	3.64 ± 0.08
GLU + GLN	1.65 ± 0.07
TAA[1]	100
THAA[2]	60.24
TEAA[3]	46.47

Results are the average of triplicate determinations ± standard deviation.
(1) TAA: total amino acids; (2) THAA: total hydrophobic amino acids; (3) TEAA: total essential amino acids.

Table 3. Set of optimal solutions for the multiobjective optimization problem: maximization of F_{2500} at a fixed content of free amino acids F_{250}.

Fraction below 250 Da F_{250}, %	**pH**	**T, ºC**	**t_{exo}, min**	**Fraction 700-2500 Da F_{2500}, %**
2.28	7.39	58.4	19	**42.4**
4	7.52	58.4	19	41.1
6	7.68	58.4	19	39.6
8	7.83	58.4	19	38.1
10	7.98	58.4	19	36.6
12	8.14	58.4	19	35.1
14	8.29	58.4	19	33.6
16	8.45	58.4	19	32.0
18	8.60	58.4	19	30.5
20	8.72	58.4	22	29.0
22	8.80	58.4	30	27.5
24	8.80	58.4	51	25.8
26	8.80	57.7	76	23.3
27.6	8.80	55.5	94	20.6
28	7.20	42.6	181	20.0
30	7.20	45.2	164	17.2
30.7	7.20	48.3	169	15.2

Figure 1. Surface plots of (a) final degree of hydrolysis, (b) percentage area of peptides in the range 700-2500 Da (F_{2500}) and (c) percentage area below 250 Da (F_{250}) as a function of pH and reaction temperature at fixed times of Flavourzyme treatment.

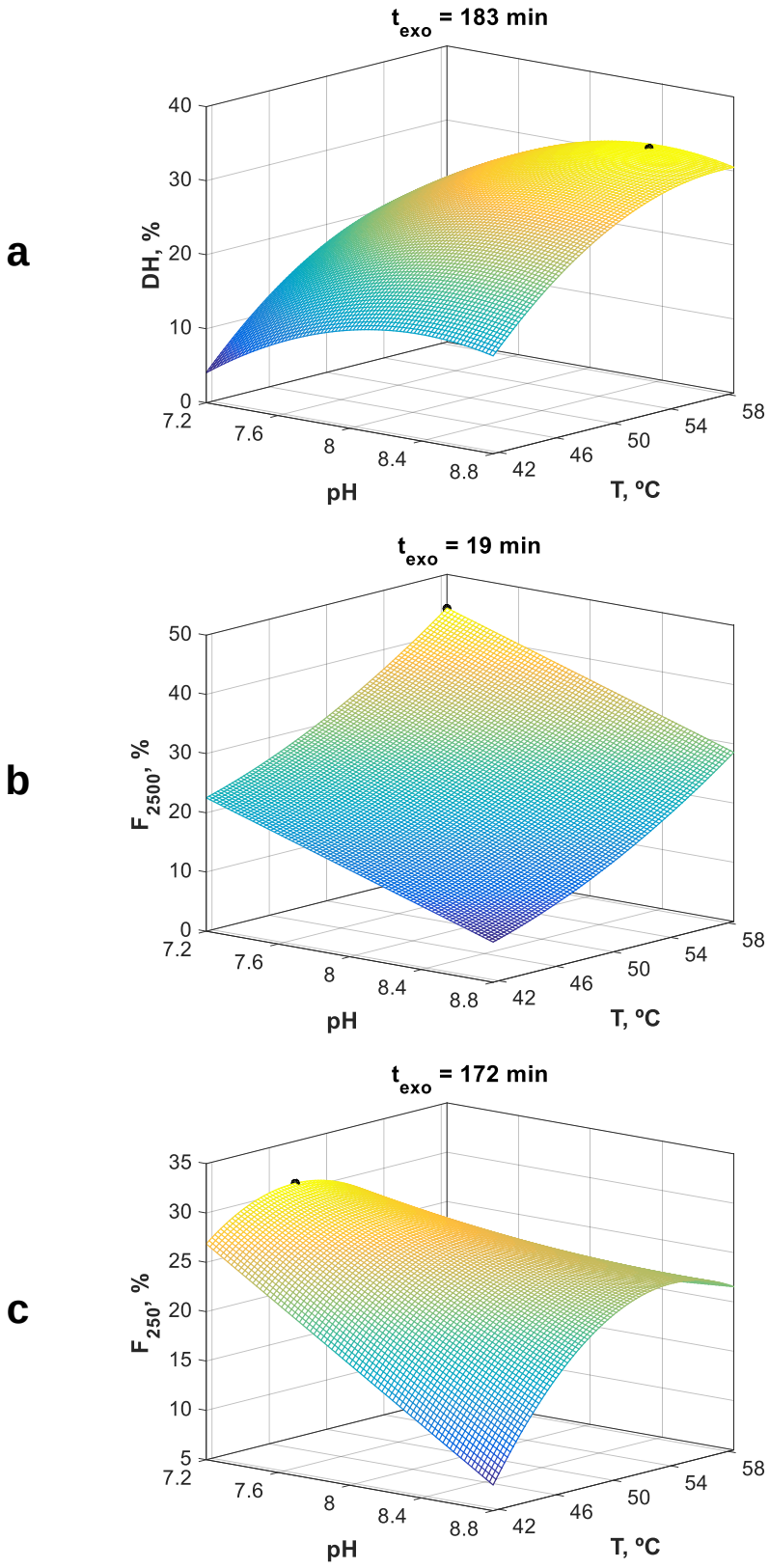

Figure 2. Size Exclusion Chromatography profiles of the hydrolysates produced at pH 8, 50ºC and increasing durations of the Flavourzyme treatment (texo).