Aquaculture 477 (2017) 28–34

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Estimating the effect of different factors on the digestive bioaccessibility of protein by the Senegalese sole (*Solea senegalensis*); combination of response surface methodology and *in vitro* assays

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ARTICLE INFO

Keywords:
In vitro
Digestibility assays
Protein
Factorial design
Solea senegalensis

ABSTRACT

The present work uses a novel approach to develop a mathematical model explaining the effect of three main factors - temperature, total reaction time and enzyme:substrate ratio - in the hydrolysis of protein by the intestinal enzymes of the Senegalese sole. The methodology combines a factorial design based in the Response Surface Methodology and *in vitro* digestibility assays adapted to the physiology and culture conditions of the species. The model revealed that, within the physiological ranges considered in the study, the efficiency of protein hydrolysis by the intestinal enzymes of the sole was linearly correlated to two of the selected factors; directly to incubation time and inversely to the amount of substrate. The effect of temperature was not linear but quadratic, hence showing a maximum at 23 °C. The information obtained can be used as a tool to maximize the biological response under variable conditions and may orientate some on-farm feeding practices.

1. Introduction

Digestion is a highly complex process providing animals with the nutrients required for their maintenance and growth. It comprises a series of physical and chemical transformations of the ingested food aimed to reduce their size and to simplify their initial structure with the objective of increasing bioavailability of proteins, fats and carbohydrates. The estimation of the efficiency of the digestion on such nutrients or on whole feeds is a key step in the evaluation of their potential nutritional value that is routinely carried out using a wide range of specific trials known as “digestibility assays”. Although these assays possess and unquestionable practical value, they follow the philosophy of the “black box”, that is, they provide results based in the changes in the composition of the initial (feeds) and end (faeces) products of the digestion, but no information on which factors and to what extent affect that final result. (Hajen et al., 1993; Rasmussen et al., 2009).

In this sense, consideration of the digestive system of a given species as a more or less complex bioreactor may give an insight on the relative influence of different factors that affect its functionality and help to provide an explanation of the results obtained after food hydrolysis, giving orientations to improve the efficiency of such process and hence to increase the potential bioavailability of the main nutrients. This can only be achieved through the total or partial modelling of the different steps involved in the digestion from either a theoretical or a practical perspective. The theoretical approach was initiated by Penry and Jumars (1986, 1987), who developed a complete theory to establish operating similitudes between animal digestive systems and reactors. These authors considered the digestion process as a problem of chemical engineering, and suggested that the different configurations of digestive tracts present in animals are the response to the need of an optimal food processing oriented to maximize gain of energy and nutrients, given the particular anatomy of each animal and the availability of food. The theory of the chemical reactors have been used by several authors to analyse the relations existing between diet composition, food processing and gut morphology, allowing to obtain interesting conclusions on how the digestion process is adapted to food nutrient availability in different animals (Karasov and Diamond, 1983; Whelan and Schmidt, 2007). Nevertheless, up to date this approach has been still scarcely applied to the study of digestive function in fish or other aquatic animals (Horn and Messer, 1992; German, 2009). On the other hand, a great number of physiologists and nutritionists have developed practical models of the guts in a wide range of species, using different types and combinations of enzymes working under conditions

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http://dx.doi.org/10.1016/j.aquaculture.2017.04.037
Received 27 February 2017; Received in revised form 17 April 2017; Accepted 18 April 2017
Available online 02 May 2017
0044-8486/

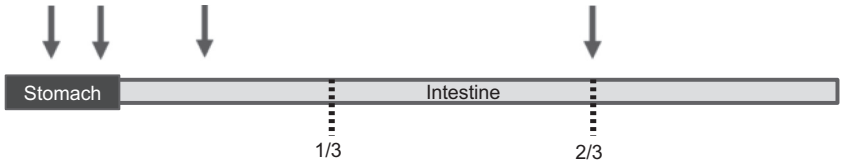

Fig. 1. Diagram of the gastrointestinal tract showing the places (arrows) in which the luminal pH was measured.

resembling those existing in the living animal. For this purpose they have designed a variety of physical arrangements; from quite simple batch reactors (Saunders et al., 1972, Parsons, 1991) to the very complex multi-compartment reactors used in human nutrition studies (Minekus et al., 1995). These models have been routinely used to perform what are generally known as "*in vitro* digestibility assays", widely used in nutritional studies of humans and terrestrial animals (Boissen, 2000).

A common approach, used by chemical engineers to optimize hydrolysis conditions in reactors, is the assessment of the influence of different variables using a factorial design. In factorial experiments, different levels of multiple factors are investigated simultaneously and one factor can be examined at different levels of the other factor or factors. Within this perspective, one of the most powerful tools is the design of such experiments using the Response Surface Methodology (RSM). RSM consists of a group of mathematical and statistical techniques that can be used to define the relationships between a selected response and several independent variables, generating a mathematical model. The graphical perspective of the mathematical model has led to the term Response Surface Methodology (Baş and Boyacı, 2007). RSM has several advantages compared to the classical experimental or optimization methods in which one variable at a time is used. Firstly, RSM offers a large amount of information from a small number of experiments. Indeed, classical methods are time consuming and a large number of experiments are needed to explain the behaviour of a system. Secondly, in RSM, it is possible to observe the interaction effect of the independent parameters on the response. Especially in biochemical processes, the interaction effect of the parameters such as synergism, antagonism and addition would be more critical. One of the more widely used designs to develop response surface models is the Box-Behnken Design (BBD) (Box and Behnken, 1960). BBD is based on the construction of balanced incomplete block designs and requires at least three levels for each factor. In BBD, the level of one of the factors is fixed at the centre level while combinations of all levels of the other factors are applied (Kocabaş, 2001; Myers and Montgomery, 2002). Considering the same number of factors, BBD approach often requires fewer experimental points as compared to other response surface designs, being therefore more suitable when there are limitations imposed by the cost of the assays or, as in the present case, by the amount of available biological material. RSM is routinely applied in the optimization of industrial processes based in enzyme hydrolysis (Zhang et al., 2013; Dey and Dora, 2014), but more recently, it has also been used in the assessment of optimal conditions to be used when modelling biological digestion *in vitro* (Hollebeeck et al., 2013). A number of recent works have focused in the development of *in vitro* digestion models for aquatic species. These models, with a variety of physical arrangements and more or less physiologically based operational parameters, are mainly oriented to test differences in the nutritional value of feeds and ingredients (see review by Moyano et al., 2014). Nevertheless, none of these studies used the *in vitro* model to get more information about the main features driving the hydrolysis within the gut of the fish from an applied perspective.

The Senegalese sole (*Solea senegalensis*) is a high market value flatfish, considered as candidate to diversify the south-western European aquaculture (Morais et al., 2016). The production of this species has been increasing in the last years in European countries and China. Accordingly, a number of recent studies have been oriented to assess optimal diets and nutritional efficiency of ingredients for this species (Costas et al., 2012; Rodiles et al., 2012; Borges et al., 2013; Marinho et al., 2014; Conde-Sieira et al., 2015; Valente et al., 2016). The aim of the present study was to develop a theoretical model of the protein hydrolysis taking place in the gut of this fish through the combination of *in vitro* assays and a BBD, considering the effects of three different variables; temperature, reaction time, and enzyme:substrate ratio. The model can be used as a preliminary tool to predict in a fast, non-invasive, and inexpensive manner the extent on which efficiency of the digestion process is affected by such factors in the species.

2. Material and methods

2.1. Biological material

Adult Senegalese sole individuals (N = 33; total biomass 9034 g; individual weight from 212 to 575 g; average weight 274 ± 106 g) were used to obtain the enzyme extracts used in the *in vitro* assays. Fish were maintained at the ICMAN experimental facilities (REGA ES110280000311) in 5000 L tanks with flow-through water system and 14 h light/10 h dark photoperiod. In order to determine physiological parameters required to establish the operative conditions for the *in vitro* assays, fish were fed *ad libitum* with one meal (commercial feed) and sampled at different moments (2, 3, 5, and 7 h after feeding) to assess postprandial variation in pH in different parts along the gastrointestinal tract (Fig. 1) as well as total production of proteases. Fish were killed by immersion in ice-cold water containing a few drops of clove oil as anaesthetic and immediately dissected to obtain the digestive tract. The experiments were carried out in compliance with the Guidelines of the European Union Council (2010/63/EU) and Spanish legislation for the use of laboratory animals, with approval of the Bioethics Committee of the Spanish National Research Council for project EFISHDIGEST (AGL2014-52888-R).

Luminal pH was measured in the duodenal portion of the intestine during a period from 2 to 7 h after feeding using a pH microelectrode (Thermo Scientific Orion) following the procedure explained in Yúfera and Darias (2007). Measurements were maintained in a narrow range, with an average value close to 7.0; for this reason, this was the pH value selected for further development of the *in vitro* assays (Fig. 2).

Pepsin activity was determined at pH 2.5 according to the methodology from Anson (1938) using haemoglobin as substrate. Total alkaline protease activity was measured at pH 8.5 according to the Kunitz's method modified by Walter (1984) using casein as substrate. One unit of enzyme activity was defined as 1 mM of tyrosine released per min, using 1280 M^{-1} cm^{-1} as the molar extinction coefficient at 280 nm. Measurements confirmed the already reported low functionality of the stomach (Yúfera and Darias, 2007), characterized by a high pH ranging from 6.2 to 6.6 and negligible pepsin activity ranging between 0.33 and 1.00 U of protease/g fish fresh weight (Fig. 2). For this reason, only the alkaline stage of the digestion was considered in the model. Total alkaline protease ranged between 1.99 and 4.71 U of protease/g fish fresh weight. Enzyme extracts used in the *in vitro* assays were prepared by mechanical homogenization of the proximal intestine in distilled water (1:10 w/v) followed by centrifugation (3220 × *g*, 20 min, 4 °C). The supernatant was then filtered through a dialysis system with a MWCO of 10 kDa (Pellicon XL, Millipore ®) to remove

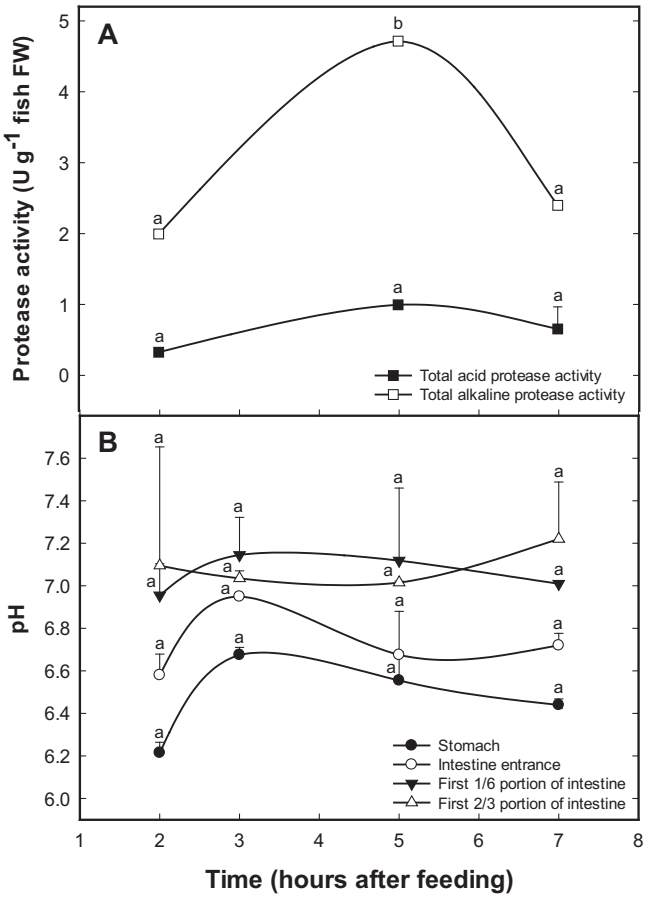

Fig. 2. Values of protease activity (A) and pH (B) in the stomach and intestine of adult Senegal sole at different moments after food supply. Values in each series not sharing a common letter are significantly different with $p < 0.05$.

small molecules and the concentrated extract was freeze dried until used in the assays.

2.2. In vitro assays

The *in vitro* hydrolysis assays were carried out using a membrane bioreactor modified from that described in Morales and Moyano (2010). The device consists in two chambers separated by a semi-permeable membrane of 3500 MWCO (ZelluTrans/Roth®). The upper part of the reaction chamber contains the mixture of the desired substrate and the enzyme extract dissolved in buffer (50 mM phosphate buffer, pH 7.0; 50 mM $NaCl_2$, 45 μmol L^{-1} sodium taurocholate) while the lower part of the reaction chamber only contains the buffer. The protein substrate used in the assays was a high quality fishmeal (BIOCEVAL, A Coruña, Spain) with a 75.4% crude protein. A magnetic stirrer maintains the upper mixture under continuous agitation (280 rpm) and the small molecules released during the hydrolysis pass across the membrane, being recovered in the lower chamber at different moments during the desired reaction time. The complete arrangement was maintained within a thermal chamber at the desired temperature. Total concentration of amino acids released as a result of the hydrolysis was measured using the o-phtaldialdehyde method as detailed by Church et al. (1983). Values measured as equivalent μg of leucine were transformed into μg of N considering the relative amount of N in the leucine molecule.

2.3. Experimental design and statistical analysis

Three key factors affecting the extent of protein hydrolysis in the intestine of Senegalese sole were examined:

- *Temperature;* it was taken into consideration because, in ectotherms, it exerts an important effect on the whole metabolism, and obviously in the digestive process, The range used in the assay was based on the average maximum and minimum seawater temperatures registered in farms where Senegalese sole is produced in south-western Europe; from 16 to 26 °C (personal communication).

- *Variations in enzyme:substrate ratio*; these were considered to reflect different situations produced as a result of a variable intake of food, and hence in the amount of available substrate present in the gut that is exposed to a given amount of enzyme production. The ratios were defined considering both the total activity of protease, measured in the different individuals used in the assay and referred to 100 g average weight, and the estimated protein intake by meal, according to the average contents in commercial feeds and rations used in the species at the above mentioned temperature range (Efico Sigma 874; Biomar Group, Denmark). The range determined was 0.48–0.84 U mg^{-1} protein and it was achieved by maintaining a fixed amount of enzyme and changing the relative amount of protein present in the assays from 700 to 400 mg.

- *Total reaction time*; changes in food transit rates and, therefore, in the gut retention time available for hydrolysis were considered. Values were estimated according both to feeding frequencies employed in common farming protocols used for growing fish (from 30 to 100 g), being from 4 to 8 h data reported for the food permanence in the anterior and medium intestine by Dias et al. (2010) for juveniles (140 g) of this species.

The effect of these factors was previously tested independently to confirm they have an effect on protein hydrolysis produced by fish enzyme extracts. A 3-level Box-Behnken factorial design was obtained with the help of the Minitab® 17 software (Minitab Inc.), to study the effects of the three above mentioned factors on two response variables: a) the net release of amino acids in each assay; and b) the relative efficiency of the hydrolysis, expressed as the percentage that such amino acids represented on the initial protein used in each assay. The combinations of variable values (coded and uncoded) are detailed in Table 1. The experimental domains of the three variable factors were defined depending on the ranges of values previously detailed. Orthogonal least-squares calculation on factorial design data was used to obtain empirical equations describing the release of amino acids as dependent variable related to the aforementioned effects. The general form of the polynomial equations is

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y is the response variable (either mg of AA or mg AA/100 mg protein) X_1, X_2, and X_3 are the independent variables and β_0, β_1, β_2, β_3, β_{11}, β_{22}, β_{33}, β_{12}, β_{13}, and β_{23} are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively.

The experimental data were fitted to a second-order polynomial model (Eq. (1)); the model generated using coded values allowed for the comparison of the effects (linear, quadratic, and interaction) of the three independent factors on the substrates of digestion (Tables 2). The ANOVA test was employed to evaluate the statistical significance of the regression coefficients. Non-significant terms ($p > 0.05$) were deleted from the second-order polynomial and a new polynomial was recalculated to obtain a simplified predictive model for each dependent variable (Table 3). Coefficients of determination R^2, adjusted R^2, and predictive R^2 were also determined for the generated model. Once the fitted regression equations were determined, contour plots were drawn

Table 1
Experimental values obtained for the response variables in the 3-level Box-Behnken factorial design.

Run order	Coded values			Uncoded values			Response variables	
	X_1	X_2	X_3	Temperature (°C)	Time (h)	Total protein as substrate (mg)	Y_1 Total amino acids released (mg)	Y_2 % amino acids released (mg/100 mg initial protein)
1	0	−1	1	21	4	733.0	116.3	15.9
2	−1	−1	0	16	4	566.5	72.9	12.9
3	−1	0	−1	16	6	400.0	82.7	20.7
4	0	0	0	21	6	566.5	152.3	26.9
5	−1	1	0	16	8	566.5	148.0	26.1
6	0	−1	1	21	4	733.0	114.4	15.6
7	1	−1	0	26	4	566.5	89.9	15.9
8	1	0	−1	26	6	400.0	118.8	29.7
9	−1	−1	0	16	4	566.5	88.9	15.7
10	0	0	0	21	6	566.5	142.9	25.2
11	0	0	0	21	6	566.5	134.1	23.7
12	−1	1	0	16	8	566.5	184.4	32.6
13	−1	0	1	16	6	733.0	124.5	17.0
14	0	0	0	21	6	566.5	131.2	23.2
15	1	1	0	26	8	566.5	207.0	36.5
16	0	0	0	21	6	566.5	166.0	29.3
17	1	0	−1	26	6	400.0	110.6	27.6
18	0	1	−1	21	8	400.0	114.2	28.6
19	−1	0	1	16	6	733.0	111.6	15.2
20	1	1	0	26	8	566.5	155.0	27.4
21	0	1	−1	21	8	400.0	115.7	28.9
22	0	1	1	21	8	733.0	251.3	34.3
23	0	−1	−1	21	4	400.0	73.8	18.5
24	1	0	1	26	6	733.0	143.1	19.5
25	0	1	1	21	8	733.0	252.6	34.5
26	0	−1	−1	21	4	400.0	68.4	17.1
27	1	0	1	26	6	733.0	141.9	19.4
28	−1	0	−1	16	6	400.0	80.6	20.1
29	1	−1	0	26	4	566.5	115.1	20.3
30	0	0	0	21	6	566.5	127.0	22.4

Table 2
Preliminary models. Regression coefficients, R^2, and Lack-of-Fit test for the two dependent variables; Y_1: total production of amino acids (mg); Y_2: % protein digestibility. Significant coefficients are highlighted in bold letters.

Model parameters	Y_1 Coef.	SE	*p*-Value	Y_2 Coef.	SE	*p*-Value
Constant	142.25	7.79	0.000	25.11	1.28	0.000
Linear						
Temperature	11.73	4.77	**0.023**	2.25	0.78	**0.009**
Time	43.03	4.77	**0.000**	7.31	0.78	**0.000**
Substrate	30.69	4.77	**0.000**	−1.24	0.78	0.128
Squared						
Temperature^2	−16.85	7.03	**0.026**	−2.34	1.15	**0.050**
Time^2	7.26	7.03	0.314	0.65	1.15	0.578
Substrate^2	−11.17	7.03	0.128	−1.61	1.15	0.178
2-Way interaction						
Temperature*time	−1.70	6.75	0.804	−0.30	1.11	0.789
Temperature*substrate	−2.14	6.75	0.755	−1.23	1.11	0.279
Time*substrate	23.19	6.75	**0.003**	1.92	1.11	0.098
Lack of fit			0.008			0.026
R^2	0.882				0.845	
R^2 (adjusted)	0.829				0.775	
R^2 (predicted)	0.719				0.633	

Table 3
Refined model for Y_2 (% protein digestibility). Regression coefficients, R^2, and Lack-of-Fit test. Significant coefficients are highlighted in bold letters.

Model parameters	Y_2 Coef.	SE	*p*-Value
Constant	24.56	0.83	**0.000**
Linear			
temperature	2.25	0.78	**0.008**
time	7.31	0.78	**0.000**
substrate	−1.24	0.78	0.123
Squared			
temperature^2	−2.28	1.14	**0.050**
2-Way interaction			
time*substrate	1.92	1.10	0.094
Lack of fit			0.062
R^2	0.816		
R^2 (adjusted)	0.778		
R^2 (predicted)	0.711		

maintaining each factor constant at a central point while the other two factors varied within the experimental range (Fig. 4).

3. Results and discussion

The present work is a first approach to test the potential and constraints of combining a particular type of factorial design and *in vitro* assays to test in a quick and easy way a variety of factors potentially affecting digestive bioaccessibility of nutrients in fish. Factorial designs are more efficient than one-factor-at-a-time experiments to estimate the effects of a factor at several levels of other factors in order to obtain valid conclusions (Dobson, 1990). Factorial designs are increasingly used in biological experiments, which conditions are quite dynamic compared to similar applications in other fields, such as in engineering where the conditions are relatively easier to control (Arun et al., 2017; Fernández-Pardo et al., 2016). The results obtained in the current different hydrolysis experiments, corresponding to the combinations of the experimental factors, are detailed in Table 1. Results varied thoroughly depending on the values of the considered factors; from 68.4 to 252.6 mg of total amino acids (aa) and from 12.9 to 36.5 mg/

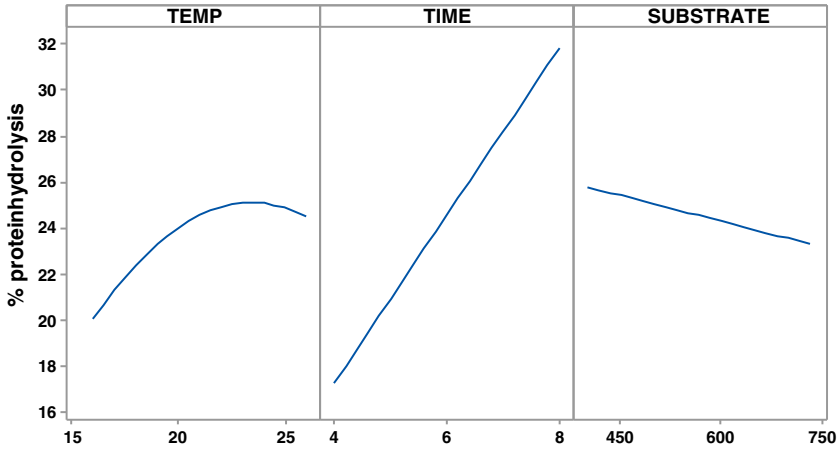

Fig. 3. Plot of the main effects of three factors, temperature, reaction time and substrate concentration within physiological ranges on the hydrolysis of protein in a simulated intestine of Senegalese sole.

100 mg protein released due to enzyme hydrolysis. The regression coefficients of the models of protein hydrolysis by the enzymes of Senegalese sole in coded form, their statistical significance and the fitting of the models are summarised in Table 2. The initial regression models with coefficients in uncoded form were:

$$total\ aa = -317 + 33.1\ \text{temp} - 36.1\ \text{time} + 0.277\ \text{substrate} - 0.674\ \text{temp}^2 + 1.81\ \text{time} - 0.000403\ \text{substrate}^2 - 0.170\ \text{temp*time} - 0.00257\ \text{temp*substrate} + 0.0696\ time*substrate. \quad (2)$$

$$\%hydrolysis = -57.9 + 5.41\ \text{temp} - 0.93\ \text{time} + 0.0546\ \text{substrate} - 0.0938\ \text{temp}^2 + 0.163\ \text{time}^2 - 0.000058\ \text{substrate}^2 - 0.030\ \text{temp*time} - 0.00148\ \text{temp*substrate} + 0.00576\ \text{time*substrate}. \quad (3)$$

Nevertheless, although fitting of such models was acceptable ($R^2 > 0.80$) they contained a number of non-significant terms that influenced the significance of the lack-of-fit test. This showed values lower than 0.05, suggesting that the model may not fit properly to the data. Lack-of-fit can occur when using replicates or if important terms from the model such as interactions or quadratic terms are not included. Hence, more simplified regression models were generated using the process of backward elimination of some of the non-significant coefficients and trying to reduce the lack-of fit. The best-simplified models obtained expressed in uncoded units were:

$$total\ aa = -198 + 30.2\ \text{temp} - 17.9\ \text{time} - 0.234\ \text{substrate} - 0.663\ \text{temp}^2 + 0.0696\ \text{time*substrate}. \quad (4)$$

$$\%hydrolysis = -23.2 + 4.27\ \text{temp} + 0.39\ \text{time} - 0.0420\ \text{substrate} - 0.0910\ \text{temp}^2 + 0.00576\ \text{time*substrate}. \quad (5)$$

The model for *total aa* in Eq. (4) was constructed excluding all the non-significant terms; it maintained a high R^2 (0.859), but still presented a significant lack-of-fit. The model for *% hydrolysis* in Eq. (5) was constructed eliminating most of the non-significant terms; it maintained a high R^2 (0.816) and the significance of the lack of fit test was $p > 0.05$. For this reason this was the only model considered for further discussion, being its parameters summarised in Table 3. The analysis of the regression coefficients of that polynomial model in coded values revealed that:

a) The factor with a higher effect on the efficiency of the hydrolysis was the total time of incubation, while the temperature had much less influence (Table 3). The ordered arrangement of the effects should be: time > temp > temp^2.

b) The efficiency of protein hydrolysis by the digestive enzymes of Senegalese sole was linear and positively correlated to incubation time, but temperature showed a more complex effect, combining both linear and quadratic terms and resulting in a non-linear response characterized by a maximum around 23 °C. The amount of available substrate showed an inverse but not significant correlation to the efficiency of the hydrolysis. All these effects can be better appreciated in Fig. 3. The non-homogeneous effect of the substrate determining the lack of significance of this factor was more evident when evaluating its interaction with time. An interaction can be deduced when the difference in the response between the levels of one factor is not the same for all levels of another factor (Montgomery, 2001). In the present case, the effect is clearly appreciated in the contour plots representing the combined effects of each pair of factors when maintaining a fixed central value for the third factor (Fig. 4). At a long incubation time (> 6.5 h) similar values of % hydrolysis were obtained irrespective of the amount of substrate used in the assay, while at shorter incubation times, a lower response was obtained when using the higher amounts of protein.

c) Other interesting conclusions may be obtained from such plots, *e.g.* for a fixed incubation time of 6 h, maximum hydrolysis was obtained for low amount of substrate within the interval 22–25 °C. When temperature is maintained constant at 21 °C, a higher hydrolysis is directly correlated to the available time, irrespective of the amount of substrate present, but for intermediate amounts of substrate better results are obtained with longer incubation times at temperatures not exceeding 23 °C (Fig. 4). This result is in agreement with the increasing growth and feed efficiency levels found by Guerreiro et al. (2012) when the temperature increased from 16 to 22 °C.

It is clear that the operation of a real gut is much more complex and that, in the living fish, a number of adaptative responses may modify to a great extent some of the results observed in these simplified *in vitro* experiments. As an example, the theoretical change in enzyme:substrate ratios used in the *in vitro* assays may not exist in the living fish if enzyme secretion is modulated to be maintained constant. On the other hand, the effect of temperature on the hydrolysis may be more complex; besides the direct and positive effect on the activity of the enzymes within the tested range (Alarcón et al., 1998; Sáenz de Rodrigáñez et al., 2005; Matus de la Parra et al., 2007; Guerrero-Zárate et al., 2014), it may exert a negative effect through the increase in the metabolic rate (Secor, 2009) and therefore in the gut transit rate (Horn and Gibson, 1990; Miegel et al., 2010), this reducing the time available

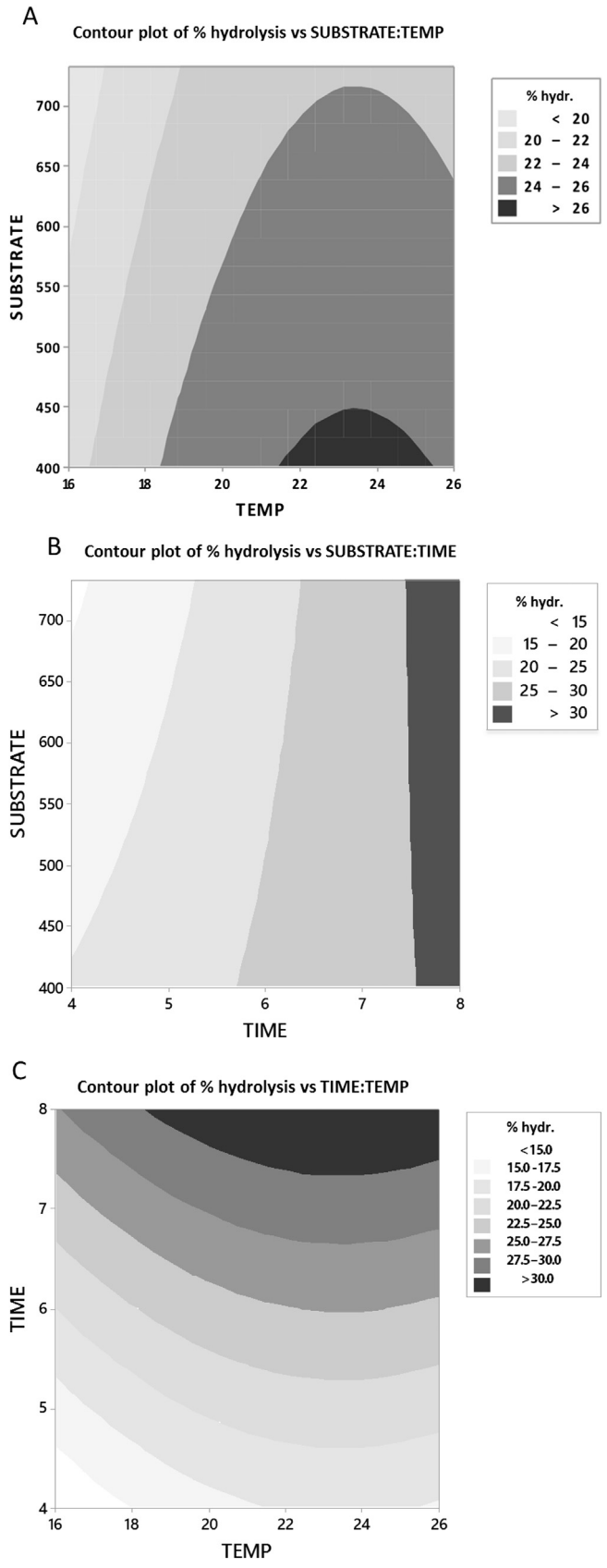

Fig. 4. Contour plots describing the combined effect of temperature (X_1), reaction time (X_2), and substrate concentration (X_3) on protein hydrolysis within a simulated intestine of the Senegalese sole. The substrate, reaction time, and temperature were fixed at 567 mg, 6 h, and 21 °C, respectively in plots A, B, and C.

for hydrolysis. Gut transit time is other critical factor that determines how long the food is being digested under given luminal conditions and that may change among species (Nikolopoulou et al., 2011). In addition, while in the present case, the pH was considered a fixed factor, in other cases it may be considered an additional variable, considering that each species has a particular acidification strategy modulated by the feeding regime (Papastamatiou et al., 2007; Yúfera et al., 2012, 2014; Hlophe et al., 2014). It is deduced that the use of this approach in other species should require adaptation to the specific features of their digestion process. In general terms, the steps to be followed should be:

a) An accurate determination of the physiological ranges of the factors to be evaluated.
b) A preliminary evaluation of the effect of each single factor on the response variable.
c) A selection of the more suitable factorial design and running of the different assays.
d) The construction and refining of the mathematical model derived from the results.
e) The validation *in vivo* of the predictions obtained with the model.

This approach presents some interesting advantages. It is possible to obtain a more detailed knowledge on the influence of different factors in the evaluated response (*e.g.* if their effect on the response is direct or inverse, if they are independent or dependent on any type of interaction, etc.). Going a step beyond, the information obtained can be used as a tool to maximize the biological response under variable conditions and may orientate some on-farm feeding practices. As an example, results obtained in the present study suggest that for the Senegalese sole, the efficiency of protein hydrolysis can decrease above an optimum temperature and also that adapting feeding frequencies to optimize gut retention times may have a very significant effect on such hydrolysis. We conclude that this combined approach may constitute the basis to develop more complex mathematical models, as those developed by Rønnestad and Conceição (2012) explaining digestive function and nutrient utilization in a different fish species. In this sense, new experiments are currently in progress.

Acknowledgments

This research was funded by the Spanish Ministry of Economic Affairs and Competitiveness (MINECO) by projects EFISHDIGEST (AGL2014-52888-R) with FEDER/ERDF contribution granted to M.Y. and WISEFEED funded by the European Union's H2020 programme (Marie Skłodowska-Curie grant No 691150). Neda Gilannejad was supported by a doctoral fellowship (BES-2015-071662) from MINECO (Spain).

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