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Optimization of enzymatic hydrolysis of red tilapia scales (*Oreochromis sp.*) to obtain bioactive peptides

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ABSTRACT

The objective of this study was to optimize the conditions of enzymatic hydrolysis (type of enzyme, pH, temperature (T), substrate (S) and enzyme concentration (E)) to increase content of soluble peptides (P), antioxidant activities and degree of hydrolysis DH (%), in hydrolysates. Also, the effect of scaling up from a 0.5 L to a 7.5 L reactor, was evaluated. Hydrolysis was carried out for 3 h in a 500 mL reactor, with Alcalase® 2.4 L and Flavourzyme® 500 L enzymes. A second experimental design was then developed with S and E as factors, where DH, P and antioxidant activity, were response variables. The Alcalase® 2.4 L was the most productive enzyme, with optimal S and E of 45 g/L and 4.4 g/L, respectively. Its hydrolysates showed antioxidant activities with IC50 of 0.76 g/L, 12 g/L and 8 g/L for ABTS, FRAP and ICA, respectively. The scale up didn't showed negative effect on the hydrolysis.

1. Introduction

In recent years, the fish industry has shown significant growth globally. Colombia is the tenth producer of red tilapia in the world, which is a species that represents 62 % of the country's fish production [1]. 60 % of red tilapia production ends up as waste, such as fillet remains (15–20 %), skin and fins (1–3 %), bones (9–15 %), heads (9–12 %), viscera (12–18 %) and scales (5%) [2]. These high volumes of waste represent a challenge from an environmental standpoint, because approximately 50 % of the waste is discarded without being used and only 30 % is used for products with a lower added value [3]. However, this waste is also an opportunity from an economic standpoint, due to the fact that some of this waste has significant levels of protein. This is the case of red tilapia scales; whose protein content ranges between 41 % and 84 % - the highest among residues of fish farms [4]. The protein is insoluble and found within a solid matrix, and enzymatic hydrolysis is used as a procedure to ensure its solubilization in the liquid medium to avoid alkaline or acid hydrolysis which produces undesirable byproducts. For this reason, enzymatic hydrolysis is one of the alternatives with the greatest potential for protein utilization, which can improve the nutritional quality of the substrate and favor the release of bioactive compounds [5]. Commercial proteases such as trypsin, chymotrypsin, pepsin, Alcalase®, Flavourzyme® 500 L, properase E, pronase, collagenase, bromelain, or papain, are usually employed in these processes [6]. Enzymatic hydrolysis allows for the possibility of obtaining peptides exhibiting biological activity, such as antioxidants, antihypertensives, antimicrobials, anticoagulants [7], and compounds with calcium binding properties [7] or antitumor properties [8].

An important parameter for hydrolysis is the type of enzyme used, because it influences the sequence of the peptides which are generated [9]. One of the most used enzymes is Alcalase®, which produces peptides exhibiting antioxidant [10], anticoagulant [11], antihypertensive [12] and calcium binding properties [13]. Another interesting enzyme is Flavourzyme® 500 L, which produces peptides capable of antioxidant activity [14] and exhibiting calcium binding properties [15]. Despite the favorable results available in the literature, there are few studies that evaluate obtaining biologically active peptides from red tilapia scales (*Oreochromis sp.)* at different scales of production. Scaling processes is a tool that allows to carry out laboratory scale processes at a greater industrial level. This discipline allows researchers to evaluate aspects such as energy consumption, productivity, efficiency, purification, and separation of products, among many others. It is usually based on criteria such as similar geometry, dynamics and dimensional analysis [16].

The objective of this study was to optimize the conditions of enzymatic hydrolysis by varying the type of enzyme, pH, temperature, substrate, and enzyme concentration, to obtain protein hydrolysates exhibiting of antioxidant activities, as well as evaluating the effect of scaling the reaction up from a 0.5 L to a 7.5 L reactor.

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2. Material and methods

2.1. Chemical and reagents

The reagents 2,2′-Azino-bis (3-ethylbenzothiazolin)-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchromo-2-carboxylic acid (Trolox), 3- (2-Pyridyl), 5–6 diphenyl acid monosodium salt hydrate (Ferrozine) were obtained from Sigma-Aldrich (Oakville, Ontario, Canada) and 2,4,6-Tri-2-pyridyl-s-triazine (TPTZ) was supplied by Merck (Darmstadt, Germany). Alcalase® 2.4 L (commercial protease obtained from the fermentation of *Bacillus licheniformis*, non-specific serine endopeptidase) was supplied by Novozymes (Bagsværd, Denmark) and Flavourzyme® 500 L (complex of fungal peptidases obtained from fermentation of *Aspergillus oryzae*, endo and exo peptidase) was provided by Sigma-Aldrich, (Oakville, Ontario, Canada). All the reagents used in the study were analytical grade.

2.2. Fish scales

The fish scales were supplied by Piscícola El Gaitero in San Jerónimo-Colombia. They were washed and disinfected with 0.2 mg/L sodium hypochlorite, to be able to store them in polyethylene bags at −-20 °C until their use. They were then dried at 60 °C for 24 h, and their size was reduced to 850 μm, by means of an endless screw mill (Corona, Colombia).

2.3. Characterization of the scales

The proximal composition (moisture, protein, fat and ash) of the scales was determined using the AOAC 930.15, 990.03, 920.38, 942.05 methods, respectively [17].

2.4. Amino acid analysis

The ground scales were subjected to an acid hydrolysis using 6 N HCl and 0.1 % phenol. Hydrolysis was carried out at 110 °C for 24 h. The derivatization of the samples was done before the pre-column, using o-phthalaldehyde (OPA) for primary AA and 9-fluorenyl methyl chloroformate (FMOC) for secondary AA [18]. The amino acids were analyzed using a HPLC Ultimate 3000 (Thermo Fisher Scientific, United States), with an UV/VIS detector with a DAD diode array, operated with a 5 μm analytical column, ZORBAX Eclipse AAA-C18, 4.6 × 75 mm (Agilent, United States). A mobile phase in the form of a gradient prepared by a quaternary pump was used to mix the water, methanol, acetonitrile and 40 mM Buffer NaH_2PO_4 solution with a pH of 7.8 [18].

2.5. Enzymatic hydrolysis

First, the protein content of the raw material was determined by Kjeldahl [17] using a 6.25 conversion factor. The content of soluble peptides from the hydrolysates were determined based on Biuret [24] using the bovine serum albumine calibration curve from MP Biomedicals (Shandong, China) as a reference.

No previous protein release processes were carried out, due to the fact that these can generate negative effects on the structure of the protein and therefore on its biological activities [20]. For this reason, the enzymatic hydrolysis was established the first process in the experiment.

A glass reactor with a water circulation jacket was used for temperature regulation, the reactor had a volumetric capacity of 1 L with a 500 mL work volume. The pH control and temperature recording were performed using a combined LL glass electrode with a fixed frosted diaphragm (temperature between 0−80 °C), connected to an automatic titrator (Titrando 842) (Metrohm, Switzerland), which was operated via a computer (tiamo software 1.2.1). The reaction system was maintained at a constant stirring of 560 rpm using a 801 magnetic stirrer for 3 h (Metrohm, Switzerland) [1]. The hydrolysis reaction was monitored based on the degree of hydrolysis (DH), because an increase in DH has been associated with a greater presence of low-molecular-weight peptides, which in turn are attributed to exhibit greater biological activity [23]. The DH was expressed as the ratio between the number of hydrolyzed peptide bonds (h) and the number of total peptide bonds in the native protein per unit weight (h_{TOT}) [21]. The DH was calculated with Eq. 1, according to the pH-stat method [21].

$$DH\ (\%) = \frac{\mathrm{B\ \ NB}}{\mathrm{Mp}\ \alpha\ \mathrm{hTOT}} \times 100 \quad (1)$$

Where B is the consumed volume of NaOH in L, M_P is the mass of the protein in kg, N_B is the concentration of NaOH, and α is the degree of dissociation of the amino groups released during the reaction. α and pK were calculated using Eqs. 2 and 3, respectively [22]. The total number of peptide bonds (h_{TOT}) was obtained from the aminogram using Eq. 4.

$$\propto = \frac{10^{\mathrm{pH-pK}}}{(1 + 10^{\mathrm{pH-pK}})} \quad (2)$$

$$\mathrm{pK} = 7.8 + \frac{(298 - \mathrm{T})}{298 * \mathrm{T}} * 2400 \quad (3)$$

According to Adler-Nissen, 1979 [19], it is possible to calculate h_{TOT} from the concentration of each of the amino acids present in the scales using Eqs. 4 and 5.

$$h_{TOT} = \frac{1}{\sum_{i=1}^{18} Fi} * (1 + Fi) * 1000 \quad (4)$$

$$Fi = \frac{[Ai]}{\sum_{i=1}^{18} [Ai]} * (PM)_i \quad (5)$$

Where the amino acid is *i*, A*i* is the concentration (μmol/mL), $(PM)_i$ is the molecular weight of amino acid *i*, and F*i* is the molar fraction of amino acid *i*. The h_{TOT} was calculated using the amino acid serine.

At the end of the hydrolysis, the enzymes were inactivated at 90 °C for 10 min. The resulting hydrolysate was centrifuged at 1160 g for 20 min at 20 °C and the supernatant containing small size peptides was collected and stored at −80 °C until use.

2.6. Antioxidant activity

2.6.1. ABTS free radical cation scavenging assay

The ABTS solution was prepared based on Zheng, Zhao, Xiao, Zhao, & Su, 2016 [25], and was mixed with 7 mM $ABTS^+$ and 2.45 mM potassium persulfate. Then, 1 mL of the solution was added to the 100 μL of sample or Trolox and left in the dark for 1 h. The absorbance was measured at 730 nm. The results were calculated using a standard Trolox curve and expressed as micromoles of Trolox equivalent (μmol TE/L).

2.6.2. Ferric-reducing antioxidant power (FRAP) assay

The FRAP was determined according to Pulido et al., 2000 [26]. The FRAP solution was prepared with TPTZ 10 mM in HCl 40 mM, $FeCl_3$ 20 mM and an acetate buffer 0.3 mM in the dark at 37 °C. 30 μL of sample or Trolox, 90 μL of distilled water, and 900 μL of the solution were added and the mix was left in the dark for 1.5 h. The absorbance was measured at 595 nm. The results were

calculated using a standard Trolox curve and expressed as equivalent micromoles of Trolox (μmol TE/L).

2.6.3. Iron chelating activity (ICA)

Ferrozine forms complexes with ferrous iron producing a red colour, and the chelating agents as peptides inhibit the formation of the complex, which leads to a decrease in the red colour. Thus, the measure of colour reduction is an estimation of the binding ability of the chelator [27]. The test was performed following the methodology of Choonpicharn et al., 2015, in which 1 mL of sample or water (blank) is mixed with 20 μL of ferrozine and 40 μL of ferrous sulfate, and left in the dark for 10 min. The absorbance was measured at 562 nm, and the iron chelating activity (ICA) was calculated as shown in Eq. 6.

$$ICA\% = \frac{Ablank - Asample}{Ablank} x100 \quad (6)$$

2.7. Optimization of the enzymatic reaction conditions for bioactive peptide production

2.7.1. The optimal pH and temperature conditions

The pH and temperature conditions depend of the substrate as reported by Hamid et al., 2015 [28]. For this reason, a central composite design was created for each enzyme (Flavourzyme® 500 L and Alcalase® 2.4 L) maintaining the same units of catalytic activity/g of substrate in both cases. This was done in order to evaluate the effect of pH and temperature on two response variables: DH (%) and content of soluble peptides (P, g/L), thus being able to determine the maximum soluble peptides in a liquid medium with a higher DH.

The substrate concentration refers to the initial content of protein in the solid matrix in the reaction, and was kept constant at 8 g/L. The enzyme was used at a 20 % w/w based on the substrate. The results were analyzed using version 7.0 of Design Expert software, (Stat Easy Inc., Minneapolis, USA). The pH levels for Alcalase® 2.4 L were between 7.5–10.4 and betwen 7–8.5 for Flavourzyme® 500 L, and the temperatures were between 40.9 °C and 69.1 °C for both enzymes. 13 random runs, 8 design points and 5 repetitions of the center point were carried out for each design. After the optimization of the models, the antioxidant and iron chelation bioactivities were evaluated at the optimum point of each of the enzymes, which was then used to calculate the productivity.

2.7.2. Determination of kinetic parameters

A saturation curve was derived for Alcalase® 2.4 L by varying the concentration of the substrate (S) from 4 g/L to 36 g/L, by means of hydrolysis carried out at the optimum pH and temperature conditions determined during the previous stage. The enzyme volume (695 μL) and agitation (560 rpm) were constant. The experiments were evaluated for over 5 min and measurements were taken every 10 s. Each curve enables the determination of the initial velocity (V_0), from which the multiplicative inverse ($1/V_0$) was plotted against the multiplicative inverse of the substrate (1/S) and the kinetic parameters maximum velocity (V_{max}) and the Michaelis–Menten constant (Km) were obtained following the Lineweaver-Burk model as shown in Eq. 7 [30].

$$\frac{1}{V_0} = \frac{Km}{V_{max}}\left(\frac{1}{S}\right) + \frac{1}{V_{max}} \quad (7)$$

2.7.3. Optimization of the substrate and enzyme concentration

After acquiring the kinetic parameters of the reaction, it was possible to define the values of the substrate (S) and enzyme (E) concentration factors for a central composite design with 5 repetitions of the central point. The levels of S and E were comprised within the range 8.8−45 g/L and 0.4–5.6 g/L respectively. The response variables were DH (%), content in soluble peptides (g/L), ABTS (μmol TE/L), and ICA (%). The results were analyzed using Design Expert software (version 7.0, Stat Easy Inc., Minneapolis, USA). Due to the relationship between DH and the antioxidant activity, this variable was included in the first design as a response variable, but in final design, the antioxidant activity was used directly, since it was the main objective of the study.

2.7.4. Determination of the time of the enzymatic hydrolysis

Once the optimal hydrolysis conditions were found, P and the ABTS, ORAC and ICA bioactivities were evaluated at different DHs such as 2, 7, 12, 17 and 18, which relate to different hydrolysis times.

2.8. Enzymatic hydrolysis scale up

The objective of scaling up the reaction was to evaluate the enzymatic hydrolysis in an industrial scale reactor with a similar geometry, in order determine if the product could be further applied in the pharmaceutical and food industries.

After the optimization of the conditions in the 500 mL reactor, the process was transferred to a 7.5 L capacity reactor – Bioflo® & Celligen 310, G628−011 (New Brunswick Scientific, USA), stirred through a Rushton turbine with a working volume of 5 L (Edinson, NJ, USA) and fitted with temperature control. Optimal conditions used at this scale were 58.5 °C, an enzyme-substrate ratio [E/S] of 0.098 g enzyme/g protein and a pH of 8.05. The pH was kept constant with the addition of 2 M of NaOH using an automatically controlled peristaltic waterfall pump and a pH sensor. The reactor was equipped with heating by using a recirculation system, it had four baffles on the tank wall and a Rushton turbine impeller with a diameter of 0.077 m. The dynamic similarity principle was used keeping the Reynolds number (Re) constant between the model (m) (500 mL) and the prototype (p) (7.5 L). Eq. 8 was used to calculate the velocity of the agitation in the prototype (p) where D is the impeller diameter, ρ is the density, μ is the viscosity and N is the velocity of agitation [31].

$$Re_m = Re_p \rightarrow \left(\frac{N.\ D_i^2.\rho}{\mu}\right)_m = \left(\frac{N.\ D_i^2.\rho}{\mu}\right)_p \quad (8)$$

In this model, N = 9.8 s^{-1} and Di=5 cm, while the prototype Di=7.7 cm. Clearing N of the protype from Eq. (8) gives a velocity of 250 rpm for the impeller in the prototype. However, to evaluate the effect of N on the hydrolysis process, a velocity of 350 rpm was also evaluated in the prototype.

2.9. Statistical analysis

The measurements were taken in triplicate. All statistical results were analyzed with a 95 % confidence level (p value <0.05).

3. Results and discussion

3.1. Characterization of the scales

Red tilapia scales (*Oreochromis sp.*) contained in dry basis 0.9 ± 0.005 % fat, 83.9 ± 1.04 % protein and 15.1 ± 0.003 % ash. These protein values show the by-product's potential for using the substrate as a source of protein. Other authors have shown lower values for protein, 49.42 %, in red tilapia scales (*Oreochromis sp.)* [4] and 45.2 % in golden goatfish scales (*Parupeneus cyclostomus*) [32].

3.2. Amino acid composition

Table 1 shows the amino acid composition of red tilapia scales and hydrolysate. The table shows that the most abundant amino acid is glycine, followed by proline, glutamic acid and hydroxyproline, which were reported to be abundant in scales from nile tilapia (*Oreochromis niloticus*) [33], tilapia (*Oreochromis sp.)* [4]. However, alanine was found in a smaller proportion compared to the results of the mentioned authors. The amino acid composition refers to the types of proteins present in the scales which are type I collagen, keratin and mucin [34]. High proline and glycine content are due to the collagen present [35]. In the case of keratin, the predominant amino acids are glycine and glutamic acid [35]. Finally, mucin is a glycoprotein rich in serine, threonine and proline [36], which are also found in high proportions in the scales.

The presence of essential amino acids (9.5 %) is indicative of the nutritional possibilities of protein in red tilapia scales (Table 1). On the other hand, the content and sequentiation of amino acids within the peptides is very important to explain their possible antioxidant activity. For example, the hydrophobic properties of amino acids can favor their interaction with the target lipids or the entry of the peptides into the target organs through an interaction with the lipid bilayer in the cell, which is an important factor to achieve the desired antioxidant effects [37]. However, P is independent from the scales, and could be associated to the amino acid composition presented in Table 1. Accordingly, the presence of aromatic amino acids (5%) and His, Pro, Met, Lys and Cys, could be related to an increase in antioxidant activity because of their ability to donate protons or electrons [38].

In relation to the hydrolysate, the study found that the proportion of aromatic and non-essential amino acids increased with respect to the raw material. Which entails that they are more chemically available to be quantified after the hydrolysis processes. This is not the case for hydrophobic and essential amino acids, which decreased in the hydrolysate due to the hydrolysis conditions of the extraction method.

Table 1
Relative concentration of amino acids.

Aminoacid	residues/1000 residues	
	Scales	Hydrolysate
ASP	55.52	33.28
GLU	105.94	56.71
ASN	2.70	3.95
SER	46.10	32.44
HIS	26.17	15.35
GLY	346.89	610.29
TRE	15.48	5.81
CIT	9.91	18.89
ARG	0.88	2.36
ALA	11.08	3.86
TYR	47.76	90.35
CYS	13.96	6.58
VAL	9.68	18.20
MET	6.89	5.86
PHE	4.22	4.06
ISO	15.60	13.09
LEU	4.97	4.77
LYS	12.03	10.04
PRO	163.54	38.08
HYP	100.67	26.02
HAA	225.73	90.44
AAA	51.98	94.42
EAA	95.94	79.54
NEAA	904.06	920.46

HAA: hydrophobic amino acids.
AAA aromatic amino acids.
EAA essential amino acids.
NEAA non-essential amino acids.

3.3. Determination of h_{TOT}

According to the aminogram shown in Table 1, the h_{TOT} was calculated using the amino acid lysine as a reference. The value found was of 8.85, which is very close to the value reported by Adler-Nissen 1986, who reported a value of 8.6, for fish in general. This result is closer to the theoretical one, compared to the 9.3 value reported for by-products of Atlantic Salmon (*Salmo salar*) [39].

3.4. Optimization of enzymatic conditions for bioactive peptide production

3.4.1. Optimization of pH and temperature conditions

An analysis of variance (ANOVA) was carried out to establish the effect of pH and T on the DH and P which is shown in Table 2. The models have acceptable R^2 values and a non-significant lack of fit adjustment for all cases. The two factors have significant effects on both responses for both enzymes. pH exerts a significant effect on DH, both in linear and quadratic terms when using Alcalase®, in addition to T and pH interactions. However, in the case of Flavourzyme® 500 L, these factors only have a linear effect. On the other hand, P is affected linearly by both variables in Alcalase®, while in Flavourzyme® 500 L only temperature has an linear and quadratic effect.

Second order polynomial models were obtained from the ANOVA (Table 2) for each response in each enzyme. The coefficients were then calculated by multiple regression. The models are the result of the exclusion of non-significant terms ($p > 0.05$), thus preserving the hierarchy of the model.

3.4.1.1. Effects of pH and temperature on Alcalase® 2.4 L. In the case of Alcalase®, Eqs. 9 and 10 describe the behavior of DH and P, respectively. The differences in signs between the linear and quadratic terms in the same equation indicate that there is an extreme point for the given response in the working range depending on the variable in question. For DH, there is a maximum point depending on pH and T, as can be seen in the graphic behavior of this polynomial Fig. 1a. For P (Fig. 1b), there is only a maximum point in terms of T, which is not as pronounced as DH. This is because the linear term ($P = 0.0002$) is much more significant than the quadratic one ($P = 0.0349$), which indicates that the maximum point is very close to the upper limit of the working range.

$$DH = -259.8 + 31.0\ pH + 5.4\ T - 0.1\ pH{*}T - 1.6\ pH^2 - 0.04\ T^2 \quad (9)$$

$$P = -9.9 - 1.1\ pH + 0.8\ T - 5.97x10^{-3}\ T^2 \quad (10)$$

Table 2
P-values of the pH and temperature models for Alcalase® 2.4 L and Flavourzyme® 500 L.

Factor	Alcalase® 2.4 L		Flavourzyme® 500 L	
	DH (%)	P (g/L)	DH (%)	P (g/L)
Model	< 0.0001	0.0002	< 0.0001	0.0017
pH	< 0.0001	0.0009	0.0002	–
T	0.2689	0.0002	< 0.0001	0.0075
pH*T	0.006	–	–	–
pH^2	0.0001	–	–	–
T^2	< 0.0001	0.0349	–	0.0029
Lack of fit	0.5752	0.0824	0.3793	0.5153
R^2	0.9904	0.877	0.8955	0.7578
Adjusted R^2	0.9836	0.836	0.8746	0.7039

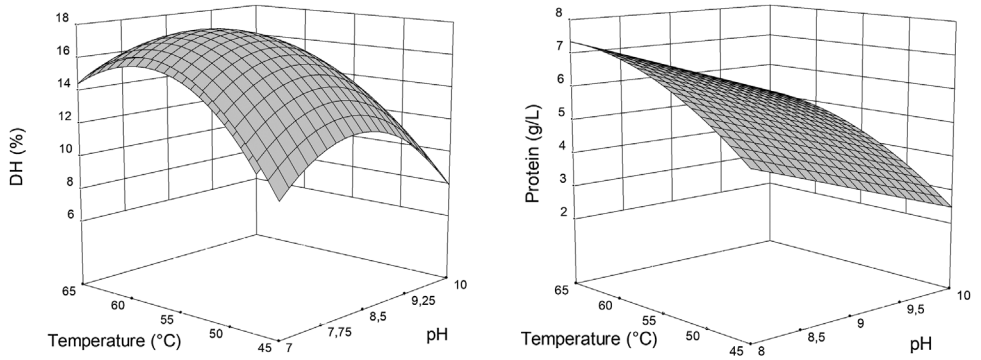

Fig. 1. Response surface for DH (a) and P (b) for Alcalase® 2.4 L.

3.4.1.2. Effects of pH and temperature on Flavourzyme® 500L. Eqs. 11 and 12 describe the behavior of DH and P using Flavourzyme® 500 L, respectively. The graphic behavior of Eqs. 11 and 12 is shown in Fig. 2a and b, respectively. These models show that DH depends on both factors linearly, while P only depends on T in quadratic terms. DH increases with pH and decreases with T in the working range. However, P is independent of pH and is function of T.

$$DH = -11.6 - 0.1\ T + 2.9\ pH \tag{11}$$

$$P = -8.6 + 0.5\ T - 4.041x10^{-3}T^2 \tag{12}$$

In general, Alcalase® 2.4 L is more affected by pH than Flavourzyme® 500 L. In the first, pH increases in disfavor of DH and P, whereas for Flavourzyme® 500 L, pH increases in favor of DH. Other studies have found that increases in pH favor the yields of protein liberation in tilapia skeletons [40]. However, very high pH values can denature proteins and damage some amino acids [41].

The effects of pH are due to changes in its level, which affects both substrate and enzyme. This is because it changes the distribution of charges and the conformation of proteins [42]. Additionally, pH can influence the dissociation of active groups of the enzyme, affecting the dynamics of association of the enzyme with the substrate [43].

The effect of temperature on both enzymes can be explained by the fact that temperature increases, generally increase the kinetic energy of molecules. However, due to the protein nature of the enzymes, their tertiary structure is compromised when certain temperature values are reached, which decreases the possibilities of union between the enzyme and the substrate by thermal denaturation of the enzyme [44]. This, in turn, leads to the loss of catalytic activity when working above a certain temperature limit [45].

3.4.1.3. Optimization of the models to maximize DH and P. The models of Eqs. (9–12) were optimized with the aim of maximizing DH and P. The results obtained for optimal conditions of pH and T for Alcalase® 2.4 L are 8.1 and 58.5 °C. For Flavourzyme® 500 L, these values were found to be 8.3 and 53.8 °C. It is worth noting the proximity of the experimental responses with the predicted ones, which corroborates the validity of the models and the power of the response surface methodology as an optimization method. However, the adjustment between predicted and experimental values is better in the case of Alcalase® 2.4 L than for Flavourzyme® 500 L. The predicted-experimental values for P were 6.9 g/L-6.7 g/L and 5.3 g/L-3.6 g/L for Alcalase® 2.4 L and Flavourzyme® 500 L, respectively. On the other hand, predicted-experimental values for DH were 17.6 %-16.7 % and 6.4 %-5.9 % for Alcalase® 2.4 L and Flavourzyme® 500 L, respectively.

Higher DH and P were obtained using Alcalase®, which agrees with Gajanan et al., [46], because when the DH is higher the yield is also increased. Herman-lara et al., [47], found similar results in hydrolysis processes with skeleton flour made from nile tilapia (*Oreochromis niloticus*), indicating that DH is higher for Alcalase® 2.4 L since it is an endopeptidase, while Flavourzyme® 500 L is exopeptidase. These higher values for Alcalase® 2.4 L could be because alkaline proteases show greater activity than neutral proteases such as Flavourzyme® 500 L when acting on fish proteins [48–51]. The higher degree of hydrolysis in these substrates is important because it may be associated with the increase in the levels of essential amino acids, hence the nutritional value of the hydrolyzed products obtained [52]. Generally, high DH in hydrolysates from fishery by-products have been linked with greater reducing capacity and radical capture [1,53]. The low molecular weights of peptides have been widely associated to the presence of biological activities [23]. The most interesting applications in this regard have been found in peptides with molecular weights between 1–4 kDa [54]. Therefore,

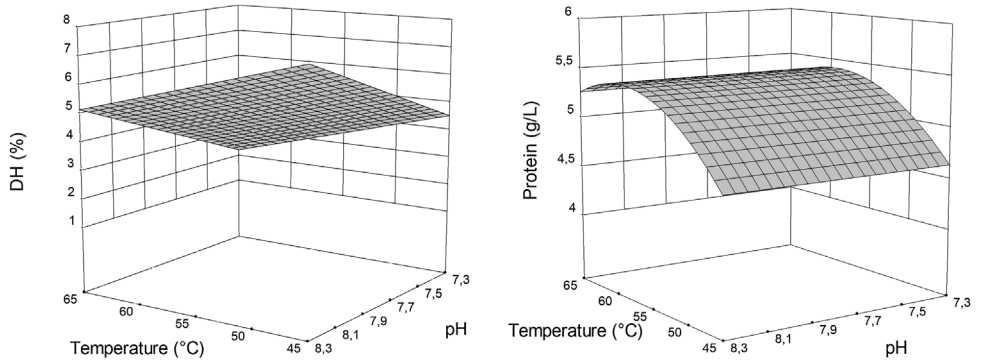

Fig. 2. Response surface for DH (a) and P (b) for Flavourzyme® 500 L.

obtaining hydrolysates with high degrees of hydrolysis (DH) seems to increase the possibility of obtaining bioactive peptides [55].

Under the optimal conditions (temperature and pH) obtained for each enzyme, the antioxidant activities were evaluated by ABTS (radical scavenging), FRAP (reducing power) and ICA (metal chelating). The productivities in each bioactivity for Alcalase® 2.4 L were ABTS (223.1 ± 4.9 μmol TE/g prot*mL enzyme), FRAP (13.1 ± 0.7 μmol TE/g prot*mL enzyme) and ICA (61 ± 1%). Meanwhile for Flavourzyme® 500 L ABTS (53.7 ± 0.6 μmol TE/g prot*mL enzyme), FRAP (6.3 ± 0.4 μmol TE/g prot*mL enzyme) and ICA (65 ± 3%).

The comparation according to the type of enzyme show that the protease affects the functionality of the hydrolysates obtained [56]. In this case, the difference is due to the fact that Alcalase® 2.4 L is an endopeptidase, which breaks peptide bonds from non-terminal amino acids, while Flavourzyme® 500 L is an exopeptidase that breaks the N-termini of chains [5]. Alcalase® 2.4 L and Flavourzyme® 500 L liberates higher residues with hydrophobic amino acids, which have been associated with antioxidant activities. Flavourzyme® 500 L liberates free amino acids [1,57], and amino acids such as proline have been associated with the increase in antioxidant potential thanks to the ability to donate protons [58]. Other amino acids with acidic residues such as glutamic acid are important contributors to the antioxidant activity of hydrolysates [59].

These results agree with those reported by Karamać, 2016 [56] who found that Alcalase® 2.4 L is superior to Flavourzyme® 500 L in terms of antioxidant activity.

Given its higher yields of protein liberation and productivity of hydrolysates with antioxidant capacity, these results suggest that Alcalase® 2.4 L is the enzyme with the greatest potential to obtain antioxidant peptides from red tilapia scales.

To fully define the operating conditions of the enzymatic hydrolysis, it was necessary to define the enzyme and substrate concentrations in which the enzyme could perform better. In this sense, it was convenient to determine the kinetic parameters of Alcalase® 2.4 L with this particular substrate, by means of a saturation curve, in which the initial velocity vs. the substrate concentration is plotted [60] as shown in Fig. 3. This figure shows that above 200 mM, increases in substrate concentration do not generate an increase in the speed of the reaction, possibly because there is a saturation of the enzyme caused by the substrate.

The kinetic parameters were determined using the Lineweaver-Burk model, by graphing the multiplicative inverses of the mentioned variables [60]. After adjusting a linear model to this curve, the slope values and the intercept can be obtained. Then, using these values, Vmax was calculated: 11.3 mM/sec and Km: 188.2 mM or 21.8 g/L. Based on this data, the range of substrate concentrations in which substrate inhibition will not occur was defined at values between 0.5Km and 2.5Km [60].

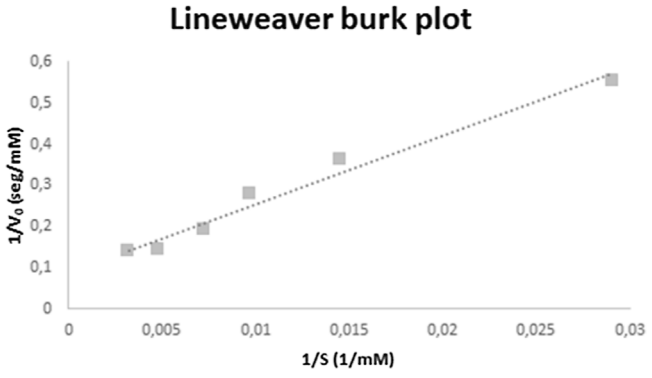

Fig. 3. Substrate saturation curve of Alcalase® 2.4 L.

$$V_{max} = \frac{1}{Intercept} = \frac{1}{0.0884} = 11.3 \ \ mM/s$$

$$Km = V_{max} * slope = 11.3 * 16.6 = 188.2 \ \ mM = 21.8 \ \ g/L$$

The high Km values indicate that the enzyme requires high substrate concentrations to reach saturation and achieve maximum speed, this parameter is associated with the affinity between the enzyme and the substrate [60]. In this sense, the Km value obtained for the substrate is greater than the obtained for Alcalase® 2.4 L in other substrates such as tilapia viscera (1.9 g/L) [61] and Salmon muscle (4 g/L) [30]. This is possibly due to the difference in the scale's structure, which has a higher mineral content, partially comprised of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$ [62], which makes it a protease resistant structure [63].

In the second experimental design, the DH and P are still shown to corroborate the assumption of the relationship between DH and antioxidant activity. The effect of enzyme (E) and substrate (S) concentrations on P, DH and the biological activities ABTS and ICA, was evaluated by means of a central composite design. The respective analysis of variance is shown in Table 3.

Table 3 shows that all of the response models were significant, without a significant lack of fit adjustment. However, for the P and ICA models, only the substrate concentration was significant, while for DH, the linear effects of both factors were significant, as well as the quadratic term of the substrate concentration. Finally, ABTS was explained by the linear and quadratic effects of the two variables, with second order interactions.

The models were obtained for each of the responses from the ANOVA (Table 3) (Eqs. 13–16) once the non-significant terms were eliminated. This was done without affecting the hierarchy of the design and the transformed variable $Y = DH^{2.55}$ was used instead of DH.

$$(DH)^{2.55} = 1163.1 - 204.9 \ S + 488.3E - 231.9 \ E^2 \quad (13)$$

$$P = 25.6 + 11.2 \ S \quad (14)$$

$$ABTS = 230.8 + 2.1 \ S + 68.7 \ E - 35.0 \ E*S + 33.0 \ S^2 - 43.9 \ E^2 \quad (15)$$

$$ICA = 82.3 - 4.2 \ S \quad (16)$$

Fig. 4 shows the response surfaces of the models found for each of the response variables. Those results indicate that S helps to increase P but decrease ICA while E improves ABTS and DH. P and S increase simultaneously because the variables are directly related.

Table 3
ANOVA of models and variables chosen on P, DH, ABTS and ICA.

Variable	P (g/L)	DH (%)	ABTS	ICA
Model	< 0.0001	< 0.0001	0.0013	0.0094
S	< 0.0001	0.0072	0.8243	0.0094
E	–	< 0.0001	0.0002	–
S^2	–	0.0103	0.0132	–
E^2	–	–	0.0060	–
S*E	–	–	0.0332	–
Lack of fit	0.3047	0.1549	0.0730	0.7405
R^2	0.9787	0.8937	0.9147	0.5065
Adjusted R^2	0.9766	0.8582	0.8538	0.4571

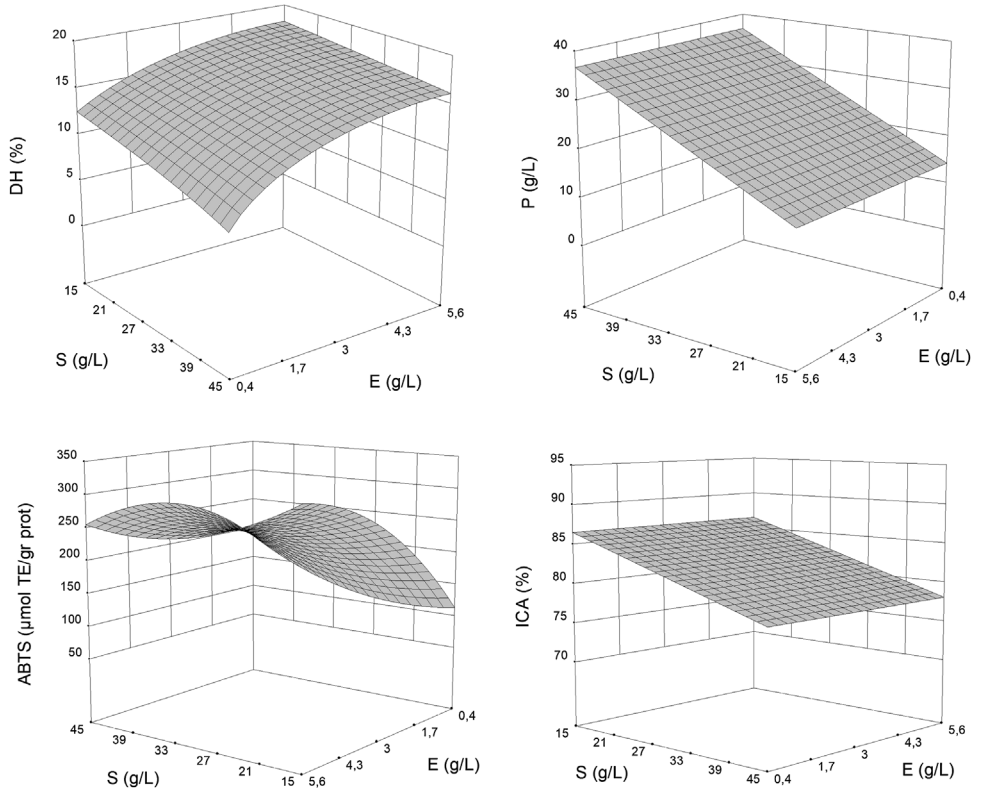

Fig. 4. Response surfaces for DH (a) and P (b), ABTS (c) and ICA (d) at different levels of S and E.

On the other hand, having a higher E in the system also favors the probability of the enzyme-substrate encounter and therefore the DH. If E increases ABTS could decrease because the enzyme would be hydrolyzing peptides. S has opposite effects on ABTS depending on the zone of E in which it is located. At low values of E, S has a positive effect on ABTS, but at high values of E, it has negative effects. These differences in ABTS as a function of S, are due to the fact that the affinity of the enzyme towards the protein is determined by the balance between the native protein and the one deployed in the solution [64]. Changes in this balance may suggest changes in hydrolysis mechanisms. It has been shown that the affinity for the intact protein was different in the hydrolysis with Alcalase® 2.4 L when using different substrate concentrations. That is, that the composition of these hydrolysates was different and that, in effect, this is caused by changes in the selectivity of the enzyme in varied operating conditions [65].

The models presented in Eqs. 5–8 were optimized to maximize DH, P and biological activities (ABTS and ICA). The study found that the substrate concentration should be at 45 g/L and the enzyme concentration at 4.42 g/L. The response variables obtained were verified experimentally (Table 4).

Protein liberation had a yield of 93 %, which was expected since Alcalase® 2.4 L allows the solubilization of fish proteins and is also highly efficient in the hydrolysis of this type of materials [66]. On the other hand, the degree of hydrolysis obtained is close to that reported by Blanco et al. [67] in the hydrolysis of skin collagen of various species using Alcalase®, which was between 12 % and 16 %. Likewise, the antioxidant activity obtained is higher than that reported by some authors such as Sai-Ut, Benjakul, Sumpavapol, & Kishimura [68], who found that the antioxidant activity by ABTS was 80 µmol TE/g sample for gelatin hydrolysates from *Aluterus monoceros*. In this study a 190 µmol TE/g sample was obtained. Respecting to ICA, the results obtained are similar to those reported by Choonpicharn & Jaturasitha [69], who found 77 % activity in hydrolysates of skin gelatin from nile tilapia.

Table 4
Predicted and experimental values for P, DH, ABTS and ICA under optimal conditions of S and E.

Variable	Predicted	Experimental	Absolute error
P (g/L)	36.78	41.9 ± 1.19	5.1
DH (%)	15.85	15.65 ± 0.33	0.2
ABTS (µmol TE/g prot)	271.15	209.8 ± 2.96	61.3
ICA (%)	78	77 ± 1.41	1.0

The hydrolysis process was carried out in optimal conditions of pH, T, S and E. Data was collected from each of the response variables as a function of time and DH to assess the moment and DH that generated the greatest biological activities.

Table 5 shows the values of the responses as a function of time and DH using Alcalase® 2.4 L. The study found that at the 10-minute point (DH of 7%) most of the protein had solubilized. However, by that time the biological activities were very low, so it was necessary to continue hydrolysis for a longer time (517 min), to reach a degree of hydrolysis of 18 %.

When comparing the evolution of the responses between 55 min (DH 12 %) and 517 min (DH 18 %), the analysis found that the increases in biological activity did not correspond to the time interval (462 min) and P had remained constant. After this discovery, the hydrolysis time was kept at 55 min, which was sufficient to bring the reaction to the limit productivity level.

The results shown in Table 5 indicate that the reaction followed a typical behavior, with a high exchange rate at first, followed by a slowdown until reaching a quasi-stationary state in the last minutes [70]. This behavior has been attributed to one or several of these three factors: (a) a decrease in the concentration of the peptide bonds that are susceptible to be hydrolysed by proteases [71], (b) possible inhibition of enzymes caused by the hydrolysis substrate [61], (c) thermal denaturation of the enzyme [72].

Table 5
Behavior of the response variables as a function of time and DH.

Time (min)	DH (%)	P (g/L)	ABTS (μmol TE/g prot)	ORAC (μmol TE/g prot)	ICA (%)
1.2	2.0	37.2 ± 1.27	115.6 ± 1.07	129.5 ± 2.00	15.8 ± 1.30
10.0	7.0	41.4 ± 1.99	200.5 ± 11.7	176.6 ± 11.78	54.9 ± 3.41
55.0	12.0	41.9 ± 1.19	257.5 ± 7.00	195.0 ± 8.78	70.7 ± 3.42
180.0	15.6	42.1 ± 0.87	209.8 ± 2.96	156.5 ± 6.77	77 ± 1.41
517.0	18.0	43.8 ± 1.19	289.6 ± 1.05	141.2 ± 18.78	85.4 ± 2.38

Table 6
DH, P, ABTS, FRAP AND ICA at different stirring rates.

Stirring rate (rpm)	DH (%)	P (g/L)	ABTS (μmol TE/g prot)	FRAP (μmol TE/g prot)	ICA (%)
250	9.9	30.3 ± 1.3	245.3 ± 10.7	38.7 ± 2.5	33.4 ± 5.1
350	12.0	37.4 ± 1.5	199.8 ± 8.5	40.3 ± 3.1	66.2 ± 4.8
Reactor 0.5 L	12.0	41.9 ± 1.2	257.5 ± 7.0	28.4 ± 5.6	70.7 ± 3.4

3.5. Scale up the optimal conditions of the enzymatic hydrolysis

During the scaling up process, it is important to prove that the same results obtained on a small scale can be achieved on the larger scale in terms of efficiency and quality. However, the scaling of fish protein hydrolysis has not been studied sufficiently [72]. When the process was scaled to 7.5 L, the analysis found that the biological activities differed from those obtained in the 0.5 L reactor. In this vein, the agitation speed was modified to determine the effect of this variable on the responses. Table 6 shows the results of the variables evaluated for the 7.5 L reactor, with two different stirring speeds (250 and 350 rpm), in contrast to the results obtained in the 0.5 L reactor. Table 6 shows that ABTS and ICA decrease when the speed of agitation is 250 rpm, however, FRAP increases. These results indicate that it is possible to obtain a hydrolysate with antioxidant activity by increasing the volume of work, as found by other authors in processes of hydrolysis of various materials such as cauliflower [73], sucrose [74] and lignocellulosic substrates [75].

It is important to note that the agitation speed favors DH and P, because it improves the homogeneity of the system, but the effect on the activities of the peptides obtained underlies the conformational changes that the enzyme undergoes when subjected to different flow conditions [76].

4. Conclusions

The amount of protein released and the degree of hydrolysis in the enzymatic hydrolysis of red Tilapia (*Oreochromis sp*) scales, is significantly affected by the pH and the temperature of the medium. This phenomenon holds true when the material is hydrolyzed with Alcalase® 2.4 L or Flavourzyme® 500 L. The productivity of the hydrolysates expressed as μmol TE/gprot* mL enzyme, is higher for Alcalase® 2.4 L than for Flavourzyme® 500 L. The kinetics of Alcalase® 2.4 L in this reaction can be described by means of the Michaelis-Menten model, with a Linewaver-Burk linearization. The antioxidant activity of the hydrolysates obtained with Alcalase® 2.4 L depend on substrate and enzyme concentration. The iron chelating activity only depends on the concentration of the substrate. The optimal conditions to obtain antioxidant peptides from red tilapia scale are pH 8.1, T 58.5 °C, S 45 g/L and E 4.42 g/L. The scaling of the reaction to a 7.5 L reactor using the dynamic similarity criterion based on the Reynolds number, allows for the reproduction of the conditions obtained in the 0.5 L reactor. However, the reaction is affected by the impeller speed in the 7.5 L reactor.

CRediT authorship contribution statement

Leidy Maritza Sierra-Lopera: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. **José Edgar Zapata-Montoya**: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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