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Research Article

Optimization of Enzymatic Hydrolysis of Viscera Proteins of Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract: This study aimed to optimize the enzymatic hydrolysis of rainbow trout (*Oncorhynchus mykiss*) viscera with the enzyme alcalase® 2.4 L to found the highest the Degree of Hydrolysis (DH). *In vitro* evaluation of the optimum conditions (pH and temperature) was performed to maximize the Proteolytic Activity (PA) of alcalase® 2.4 L with the rainbow trout viscera. The optimal conditions for maximum enzymatic activity were a pH of 8.5 and a temperature of 60°C. Response Surface Methodology (RSM) was used to evaluate the effect of the enzyme/substrate ratio (10-30%), substrate concentration (2.0-6.0%) and temperature (45-65°C) on DH. A substrate concentration of 5.53% and an enzyme/substrate level of 30% were found to be the optimum conditions to obtain the highest DH (27.6%). The Michaelis-Menten plot indicated that these conditions were not in the saturation area.

Keywords: Alcalase® 2.4 L, degree of hydrolysis, fish by-products, michaelis-menten plot, response surface methodology

INTRODUCTION

Fish consumption around of world has been increasing the last decades, mostly due to the understanding that consumers have about the health benefits associated with the intake of this source of food (Shahidi and Ambigaipalan, 2015). However, this growth is accompanied by proportional increase in by-product generation, because these industries discard between 40-60% of their total production as residues (Dekkers *et al*., 2011; Arvanitoyannis and Tserkezou, 2014; Saadi *et al*., 2015). These residues are important sources of protein and mainly consist of filleting waste (15-20%), skin and fins (1-3%), bones (9-15%), heads (9-12%), viscera (12-18%) and scales (5%) (Martínez-Alvarez *et al*., 2015). However, most of these discarded fish by-products have been used to manufacture low-value products, such as animal food, fishmeal and fertilizers (Ovissipour *et al*., 2009; Chalamaiah *et al*., 2012; Deraz, 2015). Nowadays, one of the most used options to take advantage of the visceral protein of this by-product is the enzymatic hydrolysis (Arvanitoyannis and Tserkezou, 2014) which improves the quality and functional characteristics of by-product proteins (Ovissipour *et al*., 2009; Valencia *et al*., 2015; Witono *et al*., 2016). It has been employed mainly to obtain protein hydrolysates with better nutritional characteristics and greater contribution of bioactive compounds (Chalamaiah *et al*., 2012; Shahidi and Ambigaipalan, 2015). Protein hydrolysates, apart from having an excellent equilibrium of amino acids, a rapid absorption and a good digestibility (He *et al*., 2013), also have fatty acids such as omega-3 (Ghaly *et al*., 2013; Silva *et al*., 2014). Additionally, these hydrolysates are a significant source of bioactive peptides (Chalamaiah *et al*., 2012; Liu *et al*., 2014; Valencia *et al*., 2014) that can provide health benefits, such as antihypertensive, antioxidant, immunomodulation, antithrombotic or anti-carcinogenic, depending on the sequence and number of amino acids (frequently 2-20) (Picot *et al*., 2010; Deraz, 2015; Shahidi and Ambigaipalan, 2015). Additionally, enzymatic hydrolysis can modify the physicochemical properties of proteins, such as solubility, oil/water holding, foaming capacity and sensory properties (Witono *et al*., 2016).

Several works have established the relation between biological activity of peptides and its molecular weight (Bourseau *et al*., 2009; Picot *et al*., 2010; Saidi *et al*., 2013; Liu *et al*., 2014). In particular, peptides with a molecular weight between 1 kDa and 4 kDa are most interesting (Saidi *et al*., 2014; Opheim *et al*., 2015; Soares de Castro and Sato, 2015). For this reason, to obtain peptides with high DH, namely low molecular weight, increases the possibility to get bioactive peptides (Gómez *et al*., 2013).

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Despite the prominent contribution of enzymatic kinetics to the field of enzymatic hydrolysis of fish proteins, there are very few studies involving the kinetics of these reactions (Valencia *et al*., 2014). Even fewer involving protein hydrolysis of the viscera, which is a complex raw material due to the high levels of fat (Bhaskar and Mahendrakar, 2008) and the interaction of this compound with proteins (Šližytė *et al*., 2005).

The present work evaluates the effect of pH and temperature on the Proteolytic Activity (PA) of alcalase® 2.4 L in the rainbow trout (*Oncorhynchus mykiss*) viscera. Moreover, we have also investigated the effect of the enzyme/substrate ratio, substrate concentration and temperature on the Degree of Hydrolysis (DH) of the same substrate. Additionally, we determined the Michaelis-Menten kinetic parameters for this enzymatic reaction.

MATERIALS AND METHODS

Materials: Viscera of rainbow trout were obtained from a local processing plant (Belmira Trout, Antioquia, Colombia). Those samples were packed in cold containers with ice and transported to the laboratory of the Nutrition and Food Technology research group of the University of Antioquia. Once received in the laboratory, visceral fat was extracted by heating at 90°C for 20 min, this process also aided in inactivating the endogenous enzymes. The fat was separated and the treated viscera mass was minced in a blender (Black and Decker, United States) and stored at -20°C until use.

The protease employed for the optimization studies was alcalase® 2.4 L, (Novo Industry, Denmark), a bacterial endoproteinase from a strain of *Bacillus licheniformis*, with a proteolytic declared activity of 2.4 AU/g. It has working temperatures between 30 and 65°C and pH between 7 and 9, depending on the type of substrate (Novozymes, 2011).

Proximate composition: Proximate composition (protein, fat, moisture and ash) of the fresh viscera, defatted viscera and its hydrolysates was estimated by the Standard Methods as described in AOAC (2000). The moisture content was determined in an oven at 105°C to constant weight, according to AOAC 930.15. The total fat in samples was estimated by the Soxhlet method according to AOAC 960.39. Total protein (N x 6.25) was determined using the Kjeldahl method according to AOAC 992.15 and the ash content was determined according to AOAC 942.05.

pH and temperature effects on the proteolytic activity of alcalase® 2.4 L: Response Surface Methodology (RSM) with a Central Composite Factorial Design (CCFD), has been applied to optimize Proteolytic Activity (PA) of alcalase® 2.4 L as a function of pH and temperature in the enzymatic hydrolysis of rainbow trout (*Oncorhynchus mykiss*) viscera. Table 1 shows the independent variables (pH and T) at three levels (-1, 0 and +1) and the proteolytic activity as a response, whereas Table 2 presents the randomized experimental runs. The analysis was made with Design Expert® 7.0.0 software (Stat-Ease, Inc., USA). For each run, proteolytic activity of alcalase® 2.4 L was determined using viscera of rainbow trout as substrate according to the method of Folin and Ciocalteu (1927) and Anson (1938) with a slight modification. For that, an enzyme sample of 60 μL was mixed with 600 μL of 0.5% (w/v) viscera. The mixture was incubated at the set temperature for each run for 10 min. The reaction was stopped by adding 600 μL of 110 mM TCA. After centrifugation at 10.000 rpm for 2 min, 300 μL of the supernatant was mixed with 750 μL of 0.5 M Na_2CO_3 and 150 μL of Folin and Ciocalteu's Phenol Reagent, continued by incubation at 37°C for 30 min. The absorbance of the sample was measured at 660 nm. One unit is defined as the amount of enzyme needed to produce color equivalent to 1.0 μmol of tyrosine per minute at the tested assayed condition.

Table 1: Independent factors and their coded and actual levels used in RSM for optimization of pH and temperature of alcalase® 2.4 L

	Levels		
Factor	-1	0	1
pH	7.5	8.5	9.5
Temperature (°C)	40	50	60

Table 2: Actual levels of independent variables with the observed values for the response variable, Proteolytic Activity (PA)

Run	pH	T (°C)	PA (AU/g)
1	9.5	50	8.45
2	8.5	40	10.11
3	8.5	50	10.12
4	7.5	50	8.95
5	8.5	60	10.89
6	7.5	60	9.77
7	9.5	60	9.67
8	8.5	50	10.87
9	7.5	40	8.51
10	9.5	40	8.58
11	8.5	50	11.25

Determination of the degree of hydrolysis: The Degree of Hydrolysis (DH), expressed as the percent ratio between the number of peptide bonds broken during the hydrolysis (h) and the total number of peptide bonds in the native protein per unit weight (h_{tot}). It was calculated from the volume and concentration of base (NaOH) added to keep the pH constant during the hydrolysis, according to Adler-Nissen (1986) equation:

$$DH\ (\%) = \frac{h}{h_{tot}} = \frac{B \cdot N_B}{M_P \cdot h_{tot} \cdot \alpha} \cdot 100 \quad (1)$$

where,

B = The volume of NaOH consumed (mL)
N_B = The normality of the base

Table 3: Independent factors and their coded and actual levels used in RSM for optimization of enzymatic hydrolysis conditions

Factor	Levels				
	-α	-1	0	1	α
Substrate concentration (%) (S)	0.636	2.00	4.00	6.00	7.364
Enzyme/substrate ratio (%) (E/S)	3.182	10	20	30	36.818
Temperature (°C)	38.200	45	55	65	71.800

Table 4: Actual levels of independent variables with the observed values for the response variable, Degree of Hydrolysis (DH)

Run	S (%)	E/S (%)	T (°C)	DH (%)
1	2.000	30.000	45.0	16.26
2	7.364	20.000	55.0	15.59
3	0.636	20.000	55.0	14.20
4	4.000	20.000	71.8	16.43
5	6.000	30.000	45.0	18.13
6	6.000	10.000	65.0	15.21
7	4.000	20.000	55.0	17.45
8	4.000	20.000	55.0	18.90
9	6.000	30.000	65.0	19.34
10	4.000	20.000	55.0	16.75
11	4.000	20.000	55.0	14.76
12	6.000	10.000	45.0	15.78
13	4.000	20.000	38.2	17.82
14	2.000	30.000	65.0	15.83
15	4.000	20.000	55.0	18.59
16	2.000	10.000	45.0	17.22
17	4.000	36.818	55.0	18.96
18	4.000	3.182	55.0	15.90
19	4.000	20.000	55.0	15.06
20	2.000	10.000	65.0	16.75

M_P = The mass (g) of protein (N×6.25)
h_{tot} = The total number of peptide bonds in the substrate, which was assumed as 8.6 meq/g to fish protein concentrate (Adler-Nissen, 1986)
α = The average degree of dissociation of α-amino groups released during the hydrolysis, expressed as:

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

where,
pH and pK: The values at which the proteolysis was conducted

The pK value is dependent on temperature, according to Eq. (3):

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

Hydrolysis process: Hydrolysis was conducted in a 0.5 L glass reactor for 6 h under constant stirring. The pH of the reaction was adjusted to 8.5. The variables Substrate concentration (S), Enzyme to Substrate level (E/S) and Temperature (T), were adjusted based on the experimental design (Table 3 and 4). The pH and temperature were controlled using a glass-combined electrode, connected to a Titrando 842 (Metrohm, Switzerland) operated by a PC (software Tiamo 1.2.1). The reaction was monitored with the determination of DH by the pH-stat method, which consists in maintaining the pH constant during the reaction by the addition of a base, in this case, 2N NaOH. The reaction was stopped by heating the solution at 85°C during 20 min, assuring enzyme inactivation. The hydrolysates were then centrifuged at 7740 rpm at 10°C for 20 min to precipitate the non-hydrolyzed fraction (Ovissipour *et al*., 2009). Figure 1 shows the production process of Rainbow Trout Viscera Hydrolysates (RTVH).

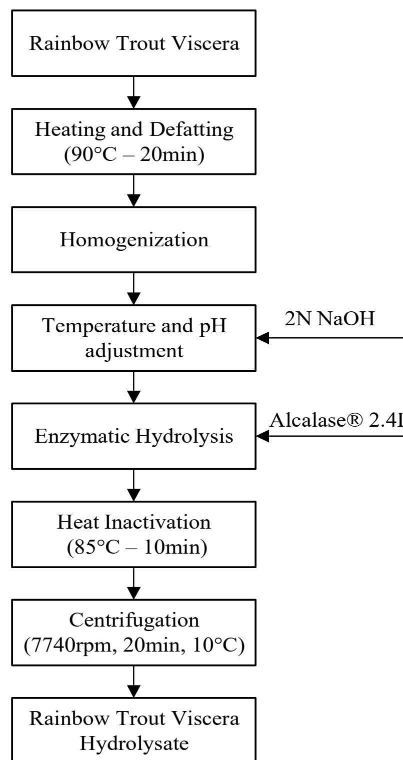

Fig. 1: Flow chart for the production of rainbow trout viscera hydrolysates

Effect of S, E/S and T on Degree of Hydrolysis (DH): Response Surface Methodology (RSM) with a Central Composite Factorial Design (CCFD) was again applied to maximize Degree of Hydrolysis (DH) as a function of S, E/S and T. Table 3 shows the independent variables (S, E/S and T) at five levels (-α, -1, 0, +1 and +α) and the DH (%) as a response, whereas Table 4 presents the randomized experimental runs. The analysis was made with Design Expert® 7.0.0 software (Stat-Ease, Inc., USA).

Determination of kinetic parameters (V_{max} and K_m): The kinetic constants V_{max} and K_m were determined graphically with initial velocity measurements obtained at varying substrate concentrations, in this case, ranging from 0.25 to 11% (w/w) (21.5-946 mM). The initial velocity of hydrolysis was calculated from the slope of the plot of α-NH versus time and then plotted against

Table 5: Proximate composition (%) of raw material and Rainbow Trout Viscera Hydrolysates (RTVH)

Material	Protein (%)	Fat (%)	Moisture (%)	Ash (%)
Fresh viscera	7.49±0.14	25.55±0.13	62.42±0.26	0.99±0.01
Defatted viscera	10.82±0.23	8.83±0.05	76.94±0.27	1.14±0.01
RTVH	5.69±0.27	2.46±0.02	88.54±0.13	3.17±0.02

Table 6: ANOVA of PA of alcalase® 2.4 L as affected by pH and temperature

Source	S.S.	DF	M.S.	F-ratio	Prob.>F
Model	9.2100	3	3.070	18.98	0.0010**
T	1.6400	1	1.640	10.12	0.0155*
pH	0.0460	1	0.046	0.28	0.6108
pH^2	7.5200	1	7.520	46.55	0.0002**
Residual	1.1300	7	0.160		
Lack of Fit	0.4600	5	0.093	0.28	0.8930
Pure error	0.6700	2	0.330		
Total	10.3400	10			
R^2	0.8905				
R^2_{adj}	0.8436				

S.S.: Sum of square; M.S.: Mean square; DF: Degree of freedom; *: $p<0.05$; **: $p<0.01$

the substrate concentration; this graph is called Michaelis-Menten plot. These experiments were carried out under the optimal conditions previously found. The kinetic parameters were estimated by a linearization using Line weaver-Burk plot (Lineweaver and Burk, 1934). MATLAB software release R2015a. (MathWorks Inc., Natick, MA, USA) was used.

RESULTS AND DISCUSSION

Proximate composition: Table 5 presents the results of the chemical composition of rainbow trout viscera, defatted viscera and its protein hydrolysate. These results are not in agreement with Taheri *et al*. (2013), who reported that the proximate compositions of rainbow trout viscera were 71.65% moisture, 15% protein, 13% lipid and 2.73% ash. However, the chemical composition of animal organs varies concerning their development, age, nutritional status and water temperature and, hence, their harvest time (Chuesiang and Sanguandeekul, 2015). Defatted viscera indicate the loss of close to 65% of lipid from the raw viscera with an increase of close to 45% of protein content. The liquid rainbow trout viscera hydrolysates had a protein content of about 6%, which is different from other published studies on fish hydrolysates, which have ranged from 68 to 87.6% protein (Gbogouri *et al*., 2004; Taheri *et al*., 2013; Morales-Medina *et al*., 2016; Wald *et al*., 2016), because they are subjected to spray drying or lyophilization processes for their subsequent use. The lipid content in RTVH decreased when compared with the defatted viscera because they were separated as a thin layer at the top of the supernatant due to centrifugation. However, the protein hydrolysates could significantly increase stability against lipid oxidation if they have a low content of these (Lassoued *et al*., 2015).

Optimization of pH and temperature of alcalase® 2.4 L: RSM was used to evaluate the interactive effect of pH and temperature on the proteolytic activity of alcalase® 2.4 L. Table 1 shows the observed values of proteolytic activity under different treatment conditions. Multiple regression analysis applied to these data generated the following second-order polynomial equation:

$$PA = -0.125 + 5.871\times10^{-5}\,T + 0.032\,pH - 1.867\times10^{-3}pH^2 \quad (4)$$

As can be seen from the ANOVA results (Table 6), the quadratic term of pH had a relatively higher significant effect ($p = 0.0002$) as compared to the linear term of T ($p = 0.0155$). The interactions between the different factors did not significantly influence ($p>0.05$). Even though the linear term of pH was not significant; this term cannot be eliminated from the model so as not to lose its hierarchy. The model was significant ($p = 0.001$), in contrast to the lack of fit, that was non-significant ($p = 0.8930$).

The response surface graph (Fig. 2) for PA as a function of pH and temperature, indicated that the proteolytic activity had a maximum as a function of pH in the range of 8.0-9.0, while as the temperature increases, the proteolytic activity increases. The model of the Eq. (4) was optimized to predict the value of the factors that maximize PA. The optimum conditions for the maximum PA (11.17 AU/g) were pH 8.5 and temperature 60°C; these values are consequent with the optimal hydrolysis conditions of alcalase according to the manufacturer's suggestions (Novozymes, 2002). Some authors found the same conditions of pH and temperature for alcalase with fish proteins, Normah *et al*. (2005) reported that the hydrolysis of Threadfin bream (*Nemipterus japonicas*) by alcalase was optimum at 60°C and pH 8.5. Benjakul and Morrissey (1997), found that alcalase showed high activity toward Pacific whiting (*Merluccius productus*) waste in a pH range of 8.5-10 with a temperature of 60°C. Other authors found similar optimum conditions, Wasswa *et al*. (2007)

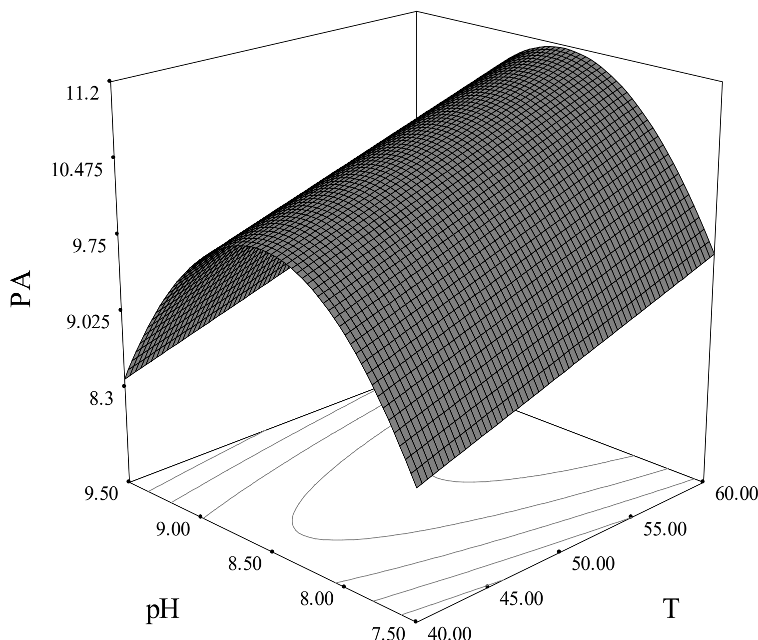

Fig. 2: Response surface graph for PA as a function of pH and temperature

Table 7: ANOVA of DH as affected by substrate concentration, enzyme to substrate level and temperature

Source	S.S.	DF	M.S.	F-ratio	Prob.>F
Model	29.1600	4	7.29	15.26	<0.0001**
S	1.6400	1	1.64	3.43	0.0852
E/S	6.9800	1	6.98	14.61	0.0019**
S*E/S	8.7400	1	8.74	18.30	0.0008**
S^2	11.8000	1	11.80	24.69	0.0002**
Residual	6.6900	14	0.48		
Lack of fit	3.3100	10	0.33	0.39	0.8955
Pure error	3.3800	4	0.84		
Total	35.8400	18			
R^2	0.8134				
R^2_{adj}	0.7601				

S.S.: Sum of square; M.S.: Mean square; DF: Degree of freedom; *: p<0.05; **: p<0.01

reported that the optimum values for temperature and pH were 59.74°C and 8.25, respectively, for hydrolysis of Grass carp skin using alcalase. Ovissipour *et al.* (2012), found that the optimum temperature was 60.4°C, for hydrolysis of Yellowfin tuna (*Thunnus albacares*) viscera with alcalase at pH of 8.5.

Optimization of enzymatic hydrolysis parameters: The influence of S, E/S and T on the DH was determined using factorial design as mentioned in the previous section. Table 4 presents the observed values for DH at different combinations of the independent variables. These data were fitted to a second-order polynomial model, whose coefficients were calculated by multiple regression. Equation (5) shows the adjusted model with the significant terms:

$$DH = 16.086 + 0.903\,S - 0.138\,E/S + 0.052S{\cdot}E/S - 0.222S^2 \quad (5)$$

The ANOVA for the response surface model of enzymatic hydrolysis conditions (Table 7), shows the coefficient of determination R^2 (0.8134) was not very high. Nevertheless, lack of fit test was not significant, indicating that the model is sufficiently accurate for predicting the DH for any combination of experimental independent variables. In the model, the effect of each coefficient on the respective response variable was determined using the F-test and p-value. As can be seen from the ANOVA results, the variable with the greatest effect on DH was the quadratic term of substrate concentration (S^2) (p = 0.0002), followed by the interaction (S·E/S) (p = 0.0008) and the linear term of the enzyme to substrate level (p = 0.0019). Temperature did not significantly influence (p>0.05). Although the linear term of substrate concentration was not significant, this term cannot be eliminated from the model so as not to lose its hierarchy. The significant effect of S in its quadratic form (p = 0.0002), geometrically implies a curvature of the behavior of the function.

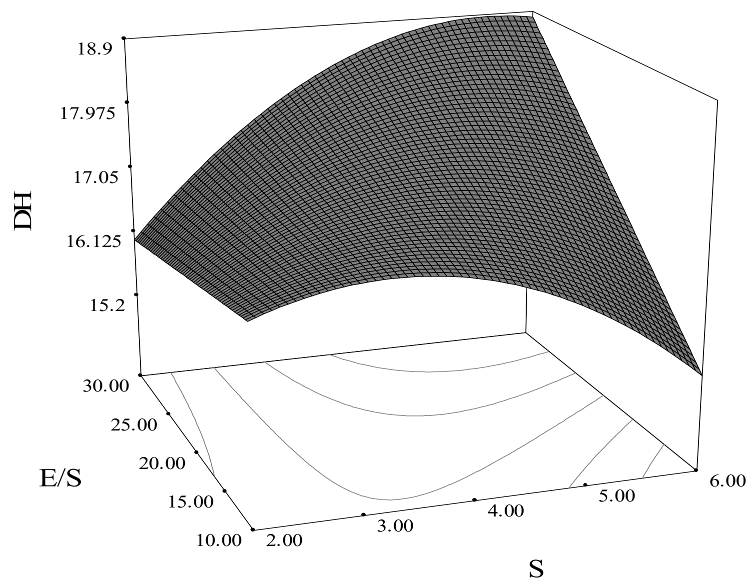

Fig. 3: Response surface graph for DH as a function of substrate concentration and enzyme/substrate ratio during hydrolysis (temperature at 60°C)

Figure 3, illustrates the response surfaces and the contour plot generated by the predictive model, varying two of the independent variables (S and E/S) within the experimental range while holding the temperature at 60°C. The experimental data display the expected behavior, namely a second order effect of S on DH. The response surface indicated that at the higher E/S ratio (30%), DH is directly proportional to substrate concentration up to values close to the upper level of the experimental range (around 5.53%) and there is a slight decrease in DH for higher values of S. Several studies on enzymatic hydrolysis of fish protein has also observed that an increase in enzyme/substrate ratio leads to a rise in DH (Guerard *et al*., 2001; Klompong *et al*., 2007; Bhaskar *et al*., 2008; Batista *et al*., 2010). Therefore, an increase in enzyme concentration has a positive effect on overall proteolysis, with subsequent improvements in the solubilization of fish protein. Also, it is more likely that proteases can localize close to the substrate, thereby cleaving peptides to a higher degree (Benjakul *et al*., 2014). As a consequence, with increasing enzyme concentration in the reaction mixture, the substrate must be present in high amounts (Benjakul *et al*., 2014). Our results are in agreement with these observations, which is mainly due that the quantity of product formed over a specified time is dependent on the enzyme present (Klompong *et al*., 2007).

A maximization of DH was conducted using Eq. (5), as a function of substrate concentration and enzyme/substrate ratio. The optimum values of the process variables for the maximum DH (18.84%) were E/S ratio, 30% and S, 5.53%. Three experimental runs of hydrolysis were carried out under the optimum conditions established, to verify the validity of the results. The pH and temperature of the reaction were adjusted to 8.5 and 60°C, respectively, according to our previous findings in the proteolytic activity of alcalase® 2.4 L with the rainbow trout viscera.

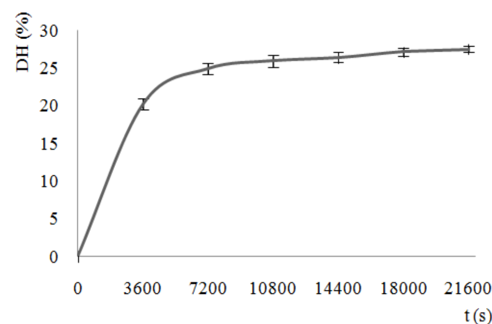

Fig. 4: Degree of hydrolysis at optimum conditions

The experimental data obtained are shown in Fig. 4 and the results indicated that the maximal DH, 27.6%, was in good agreement with the value predicted from the model. Therefore, the strategy to optimize the enzymatic hydrolysis conditions to obtain the maximal DH by RSM in this study was successful. These values of DH were higher than those of Taheri *et al*. (2013), who found a DH of 15.4±0.3% for the enzymatic hydrolysis of rainbow trout viscera with alcalase and Kim and Byun (2012) who reported a degree of hydrolysis close to 20% for rainbow trout muscle hydrolyzed with alcalase. The difference with these studies may be the result of the different enzymatic conditions used in those works, in addition to using a different method to control the hydrolysis process.

Determination of kinetic parameters (V_{max} and K_m): A series of experiments were conducted at substrate

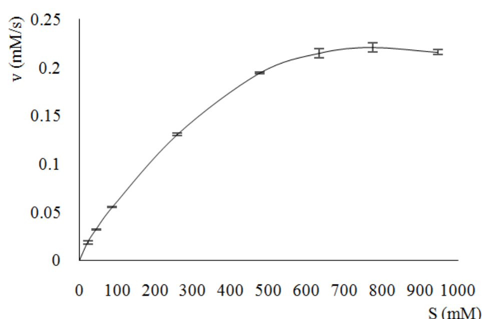

Fig. 5: Michaelis-menten plot of hydrolysis of rainbow trout (*Oncorhynchus mykiss*) viscera with alcalase® 2.4 L

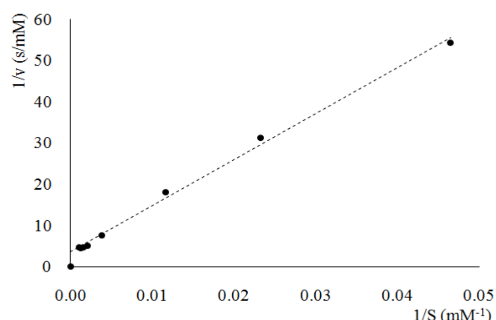

Fig. 6: Lineweaver-burk plot of hydrolysis of rainbow trout (*Oncorhynchus mykiss*) viscera with alcalase® 2.4 L

concentrations varying between 21.5-946 mM, at the optimal conditions previously found, to examine the fitting of the enzymatic hydrolysis of viscera of rainbow trout to Michaelis-Menten kinetics Eq. (6). Figure 5 presents the effect of the substrate concentration on the reaction rate of hydrolysis of rainbow trout viscera catalyzed by alcalase® 2.4 L. The hydrolysis shows a characteristic feature of the enzymatic reactions that follow Michaelis-Menten kinetics, namely the saturation of the enzyme by the substrate. Also, at low substrate concentrations, the reaction rate is proportional to this concentration, but at higher values, the reaction rate is independent of substrate concentration:

$$v = \frac{V_{max} \cdot [S]}{[S] + K_m} \quad (6)$$

The Lineweaver-Burk expression Eq. (7) was used to determine the kinetic parameters and the plot are shown in Fig. 6. Values for, K_m and V_{max}, were 316.21 mM and 0.283 mM/s, respectively. The coefficient of determination was 0.9965, which indicates the validity of double reciprocal transformation of Lineweaver-Burk to represent the data from this study:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \quad (7)$$

The value of K_m is high when compared to the values reported for other substrates with this same enzyme (Tardioli *et al*., 2005; Valencia *et al*., 2014). Which may indicate that there is a low affinity of the enzyme for the substrate, suggesting the presence of some negative effect of the substrate on the activity of the enzyme.

CONCLUSION

The pH and the temperature significantly influence the enzymatic activity of alcalase® 2.4 L with rainbow trout viscera (*Oncorhynchus mykiss*). The optimal operating conditions to maximize the proteolytic activity are pH of 8.5 and temperature of 60°C. The DH is significantly influenced by hydrolysis conditions (i.e., substrate concentration and enzyme/substrate ratio). Under optimal conditions, the hydrolysis of rainbow trout viscera (*Oncorhynchus mykiss*) using alcalase® 2.4 L resulted in a DH about 27%. The Michaelis-Menten plot confirmed that the optimum hydrolysis conditions are not in the region of saturation of the substrate. Consequently, the enzymatic reaction for the hydrolysis of rainbow trout viscera with alcalase® 2.4 L follows first order kinetics.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this study.

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