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Production of fish protein hydrolisates from *Oreochromis niloticus* fillet trimmings

Produção de hidrolisados de proteína de peixe a partir de aparas de filé de *Oreochromis niloticus*

Producción de hidrolizados de proteína de pescado a partir de recortes de filetes de *Oreochromis niloticus*

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Abstract
Process optimization is essential for the large-scale viability of the food industry. Central composite rotational design (CCRD) followed by response surface analysis was used to optimize the production of fish protein hydrolysate (FHP). FHP was obtained from tilapia filet trimmings hydrolyzed subjected using Alcalase 2.4L™, Neutrase™, and Novo-Pro™ D enzymes under temperature, enzyme concentration, and pH-controlled conditions. A 23-3 full factorial design (FFD) was initially employed to select the most influential variables in the process (with each enzyme used in the CCRD). From the FFD, temperature and enzyme concentration for Alcalase 2.4L™ and Novo-Pro™ D, and temperature and pH for Neutrase™ were selected. The estimated maximum degree of hydrolysis (DHmax) using Alcalase 2.4L™ was 60.05% during 180 min of processing at 39.03°C and 0.65% enzyme concentration. A DHmax of 56.96% was reached using Neutrase™ during 120 min at 39.46°C and 6.039 pH. Novo-Pro™ D was associated with a DHmax of 54.76% during 60 min at 47.95°C and 0.866% enzyme concentration. The three enzymes showed promising results for obtaining FHP with high DH from Nile tilapia filet trimmings.
Keywords: Enzyme hydrolysis; Optimization; Fish co-products; Protein; Protease.

Resumo
A otimização de processos é essencial para a viabilidade em larga escala na indústria alimentícia. Aqui, aplicamos a metodologia de Delineamento Composto Central Rotacional (DCCR) seguido de análise de superfície de resposta para otimizar a produção de hidrolisado de proteína de peixe (HPP). O HPP foi obtido a partir de aparas de filé de tilápia submetidas ao processo de hidrólise utilizando as enzimas Alcalase 2.4L™, Neutrase™ e Novo-Pro™ D, sob condições controladas de temperatura, concentração de enzima e pH. Primeiramente, aplicamos um Planejamento Fatorial Fracionado (PFF) 23-3 para selecionar as variáveis mais influentes no processo com cada enzima a ser aplicada no DCCR. A partir do PFF, selecionamos temperatura e concentração de enzima para Alcalase 2.4L™ e Novo-Pro™ D, temperatura e pH para Neutrase™. O grau máximo estimado de hidrólise (GHmax) usando Alcalase 2.4L™ foi de 60,05% em 180 min de processo a 39,03°C e concentração de enzima de 0,65%. Neutrase™ atingiu

GHmax de 56,96% durante 120 min a 39,46°C e pH 6,039, enquanto Novo-Pro™ D teve GHmax de 54,76% durante 60 min a 47,95°C e 0,866% de concentração de enzima. Assim, as três enzimas apresentaram resultados promissores para obtenção de HPP com alta GH a partir de aparas de filés de tilápia do Nilo
Palavras-chave: Hidrólise enzimática; Otimização; Coprodutos de pescado; Proteína; Protease.

Resumen
La optimización de procesos es esencial para la viabilidad a gran escala en la industria alimentaria. Aquí, aplicamos la metodología de diseño rotacional compuesto central (DRCC) seguida de un análisis de superficie de respuesta para optimizar la producción de hidrolizado de proteína de pescado (HPP). El HPP se obtuvo a partir de recortes de filete de tilapia sometidos al proceso de hidrólisis utilizando las enzimas Alcalase 2.4L™, Neutrase™ y Novo-Pro™ D, en condiciones controladas de temperatura, concentración de enzimas y pH. Primero, aplicamos un Diseño de Factor Fraccionario (DFF) 23-3 para seleccionar las variables más influyentes en el proceso con cada enzima a aplicar en el DRCC. Del DFF, seleccionamos la temperatura y la concentración de enzimas para Alcalase 2.4L™ y Novo-Pro™ D, la temperatura y el pH para Neutrase™. El grado máximo estimado de hidrólisis (GHmax) con Alcalase 2.4L™ fue del 60,05 % en un proceso de 180 min a 39,03 °C y una concentración de enzima del 0,65 %. Neutrase™ logró una GHmáx del 56,96 % durante 120 min a 39,46 °C y un pH de 6,039, mientras que Novo-Pro™ D tuvo una GHmáx del 54,76 % durante 60 min a 47,95 °C y una concentración de enzima del 0,866 %. Por lo tanto, las tres enzimas mostraron resultados prometedores para obtener HPP con alto contenido de GH a partir de recortes de filete de tilapia del Nilo.
Palabras clave: Hidrólisis enzimática; Optimización; Subproductos de pescado; Proteína; Proteasa.

1. Introduction

The global demand for protein has increased owing to the recognition of its importance in the diet for the maintenance of human health, especially for people with diet restriction issues, athletes, and the elderly (Jafarpour *et al*., 2020; Egerton *et al*., 2018). Approximately 12% of the fish produced in the world, which corresponds to species of low commercial value and co-products of the processing industry, is underutilized for non-human food purposes, mainly in the form of fish meal and fish oil for animal feed (FAO, 2020; Ucak *et al*., 2021). The use of biotechnologies is being increasingly explored to optimize protein recovery from animal by-products and increase their market value (Mullen *et al*., 2017; Toldrá *et al*., 2016).

Tilapia (*Oreochromis niloticus*) is one of the most popular commercial fish species in several countries (FAO, 2020). The filet is the main commercialized product (Silva *et al*., 2014). Consequently, considerable filet trimmings are generated (Roslan *et al*., 2014b). Generally, these trimmings are used to make breaded products, sausages, and other lower-value products. However, because of their nutritional potential and the need to optimize raw material use in the fish processing industry (Shen *et al*., 2012; Roslan *et al*., 2015), tilapia filet trimmings are excellent to produce fish protein hydrolysates (FPHs).

FPHs are obtained through enzymatic hydrolysis. Advantages of this process include a relatively low manufacturing cost, high nutritional value, functional properties suitable for application in the food industry, and the potential to obtain peptides with specific biological activities (Giannetto *et al*., 2020; Roslan *et al*., 2014b; Vázquez *et al*., 2017; Yarnpakdee *et al*., 2015). The technical and economic feasibilities of enzymes lie in the mild conditions in which the reactions occur, minimization of undesirable products, quality, and high yield of the final product (Maluf *et al*., 2019; Roslan *et al*., 2014b). These advantages are based on the selectivity of the enzymes for substrates and the possibility of controlling process conditions, such as temperature and pH, to obtain products with specific characteristics and purposes (Amiza & Masitah, 2012; Roslan *et al*., 2014b; Silva *et al*., 2014).

Enzymatic hydrolysis is performed by proteases that cleave peptide bonds. This transforms high molecular weight protein chains into smaller molecules and free amino acids, thereby increasing the reactivity and improving the functional properties of proteins (Giannetto *et al*., 2020; Foh *et al*., 2010; Nelson & Cox, 2014). The proteolytic enzymes most used for fish protein hydrolysis are Alcalase 2.4L™, papain, pepsin, trypsin, chymotrypsin, pancreatin, Flavourzyme™, Pronase™, Neutrase™, Protamex™, bromelin, Protease N™, thermolysin, and Validase™ (Messina *et al*., 2021; Amiza *et al*., 2017; Foh

et al., 2010; Hsu, 2010; Ngo *et al*., 2010; Raghavan & Kristinsson, 2008; Roslan *et al*., 2014a; Roslan *et al*., 2014b; Roslan *et al*., 2015; Yarnpakdee *et al*., 2015).

In general, enzymes with high activity/cost ratios are employed in the industry (Maluf *et al*., 2019). The endopeptides Alcalase 2.4L™ and Neutrase™, both developed by Novozymes Latino Americana Ltd. (Paraná, Brazil), deserve special mention. Alcalase 2.4L™ from *Bacillus licheniformis* is active at moderate alkalinity and temperature conditions and provides a high degree of hydrolysis (DH) in a relatively short time (Adler-Nissen, 1986; Amiza *et al*., 2017; Egerton *et al*., 2018; Foh *et al*., 2010). In contrast, Neutrase™ from *B. amyloliquefaciens* is utilized commercially to enhance proteins of animal and plant origin. The enzyme acts at neutral pH under mild temperature conditions (Amiza *et al*., 2017; Egerton *et al*., 2018; Foh *et al.*, 2010). Novo-Pro™ D produced by genetically modified *B. licheniformis* has also shown potential applications in industrial fermentation (Lopes *et al*., 2020; Maluf *et al.*, 2019).

For enzymatic hydrolysis to occur efficiently and yield a product with characteristics suitable for a given application, selecting an appropriate enzyme and the control of the procedural conditions are essential (Ishak & Sarbon, 2018). The most important variables in enzymatic reactions are the concentration and specificity of the enzyme, the temperature and pH of the reaction, and the nature of the substrate (Tkaczewska *et al*., 2020; Amiza *et al*., 2017; Roslan *et al*., 2015). The efficiency of the hydrolysis process is measured by the DH, defined as the percentage of peptide bonds broken throughout the reaction (Adler-Nissen, 1979). Knowledge of DH enables the elaboration of products with defined properties and higher yields (Foh *et al*., 2010).

Highly specific products with a maximum DH (DH_{max}) can be obtained by optimizing the protein hydrolysis conditions (Tkaczewska *et al.*, 2020; Ishak & Sarbon, 2018; Kristinsson & Rasco, 2010). For industrial applications, the process must be technically and economically feasible, with maximum utilization of available resources (Ishak & Sarbon, 2018). In the scientific environment, the technique of central composite design (CCD), followed by response surface statistical analyses, are commonly employed methodologies to optimize chemical process conditions (Amiza *et al*., 2011; Amiza & Masitah, 2012).

The present study evaluated the conditions of enzymatic hydrolysis of tilapia filet trimmings using Alcalase 2.4L™, Neutrase™, and Novo-Pro™ D enzymes. Conditions that resulted in higher protein cleavages were identified for each enzyme, optimizing the yields of FPHs.

2. Methodology

2.1 Raw material

The raw material used was the filet trimmings of Nile tilapia (*O. niloticus*) provided by a slaughterhouse in Toledo-Paraná-Brazil. The fish came from male batches raised in ponds under semi-intensive confinement. The creation period was from April to November, with an average water temperature of 23 °C. The feed provided contained 32% crude protein and the average slaughter weight was 700g.

2.2 Preparation of raw material

The trimmings were ground in an electric mill with a 5 mm sieve, separated into 2 kg packages, and stored by freezing at -18°C until use in the preparation of hydrolysates.

2.3 Protein content of raw material

The raw material was characterized in terms of the total protein content (928.08) (AOAC, 2016; Brasil, 2019). The analyses were performed in triplicate.

2.4 Enzymes

The enzymes used for the hydrolysis of the raw materials were provided by Novozymes Latin America Ltd. Alcalase 2.4L™ is a serine-protease type endoprotease produced by *B. licheniformis*, with Carlsberg subtilisin as its main component. The specific activity is 2.4 Anson Units/g.

Neutrase™ is a bacterial endoprotease produced by *B. amyloliquefaciens*. It is used commercially to improve animal and vegetable proteins. It is a metalloprotease and requires zinc ions for activity. This enzyme randomly hydrolyzes internal peptide bonds.

Novo-Pro™ D is an alkaline endoprotease produced by genetically modified *B. licheniformis*. It is mainly used in the pet food industry for the processing of animal proteins for flavor improvement. The specific activity declared by the manufacturer is 16 kilo new protease units (KNPU-S)/g.

2.5 FPH production

FPHs were prepared in an experimental borosilicate glass reactor jacketed with a thermostatic bath. For each trial, a suspension was prepared using 2 kg of raw material and 20% water. The pH and temperature of each suspension were adjusted according to the specific conditions of each test. The pH was adjusted using 10% anhydrous sodium carbonate solution. Then, enzyme was added to the suspension with constant agitation. The amount of enzyme added varied according to the experiment.

2.6 Enzymatic inactivation

At the end of the hydrolysis process, the enzyme was inactivated in a thermostatic water bath at 90°C for 20 min. The suspension was then cooled in an ice bath and the hydrolyzed contents were stored at - 4°C.

2.7 Soluble protein and DH

For the determination of soluble protein of FPHs, samples of each hydrolysate were centrifuged (Excelsa Baby I Centrifuge) at 3023 x g for 20 min. The supernatant was collected, diluted with 6.25% trichloroacetic acid (TCA) in a 1:1 ratio, and left to stand for 30 min. After precipitation of the TCA insoluble material, the samples were filtered through Whatman No. 40 paper to remove impurities. The soluble protein content of the filtrate was determined using the method of Lowry *et al*. (1951) using a spectrophotometer at a wavelength of 750 nm. The values obtained were expressed as milligrams of albumin. DH was estimated as described by Hoyle and Merritt (1994) and Liceaga-Gesualdo and Li-Chan (1999), which relates the proteins solubilized in TCA present in the sample and the total proteins initially present in the raw material:

$$DH = \frac{SP\ em\ 6.25\%\ TCA}{TP} x100$$

where DH is the degree of hydrolysis, SP is the soluble protein of FPH (mg/g), and TP is the total protein of the substrate (mg/g).

2.8 Optimization of the FPH process

Initially, a PFF 2^{3-3} Fractional Factorial Design (FFD) comprising three center points and a total of 11 trials was applied to evaluate the effect of pH, temperature (°C), and enzyme concentration (E; % relative to substrate) on the DH. The hydrolysis time was fixed at 1 h. The conditions varied from pH 7.5 to 9.5, temperature of 55 to 75°C, and E from 0.05 to 0.25% for the hydrolysis with Alcalase 2.4L™; from pH 6 to 8, 40 to 60°C, and 0.1 to 0.5% E for Neutrase™; and from pH 7.5

to 9.5, 60 to 80°C, and 0.5 to 2.5% E for Novo-Pro™ D. These ranges were selected according to the enzyme technical data sheets provided by the manufacturer.

Subsequently, a new central composite rotational design (CCRD; 2-2, comprising four trials, four axial points, and three central points, totaling 11 trials) was applied to optimize the hydrolysis process of Nile tilapia trimmings. The influence of the significant variables was evaluated using DH response.

According to the results obtained with the first planning, the variables that significantly influenced the hydrolysis of Nile tilapia filet trimmings with were T and E for Alcalase 2.4L™, pH and T for Neutrase™, and T for Novo-Pro™ D. In the case of Novo-Pro™ D, the variable E was evaluated in the second planning, although it was not significant in the first based on prior studies (Dey & Dora, 2011; Fountoulakis & Lahm, 1998; He *et al*. 2013). Table 1 summarizes the actual and coded levels of the variables studied in CCRD 2-2 for DH of Nile tilapia filet trimmings hydrolyzed with Alcalase 2.4L™, Neutrase™, and Novo-Pro™ D.

Table 1. Actual and simulated values of each variable (temperature (T, ºC), enzyme concentration (E, %) and pH) studied in CCRD 2-2 for hydrolysis of Nile tilapia filet trimmings using Alcalase 2.4L™, Neutrase™ and Novo-Pro™ D.

Encoded levels	Alcalase 2.4L™		Neutrase™		Novo-Pro™ D	
	T (x_1)*	E (x_2)*	T (x_1)	pH (x_2)	T (x_1)	E (x_2)
-1.41	29.9	0.1	27.5	5.2	35.9	0.2
-1	34.0	0.3	30.0	5.4	40.0	0.4
0	44.0	0.6	36.0	5.8	50.0	0.8
1	54.0	0.9	42.0	6.2	60.0	1.2
1.41	58.1	1.0	44.5	6.4	64.1	1.4

*x_1 and x_2 are the symbols representing the variables studied for each enzyme in the equations of the applied response surface models. Source: Authors.

The Alcalase 2.4L™ and Novo-Pro™ D assays were performed at pH 7.5 and varying T and E. Neutrase™ assays were performed with E fixed at 0.5% and varying T and pH. The durations of the Alcalase 2.4L™, Neutrase™, and Novo-Pro™ D assays were 3, 2 and 1 h, respectively. For a 2-factor system, the equation of the second-order response surface model is:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2$$

where Y is the predicted response, b0 is the intercept, b1 and b2 are the linear coefficients, b12 is the interaction coefficient, and b11 and b22 are the quadratic coefficients. The right-hand side of the model is represented by the SO (x1, x2).

The values obtained from the experimental trials were analyzed in R software (R Core Team, 2019), with the aid of the RSM package (Lenth, 2009). Response surface graphs were developed to demonstrate the effects of the independent variables on the evaluated response variables.

3. Results and Discussion

3.1 Protein content of raw material

The proportion of total protein in the filet trimmings of Nile tilapia was 16.53 ± 0.32%. This amount of protein was slightly lower than the previously reported value of value 18.65% in filets (Herath *et al*., 2016) and 19.95% in muscles (Wu *et*

al., 2017) of this species. This small variation in the chemical composition of fish is natural and varies with the size, age, season, and nutritional status of the fish (Nollet & Toldrá, 2011; Ogawa, 1999). In addition, the proportions of water, fat, and protein vary throughout the fish body, even between the edible parts (Nollet & Toldrá, 2011).

3.2 Optimization of FPH

DH in FPH from tilapia filet trimmings ranged from 36.81 to 60.58% for Alcalase 2.4L™; 32.84 to 46.29% for Neutrase™, and 46.08 to 55.36% for Novo-Pro™ D (Table 2).

Table 2. Degree of hydrolysis (DH) of the protein hydrolysate of filet trimmings from Nile tilapia in tests of temperature (T), enzyme concentration (E), and pH using Alcalase 2.4L™, Neutrase™ and Novo-Pro™ D.

Assay	Alcalase 2.4L™			Neutrase™			Novo-Pro™ D		
	T	E	DH	T	pH	DH	T	E	DH
1	34.0	0.3	43.18	30.0	5.4	33.04	40.0	0.4	46.95
2	54.0	0.3	50.72	42.0	5.4	40.87	60.0	0.4	49.85
3	34.0	0.9	52.17	30.0	6.2	37.53	40.0	1.2	50.14
4	54.0	0.9	40.58	42.0	6.2	46.29	60.0	1.2	46.95
5	29.9	0.6	57.09	27.5	5.8	32.84	35.9	0.8	52.75
6	58.1	0.6	45.50	44.5	5.8	42.46	64.1	0.8	49.27
7	44.0	0.1	36.81	36.0	5.2	34.58	50.0	0.2	46.08
8	44.0	1.0	53.59	36.0	6.4	43.56	50.0	1.4	50.14
9	44.0	0.6	58.55	36.0	5.8	45.01	50.0	0.8	54.49
10	44.0	0.6	57.97	36.0	5.8	44.63	50.0	0.8	54.20
11	44.0	0.6	60.58	36.0	5.8	44.78	50.0	0.8	55.36

Source: Authors.

As expected, the DHs in the FPHs obtained using Alcalase 2.4L™, Neutrase™, and Novo-Pro D™ displayed significant ($p<0.05$) second-order relationships with the variables used in CCRD 2-2, with R^2 values > 85% (Table 3).

Table 3 - Statistics of the second-order response surface model (SO (x1, x2)) applied for optimization of hydrolysis degree in fish protein hydrolysate using Alcalase 2.4L™, Neutrase™ and Novo-Pro™ D.

Method	$F_{(5, 5)}$	p	R^2	Stationary points	DH_{max}
Alcalase 2.4L™	8.890	0.0158	0.8989	(T=39.93; E=0.677)	60.05
Neutrase™	88.00	<0.0001	0.9888	(T=39.46; pH=6.039)	56.96
Novo-Pro™ D	8.343	0.0181	0.8930	(T=47.95, E=0.866)	54.76

Stationary points are temperature (T, ºC), enzyme concentration (E, %) and pH. DH_{max} is the maximum degree of hydrolysis estimated at the stationary points. Source: Authors.

The observations were adequate to explain the variability of the data (Joglekar & May, 1987). All the methods employed showed maximum estimated values > 50% DH_{max}. However, Alcalase 2.4L™ was more efficient at cleaving proteins from Nile tilapia filet trimmings, followed by the enzymes Neutrase™ and Novo-Pro™ D (Table 3). The superiority of Alcalase 2.4L™ over Neutrase™ was also observed for the hydrolysis of muscle from *O. niloticus* (Foh *et al*., 2010) and *Collichthys niveatus* (Shen *et al*., 2012). However, Egerton *et al*. (2018) evaluated the use of different enzymes in the hydrolysis of *Micromesistius poutassou* and found that Alcalase 2.4L™ yielded a lower DH compared to Neutrase™, possibly due to conditions of pH, temperature, enzyme concentration, and substrate type. These conditions differed from those explored in the present study.

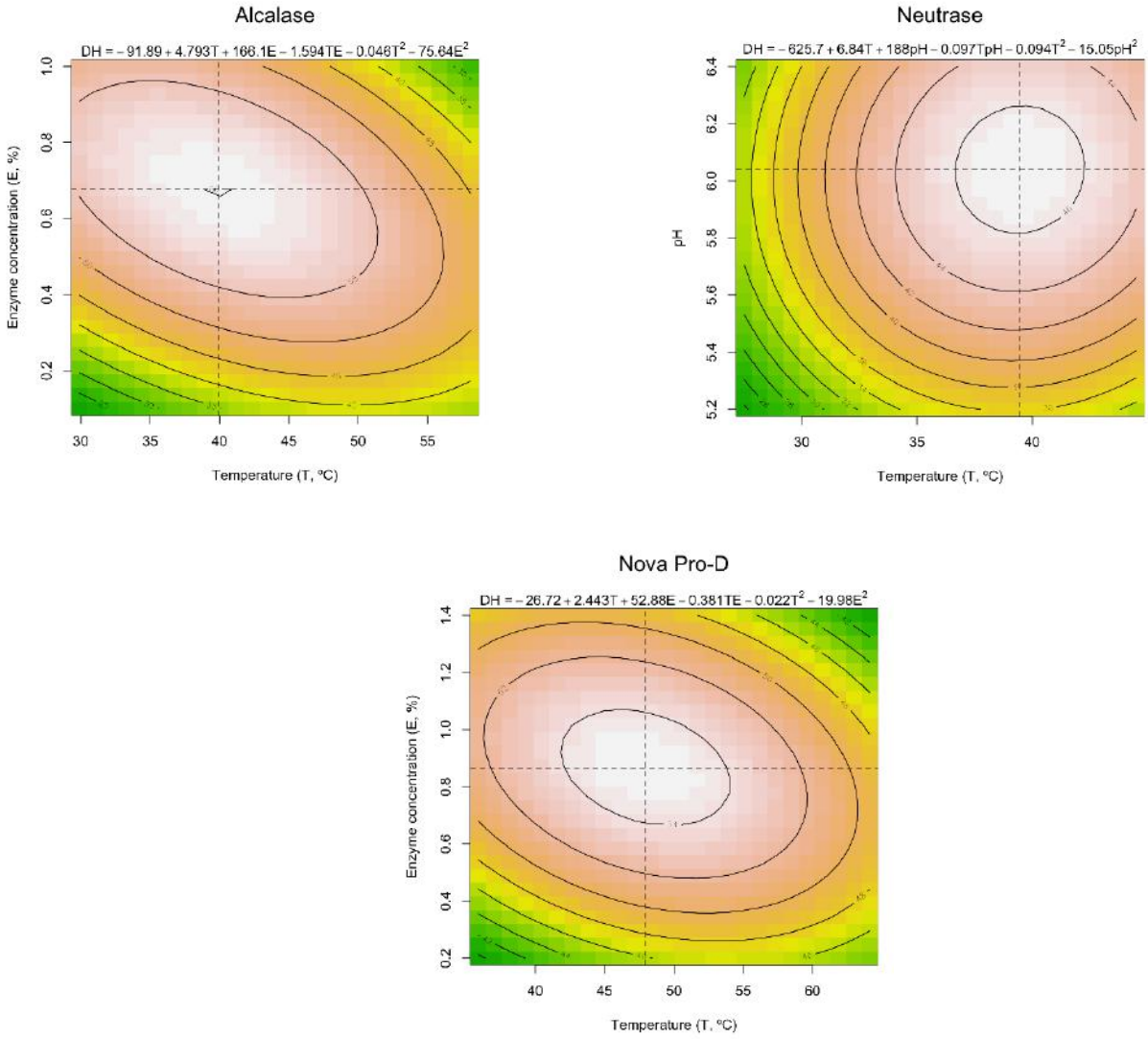
Temperature affects the reaction speed during protein cleavage, activation energy of the catalytic reaction, and thermal stability of the enzyme and substrate (Shen *et al*., 2012). These authors described that DH increases with temperature until it reaches an optimal value. Above this value the DH begins to decrease because higher temperatures disrupt the force that maintains the shape of the enzyme molecule, which is gradually denatured. At relatively low temperatures, the rate of enzyme reaction is slower, the kinetic energy of the molecules decreases, and collisions between them are less frequent. Thus, the optimum temperature for enzyme activity results from the acceleration of the reaction and increased denaturation due to temperature rise (Nelson & Cox, 2014; Whitaker & Dekker, 1994).

The concentration of the enzyme can affect the efficiency of the enzymatic reaction with the substrate in different ways. In general, the speed of the enzymatic reaction increases with increasing enzyme concentration. However, with the progression of the reaction, enzyme activity begins to be inhibited owing to the formation of compounds that compete for bonds in active sites or allosteric sites of the enzyme and substrate (Nelson & Cox, 2014). In addition to this competition, substrate reduction occurs due to successive cleavage, which increases the likelihood that an inhibitor molecule will bind to the enzyme (Nelson & Cox, 2014).

In addition to temperature and enzyme concentration, enzyme activity is influenced by pH. According to Shen *et al*. (2012), high or low pH conditions cause a decrease in enzyme activity, since the active site becomes progressively distorted, which negatively influences enzyme function. According to Whitaker and Dekker (1994), pH affects the active site of the enzymes by modulating the hydrophobic and hydrophilic interactions and can reversibly reduce enzyme activity or even denature the enzyme.

Thus, the polynomial relationships obtained in the response surface models indicate that the experimental trials were performed properly, allowing the identification of the optimal conditions with maximum DH for FPH production in relation to the factors evaluated (Figure 1).

Figure 1 - Contour plots of the response surface model generated for the degree of hydrolysis (DH) of FHPs obtained using Alcalase 2.4L™ (a), Neutrase™ (b), and Novo-Pro™ D (c). The model equations are presented as subtitles. Dashed lines indicate stationary points.



Source: Authors.

A DH_{max} of 60.05% was attained with Alcalase 2.4L™ at a temperature of 39.93 ºC and enzyme concentration of 0.677% within 180 min of process; this value was relatively higher than that previously reported with Alcalase 2.4L™. When temperature, enzyme concentration, and pH were optimized to generate Nile tilapia hydrolysates, Foh *et al*. (2010) and Roslan *et al*. (2014) estimated DH values for FPH prepared from the muscle to be 25.16% and 25%, respectively. From co-products, Roslan *et al*. (2014b) and Roslan *et al* (2015) estimated 20.42% and 20.20% yields, respectively. For FPH prepared from viscera and carcass, Silva *et al*. (2014) estimated a DH value of 34.73%. Our results were also superior to those obtained using Alcalase 2.4L™ for hydrolysis in other tissues of various species, such as whole *Micromesistius poutassou* (18% DH) (Egerton *et al*., 2018) and viscera of tuna (*Katsuwonus pelamis*; 35% DH) (Klomklao & Benjakul, 2016). Therefore, enzymatic hydrolysis is strongly affected by the operating conditions.

Despite the superiority of the DH achieved with Alcalase 2.4L™ with respect to other proteases, Shen *et al*. (2012) pointed out the possibility of the formation of unwanted products. This is because at high DH more phenylalanine is formed,

peptides are shorter, and amino acids with hydrophobic groups are present. These are responsible for the bitter taste of the product and bind to the active sites of Alcalase 2.4L™.

Shen *et al*. (2012) evaluated the use - among other enzymes - of Alcalase 2.4L™ and Neutrase™ for the hydrolysis of *Collichthys niveatus* muscle. DHs of 17.03 and 15.04%, respectively, were obtained. Although Neutrase™ yielded a lower DH compared to Alcalase 2.4L™, the authors emphasized that in terms of taste of the final product, Neutrase™ was superior owing to the formation of alanine, aspartic acid, and glutamic acid, which are responsible for the umami and sweet flavors. Therefore, the choice of one enzyme or another - in addition to DH - should be based on the application objective of the final product.

With respect to Neutrase™, the conditions for the hydrolysis of the Nile tilapia filet trimmings were optimized at a temperature of 39.46°C and pH of 6.039, resulting in an estimated DH_{max} of 56.96% within 120 min of processing. This DH_{max} is more than double of that reported by Egerton *et al*. (2018), who used this enzyme for the hydrolysis of *M. poutassou*. In a study by Foh *et al*. (2010), the hydrolysis of Nile tilapia muscle with Neutrase™ resulted in a DH of only 5% within 120 min of processing. These discrepancies between the reported DHs may be attributed to the fact that the temperature and pH used in these studies were above the optimums determined in the present study, a feature that negatively affects the efficiency of protein cleavage, even at higher enzyme concentrations.

The estimated DH_{max} for Novo-Pro™ D (54.76%) was close to that of Neutrase™ at 47.95ºC and enzyme concentration of 0.866%, in a processing time of 60 min. There is little scientific literature on the Novo-Pro™ D enzyme. Novo-Pro™ D is an endoprotease and therefore acts randomly on peptide bonds within the polypeptide chain (Whitaker & Dekker, 1994). Furthermore, it has slightly lower than the activity of the enzyme Alcalase 2.4L™, although the latter has a lower initial reaction speed than Novo-Pro™ D (Kamnerdpetch *et al*., 2007). Both Novo-Pro™ D and Alcalase 2.4L™ are classified as serine proteases. However, Novo-Pro™ D preferably cleaves at amino acids containing aromatic rings and sulfur, namely, histidine, phenylalanine, tryptophan, and tyrosine. Alcalase 2.4L™ exhibits a high specificity for cleavage at aromatic amino acids (Tavano, 2013; Waglay & Karboune, 2016).

Among the enzymes evaluated to obtain FPHs from tilapia filet trimmings, Novo-Pro™ D displayed the highest reaction speed, which was also reported by Kamnerdpetch *et al*. (2007). Under optimal process conditions with the enzyme, the predicted DH was 54.76% after 60 min of hydrolysis. Alcalase 2.4L™ exhibited a slower reaction than Novo-Pro™ D, while the latter required a higher temperature. Both factors are limiting factors in the viability of a process to generate FPHs on an industrial scale. Both should be taken into consideration when choosing the enzyme to be used in production.

To reaffirm the results predicted under the optimized conditions, new experimental trials were performed (data not shown), which corroborated the responses predicted for the process under optimal conditions. This finding indicates that the strategy adopted to optimize the enzymatic hydrolysis conditions and obtain the highest DH employed in this study was successful.

4. Conclusion

The three enzymes evaluated showed promising results with DHs > 50% for obtaining FPHs from Nile tilapia (*O. niloticus*) filet trimmings. The hydrolysis process with Alcalase 2.4L™ was optimized at 39.93°C and 0.677% enzyme concentration, generating 60.05% hydrolysis grade in 180 min at pH 7.5. The hydrolysis process with Neutrase™ was optimized at 39.46ºC and pH 6.039, generating 56.96% hydrolysis grade in 120 min, at 0.5% enzyme concentration. The hydrolysis process with Novo-Pro™ D was optimized at 47.95ºC and 0.866% enzyme concentration, yielding 54.76% hydrolysis grade in 60 min at pH 7.5.

Studies regarding the nutritional and functional characterization and chemical composition should be performed to determine the potential application of each of the developed FPHs under the optimized conditions.

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